A NOVEL APPLICATION OF TWO BIOMATERIALS FOR THE
DELIVERY OF GROWTH HORMONE AND ITS EFFECT ON
OSTEOBLASTS

by

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of Philosophy in the Faculty of Science at the University of London.

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Institute of Orthopaedics
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"What sets us against one another is not our aims - they all come to the same thing - but our methods, which are fruits of varied reasoning."

Antoine de Saint-Exupéry
Wind, Sand and Stars (1939)
Acknowledgement

This thesis is dedicated to my parents
(Questa tesi é dedicata ai miei genitori)

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Abstract

Hormonal stimulation of osteoblasts can provide better focal integration of orthopaedic implants. In this study both direct and indirect actions of growth hormone on primary human osteoblasts have been demonstrated. Growth hormone significantly increased cell proliferation and alkaline phosphatase activity and stimulated the synthesis of Insulin-like Growth Factor-I and the release of Insulin Growth Factor -binding protein 3.

A drug delivery system releasing biologically active growth hormone targeted to osteoblasts could be used to improve tissue repair in orthopaedics. Two biomaterials have been successfully developed for this novel application. The first is based on natural biodegradable, biocompatible gelatin, in the form of microspheres, where the amount of growth hormone released was significantly increased following ultrasound treatment. The second, a non-degradable polymer based on polyethylmethacrylate and tetrahydrofurufurylmethacrylate (PEM/THFMA) showed an initial rapid release followed by a prolonged sustained release of growth hormone. The preparation method used, significantly affected the amount of growth hormone released. Varying the ratio of PEM/THFMA and gelling it with hydroxyethylmethacrylate changed the profiles and increased the amount of growth hormone released, exposure to various temperatures did not affect the amount or bioactivity of the GH released.

The initial rapid release of growth hormone locally from both systems is desirable post-operatively to stimulate osteogenic cells at the polymer interface and accelerate repair during the critical early wound healing period, whilst the subsequent slow release would enhance osteoid deposition and bone mineral formation. Both systems have advantages, the choice is dependent on the intended site of use. The microspheres are biodegradable and the GH released can be increased in a physiological manner depending on clinical requirements. The PEM/THFMA can be used where stability is required and can absorb tissue fluids and growth factors from the bone matrix, marrow and synovial fluid, thereby creating an optimal environment for rapid bone remodelling.

This study has demonstrated a level of control for GH delivery in both systems and has confirmed that targeted osteoblasts respond to GH. Numerous clinical applications could benefit from these controlled systems, for example, enhancing the fixation of total joint prostheses, bone defects in non-union fractures, and in pathological conditions where local growth promoting effects would be beneficial.
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Abbreviations

1,25(OH)2D3  1,25 dihydroxy vitamin D3
125I-GH  125-Iodinated growth hormone
ALP  Alkaline Phosphatase
B/F  Bound counts/Free counts
BMP  Bone Morphogenic Protein
BP  Binding protein
BrdUrd  Bromodeoxyuridine
BSA  Bovine Serum Albumin
cAMP  Adenosine 3' 5'-Cyclic-Monophosphate
CB  Coomassie Blue
DEPC  Diethyl pyrocarbonate
DMEM  Dulbecco's modified Eagles medium
DMSO  Dimethyl sulphoxide
DNA  Deoxyribonucleic acid
DPX  Distrene/tricresyl phosphate plasticizer with xylene
E.Coli  Escherichia Coli
ECM  Extracellular matrix
EGF  Epidermal Growth Factor
ELISA  Enzyme Linked Immunoabsorbent Assay
ESTA  Eluted Stain Assay
EVA  Ethylene-vinyl acetate
FGF  Fibroblast Growth Factor
GF  Growth Factor
GH  Growth Hormone
GHBP  Growth hormone binding protein
HEMA  Hydroxyethyl methacrylate
hGH  Human Growth hormone (synthetic 22K)
HOBS  Human Osteoblasts
HOS  Human Osteosarcoma
HS  Horse Serum
IFN-r  Interferon -r
IGF  Insulin-like Growth Factor
IGFBP  Insulin-like Growth Factor Binding Protein-3
IRMA  Immunoradiometric assay
LHRH  Luteinizing hormone releasing hormone
m  Monolayer
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>MGF</td>
<td>Macrophage Growth Factor</td>
</tr>
<tr>
<td>mm</td>
<td>Micromass</td>
</tr>
<tr>
<td>MOPS</td>
<td>(3-[N-Morpholino]propane-sulfonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NR</td>
<td>Neutral Red</td>
</tr>
<tr>
<td>NSB</td>
<td>Non-specific binding</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PDS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PEM</td>
<td>Polyethylmethacrylate</td>
</tr>
<tr>
<td>PEM/THFMA</td>
<td>Polyethylmethacrylate-Tetrahydrofurfuryl methacrylate</td>
</tr>
<tr>
<td>pHEMA</td>
<td>Polyhydroxyethyl methacrylate</td>
</tr>
<tr>
<td>PICP</td>
<td>Carboxyterminal propeptide type - I procollagen</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethylmethacrylate</td>
</tr>
<tr>
<td>Poly(EM:THFMA)</td>
<td>Copolymers for EM/THFMA</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum free medium</td>
</tr>
<tr>
<td>SGF</td>
<td>Skeletal Growth Factor</td>
</tr>
<tr>
<td>SR</td>
<td>Swelling Ratio</td>
</tr>
<tr>
<td>SSC</td>
<td>Stock solution</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>THFMA</td>
<td>Tetrahydrofurfuryl methacrylate</td>
</tr>
<tr>
<td>THR</td>
<td>Total hip replacement</td>
</tr>
<tr>
<td>TMB</td>
<td>Trimethyl Benzoate</td>
</tr>
<tr>
<td>GHBP</td>
<td>Growth hormone binding protein</td>
</tr>
<tr>
<td>TNF-a</td>
<td>Tumour Necrosis Factor-a</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasound</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
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CHAPTER 1

General Introduction
The surgical application of polymeric materials dates back to 600 B.C when Indians used natural polymers such as horse hair, cotton, and leather for wound repair and the Egyptians used linen and silk as sutures. The use of natural polymers for biomedical applications continued well into the twentieth century and interest in materials from biological sources is now being revived for applications in soft and hard tissue replacement, for dental applications, and adhesives (Byrom, 1991).

Many biomaterials in current clinical use were not originally designed as such, but were found to be useful in other medical applications. Dacron, for example, derived from textiles, has been used for vascular grafts, and commercial-grade polyurethanes have been used in the manufacture of artificial heart valves. Advances in the development of synthetic materials over the last fifty years have resulted in a significant improvement and proliferation in the biomaterials available for biomedical applications (Shalaby et al., 1984).

In the 1960s the incorporation of drugs into polymers was extended from agricultural products to medical applications, with drugs being incorporated into silicone rubber tubing or into a polyethylene matrix. This led to new challenges, which were reflected in the use of biomaterials in various branches of medicine in the treatment or replacement of diseased or traumatised organs.

From 1970 onwards a considerable growth in the research of polymeric materials occurred, and a variety of different methods became available for their synthesis. This resulted in a new generation of biomaterials with a shift towards natural biodegradable materials in particular, for use as drug carriers for targeted and controlled delivery (Kasuya et al., 1993, Peppas and Langer, 1994). Biomaterials are now being designed to elicit a specific response from cells and tissues within the body. Examples include poly(lactic/glycolic acid) polymers (Schakenraad et al., 1988), cellulose (Ghorab and Luzzi, 1990), hydrogels such as poly(2-hydroxyethyl methacrylate) (Chirila et al., 1993), poly(ethylmethacrylate) tetrahydrofurfuryl-methacrylate (PEM/THFMA) and gelatin (Di Silvio et al., 1994).

There has always been a need for the development of biologically compatible controlled drug delivery systems. Although a wide range of biomaterials are now available for the development of drug-delivery systems, the material in question must be able to function safely and efficaciously within the complexity of the biological environment in which it is placed. This makes the design of controlled systems a difficult, but challenging task for the researcher. Present methods are inefficient, mainly due to the difficulties in placing and maintaining an adequate non-toxic amount of drug at the intended site for a sufficient time. Controlled release systems offer the opportunity to overcome these problems and allow the local delivery of drugs for tissue repair and/or healing to take place focally.
New materials are now being considered, primarily for their orthopaedic function and secondly as drug-delivery systems with the incorporation of drugs for local release. Pritchett (1992) incorporated human growth hormone in polymethylmethacrylate (PMMA) in a controlled study of hip arthroplasties, and Salked et al., (1995) used synthetic polymers as a carrier system for the release of osteogenic proteins. The use of biomaterials as local drug delivery systems for bone repair and tissue regeneration has opened a new and challenging field in orthopaedic research.

Concept of Drug Delivery

Drugs are conventionally administered either orally or via injections, and often at a site remote from the target tissue, requiring frequent and repeated administration. This poses several problems; the drug level and duration of bioavailability cannot be controlled independently, and the drug is free to diffuse throughout the body, and, it is often difficult to achieve an optimal local dose at the desired site. The result of this is that there are "peak" and "trough" plasma concentrations and the peaks may be above the toxic level or the troughs may be below the therapeutic level. The dose size and frequency of administration are the only parameters that can be controlled.

Protein and Peptide Delivery

The majority of proteins and polypeptide drugs are usually unsuitable for oral administration due to their rapid degradation and deactivation by proteolytic enzymes in the digestive tract. Their high molecular weight also makes them unsuitable for intestinal absorption and the parenteral route is usually the most effective means of administration. In many cases, one or more injections daily may be required, and the initial delivery of the protein/peptide to the target tissue results in high concentrations followed by a continuous decline due to the short half-life of the agents.

There is now an increasing awareness for the need to administer certain drugs and hormone preparations, not only in the correct dose, but also at the right time. Certain drugs such as antihistamines, antiasthmatics, cardiovascular drugs, and some hormones have been shown to have an optimal effective level and time of administration. The effects and/or pharmacokinetics of drugs can display significant daily variations. It is imperative therefore, to consider biological rhythms when evaluating drug delivery systems, the formulations and the release kinetics as a basis for drug treatment (Stolar and Baumann, 1986). Significant daily variations in the pharmacokinetics of the drug have been observed depending on physiological changes in the circadian rhythm (Conte et al., 1993, Lemmer, 1991). In the diseased condition where there is a hormone deficiency, genuine replacement therapy should mimic the natural state of control. The importance of rate-controlled drug
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and hormone administration can be demonstrated by the increase in the release of insulin in response to an increase in glucose concentration in the blood. There is increasing evidence that better glycaemic control can be achieved with the use of continuous insulin-infusion systems (Goosen et al., 1983). Another example is growth hormone (GH), which is secreted in episodic bursts and as a result its concentration fluctuates over a wide range and is age-related. Various physiological stimuli cause an increase in GH secretion; these include sleep, exercise, stress and the postprandial fall in blood glucose (Finkelstein et al., 1972, Drobny et al., 1983, Stolar and Baumann, 1986).

If drug-delivery systems are to be used to treat diseases with drugs and hormones, the release pattern should be precisely controlled to mimic normal physiological release patterns. To fulfil the therapeutic needs of diseases which depend on circadian rhythmicity or pulsatility, or where the drug in question could be released on demand, a controlled-drug delivery system capable of releasing the drug locally would be very desirable.

Controlled release dosage forms can only be injected or implanted at or near the target site. Attempts have been made to try and overcome these problems by the development of "sustained-release" systems in the form of capsules, or slowly dissolving coatings of cellulose or similar materials, or by complexing the drug to the polymeric vehicle in an attempt to decrease its solubility (Rhine et al., 1980, Okada et al., 1991, Conte et al., 1993). An example is the use of photopolymerised microcylinders as a slow-release method for delivering cytokines to target sites using a lipid vehicle to allow the possibility of sustained release and reduce the rate of proteolytic degradation (Cliff et al., 1992).

A more desirable regime would be to release the active agent from the polymer for a sufficient time period directly at the target site and not indiscriminately to the whole body, thus improving efficacy and minimizing systemic effects; furthermore, this would eliminate patient compliance problems (Graham, 1978, Conte et al., 1993, Caramella et al., 1995). By using different polymer systems as the carrier, and by altering polymer-drug incorporation, it is possible to achieve variable release rates. The advantages of a controlled drug-release system are many, but the major one is the maintenance of a desirable therapeutic level which can be altered depending on clinical requirements.

Although polymer systems have been described for extracorporeal uses, such as for ocular therapy and transdermal patches, the majority of polymer drug delivery systems are intended for implantation within the body, and this disadvantage must be weighed against the advantages. As the polymer is to be implanted it must be non-toxic, free of elutable impurities or harmful degradation products; and its presence must not cause discomfort to the patient. The biological requirements of the polymer are related to the specific requirements of the drug-release systems and the physiological environment in which it has
to operate. With the exception of biodegradable systems, the physical, chemical and mechanical properties of the polymer should not be altered by the biological environment in which it is placed and it should not induce an inflammatory response when placed at the target site. Biocompatibility is a very important issue and refers to the tissue/device interactions of the polymer and additives; and implies that the polymer should remain biologically inert and not have any detrimental effect. Biocompatibility has been defined previously as "the ability of a material to perform with an appropriate host response in a specific application" (Williams, 1989). This definition addresses the need of a potential biomaterial not only to be free from damaging effects on the host, but to elicit those host responses necessary for optimal functioning of the medical device. The consideration of what constitutes an 'appropriate host response' in the orthopaedic situation will depend upon the biomaterial in question and its intended site of use. The maintenance of cell viability in the presence of the material has been considered a good indicator of biocompatibility but, when early bone remodelling is the desired response, it seems more appropriate to select those materials which will promote cell proliferation.

There are numerous situations where an augmentation in the release of a particular drug on demand is beneficial, examples include, the delivery of insulin in diabetics, nitrates for patients with angina, anticancer therapy, the release of hormones for controlling childbirth, hormone replacement and many more. Delivery systems which allow drug release rates to be modulated are still experimental, and include magnetically triggered or ultrasonically treated systems, where release can be activated by an external stimulus. In magnetically triggered systems, the drug is usually dispersed within the polymer matrix with magnetic beads and release rates are controlled by an oscillating external magnetic field, as described in detail by Kost and Langer (1990). In further studies Kost (1993) has shown that the release rate of biologically active agents from a polymeric matrix can be modulated externally by ultrasonic energy. The ultrasound affects the degradation rate of degradable polymers as well as the permeation through non-degradable polymers. Unlike the magnetically triggered system, the use of ultrasound has the advantage that it does not require the addition of any substance (Kost et al., 1988, 1989). A 5-fold increase in polymer degradation rate and up to a 20-fold increase in drug release rate was observed with biodegradable polyanhydrides, polyglycolides and polylactides when the polymer system was exposed to ultrasound.

The possibility of using ultrasound on demand, as a trigger to alter the release of contraceptive agents from biodegradable implants has also been investigated and the results suggest that it may be possible to externally control the release rates of implantable devices (Kost et al., 1992). A similar observation has been described by Miyazaki et al., (1985), who evaluated ultrasound as a method for increasing drug release at desired times.
depending on clinical requirements for the release of drugs in chemotherapy to maximize
the effectiveness of the anticancer drug and to minimize toxic side-effects.

Ultrasound is an attractive non-invasive method of being able to externally modulate the
amount of drug released from the delivery system depending on clinical requirement at the
time. This was tested as a potential method for use in enhancing the release of GH from
gelatin microspheres on demand, and will be discussed in detail in Chapter 4.

Biomaterials

Many natural polymers occur extensively within the body and perform many roles
including support, function and protection. It would appear appropriate therefore, to try
and imitate natural polymers and use them in the design of non-toxic synthetic polymers for
use as implants and drug-delivery systems (Giusti et al., 1993, Brandeis et al., 1993).
Such an example is collagen, a fibrous protein which constitutes a large proportion of the
total protein in animals. The use of collagen in medicine can be traced back to the physician
Galen 175 A.D who used absorbable catgut sutures (Katz and Turner, 1970). It is an
excellent naturally occurring biomaterial, uniquely designed to transmit tensile and
compressive forces of great magnitude which acts as a 'scaffold' for general skeletal
support. It has very low antigenic properties, controllable biodegradation (Chapvil et al.,
1973) and furthermore, has the ability to promote cellular growth and attachment (Chirila et al.,

Collagen based materials have been used as a support for cell cultured wound healing and
implants (Doillon and Silver, 1986) In another study, this group showed that a porous
collagen sponge seeded with fibroblasts could be used as an 'artificial skin' support to
encourage epidermal cell replication (Doillon et al., 1987, 1988). The fact that collagen can
be reconstituted in different forms, such as films, sponges and injectables, makes it useful
for numerous clinical applications. Geggel et al., (1985) proposed the use of collagen gels
in bioartificial ocular surfaces, and Tachibana et al., (1985) described the use of collagen
sponge tube grafts for ureteric replacements, where the structural design was achieved by
growing cells derived from the bladder on the matrix. There has also been extensive use of
collagen in periodontology, and Minabe et al., (1989), demonstrated the benefits of using
collagen film to immobilize tetracycline in periodontal treatment. Their results showed that
this method of release was effective for a period of 2-3 weeks. Collagen can interact with
various substances to form complexes which vary in stability and this feature has been
exploited for drug delivery systems (Kincl et al., 1984, Phinney et al., 1988, Rao et al.,
1994i ).
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The key factor in the design of a delivery system is the choice of polymeric material used as the vehicle, as this determines the kinetics of drug release. Optimal design or selection of a biomaterial for drug release systems must take into consideration the structure and morphology of the polymer matrix per se (Roseman and Yalkowsky, 1976, Mathiowitz and Langer, 1987, Forni et al., 1992). The biocompatibility and physicochemical integrity of a drug delivery vehicle are critical determinants of its effectiveness and practicality, and these characteristics in turn, are directly related to the surface chemistry of the material. For example, porous structure, degree of swelling, crosslinking, additive concentration and thermodynamic transitions will all affect the diffusion process and the release kinetics; this has been reviewed in detail by Langer and Peppas (1981), Kim et al., (1980) and Vert, (1987). The ability of polymers to exist in various rubbery, glassy or solvent swollen forms with different diffusion coefficients renders them suitable for the effective release of incorporated drugs (Olanoff et al., 1981, Good and Mueller, 1981, Heller et al., 1981).

A variety of degradable and non-degradable controlled release systems have been described in the literature. Liposomes, for example, have the potential to be tailored in a variety of ways and this has been reflected in their use as delivery systems in areas such as cancer therapy, antimicrobial therapy and for various ophthalmic disorders (Gregoriadis and Florence, 1993). Weiner and co-workers (1985), used a liposome-collagen gel matrix for the delivery of insulin and GH, which were encapsulated in vesicles within the matrix. Hydrogels have also been investigated for the controlled release of drugs, Cardinal et al. (1981) described the use of polyhydroxyethyl methacrylate (pHEMA) for the release of progesterone from monolithic and reservoir devices. More recently synthetic polymers, such as poly(vinyl alcohol) and poly(acrylic acid) have been blended with biological polymers, collagen and hyaluronic acid, to produce sponges and hydrogels, and their ability to release macromolecules such as growth hormone has been investigated (Cascone et al., 1994).

Okada et al., (1991) developed copoly (DL-lactic/glycolic) injectable microspheres for the administration of depot formulations which released drugs at a zero-order rate over a one-month period. This type of application aims to improve patient compliance and achieve a more constant plasma concentration than daily injections. Benagiano and co-workers (1973), studied sustained contraceptive effects using subcutaneous polydimethylsiloxane (PDS) implants, and found that they were able to maintain constant body levels of the hormones, but this was achieved, however, three months post-insertion. Using the same technology for the continuous release of steroids, this group found an initial decrease in the diffusion from the capsules during the first few months, suggesting that there may be some local factor affecting the absorption of the steroids from the subcutaneous implant. Following exhaustion of the drug, the capsule was removed and a continuous fibrotic
covering was observed around the capsule; the cause of the decrease in the amount of drug released (Benagiano et al., 1973).

Polymer degradation is an important consideration in the production and use of implantable and other medical devices and the majority of biodegradable polymers have been designed to have hydrolytically unstable bonds in the polymer backbone (in 't Veld et al., 1993, Zhong et al., 1994). The choice of material is dependent on its function; it has to remain in-situ long enough to allow healing to take place, but consideration has to also be given to the fact that it will gradually lose its strength and produce breakdown products as degradation takes place (Ali et al., 1993, Golomb et al., 1993). In the case of collagen, for example, when used for haemostasis, its function is required only until a clot is formed but, in situations where the collagen is used for tissue augmentation and for burn and wound dressing, a much slower rate of absorption is required.

In order to consider all these factors more sophisticated polymer drug-delivery systems are required that allow controlled sustained delivery, in particular for the release of larger molecular weight polypeptide drugs and proteins to local targets (Rhine et al., 1980, Hsieh et al., 1983, Langer et al., 1980, 1991). As materials science moves forward and the knowledge of cell biology increases, a new generation of materials is emerging, for example, genetically engineered biological bandages consisting of Keratinocytes cells (SCC-13) have been engineered by gene transfer to produce high levels of growth factors directly into the wound site (Andreatta-van Leyen et al., 1993). The use of hydrophilic, water-soluble polyacrylates for the microencapsulation of mammalian cells, has been investigated by Lamberti et al., (1984). This type of technology has led to the production of a durable implantable metabolic prostheses for use in insulin-dependent diabetes. Similar work was also carried out by Cole et al. (1993) who compared the biocompatibility of alginate-polylysine-alginate microcapsules in two animal models of insulin-dependent diabetes. Cohen and co-workers (1990) investigated the use of a gel matrix-polyphosphazene to encapsulate mammalian cells, liposomes and proteins. Various copolymers have been characterised for the encapsulation of mammalian cells and bone growth factors as a possible means for improving fracture healing (Schlameus et al., 1990, Eisa and Sefton 1993, Zielinski and Aebischer 1994).

PMMA has important areas of application both in the dental and medical fields. It is has been extensively used as a bone cement in orthopaedic surgery to fix prostheses, but it has been shown to have a secondary function as a carrier for therapeutic agents (Marks et al., 1976, Downes and Maughan, 1989, Downes et al., 1990, Downes 1991, Pritchett 1992). The work of Buchholz (1970) led to the incorporation of antibiotics in PMMA to prevent deep wound infection, similarly, Marks et al. (1976) used antibiotic-impregnated acrylic bone cement to reduce post-operative infection. Although extensively used, PMMA has
several disadvantages for clinical use. It has a high exotherm; in orthopaedic bone cements temperatures in excess of 100°C can occur in the femoral cavity. This makes it unsuitable for the incorporation of proteins and peptides. Furthermore it has high polymerization shrinkage which can result in loosening around the implant, and can cause the production of an allergic reaction.

In view of the disadvantages of PMMA as a non-degradable drug delivery system, various other methacrylates have been studied (Bhusate and Braden 1985, Patel et al 1987, 1991i,ii,iii). Tetrahydrofurfurylmethacrylate (THFMA), a heterocyclic monomer emerged as a suitable candidate (Patel and Braden, 1991ii). The room temperature polymerising system is based on PEM polymer powder and THFMA liquid monomer, it has lower shrinkage than conventional acrylic systems such as PMMA, whilst remaining rigid. In addition, this new polymer, unlike PMMA which takes up 2-3% water in two years, is able to take up 34% water in two years (Patel and Braden, 1991iii). Although this high water uptake appears in the first instance to be a disadvantage (as water absorption reduces the strength of polymers), but it may prove to be an advantage in allowing the release of drugs. If the high water uptake of this polymer is maintained in-vivo then the polymer will be able to absorb tissue fluids and growth factors from the surrounding bone matrix. The properties displayed by this new polymer make it a challenging candidate to study for use as a potential non-degradable drug delivery system. The properties of this polymer were exploited to evaluate its use as a non-degradable drug delivery system for bovine serum albumin (BSA) and for GH.

Non-degradable polymers, however, have the disadvantage of requiring surgery for both insertion, and removal where appropriate, or remaining in-situ, which can present problems such as fibrotic tissue growth around the implant (Benagiano et al., 1973, Jeyaseelan et al., 1977). The use of degradable materials eliminates this problem, but the number of currently available biodegradable and absorbable polymers is limited. A degradable system only needs insertion and is therefore potentially more attractive for use in the design of controlled drug-delivery systems. The choice of biodegradable polymer involves its biocompatibility in physiological environments and degradation to toxicologically acceptable products that are eventually eliminated from the body; they therefore require extensive toxicological testing. The majority of biodegradable polymers are usually hydrophilic and, once the water has penetrated the matrix, the more hydrophilic the drug, the more easily it is released.

It appears therefore, that biodegradable polymers may have some advantages over non-degradable ones, and one of the aims of this study was to develop a delivery system using a natural polymer. Gelatin was used; this has the advantage of being a biodegradable hydrophilic polymer and is non-toxic and non-immunogenic. Gelatin has previously been
used for incorporating drugs (Jeyanthi and Rao, 1987, Oppenheim 1987), but it has not been used to release growth factors.

**Mechanism of Drug Delivery**

Diffusion controlled systems are the most widely used drug-delivery systems, and are generally classified according to the mechanism that controls the release of the incorporated drug. The different systems have been extensively reviewed by Langer and Peppas (1981), but a brief mention will be given of the different systems available in order to explain the various mechanisms involved in drug delivery.

Reservoir or 'depot' systems have a rate controlling membrane which surrounds the active agent; this may or may not be porous and, in some cases, the reservoir may contain a suspension of the active agent thereby creating a steady-state release rate by diffusion across the membrane (Kissel and Traechslin 1992). If the drug is totally dissolved then the release rate will decay in an exponential manner with time, as expected for 'first-order' kinetics, according to Fick's law. These systems include membranes, capsules and hollow fibres. The polymers most widely used include silicone rubber, hydrogels and ethylene-vinyl acetate copolymers. Sanders et al., (1985) used this type of system for the microencapsulation of Nafarelin - an analogue of luteinising hormone releasing hormone. The release of non-steroidal anti-inflammatory drugs has also been investigated using this type of system (Wagenaar and Muller, 1994). Although these systems do not always achieve zero-order release rates, they can be designed to produce different kinetics. This is usually achieved however, using non-degradable polymeric materials which require implantation and surgical removal, thus rendering them unsuitable for long-term use in the delivery of high molecular weight drugs such as GH or other polypeptides. The choice between a degradable or a non-degradable material will depend upon the intended site of use, as different polymers will have different mechanical and degradation properties (Wu et al., 1994, Cremers et al., 1994).

Generally, drugs released from the systems currently available are small molecules with low molecular weight, and macromolecules, such as polypeptide proteins, are not easily released using these systems because of their small permeability coefficients through polymers.

In matrix or monolithic systems, the drug is uniformly dispersed within the polymer matrix, and as in reservoir systems, drug diffusion through the polymer is the rate-limiting step. Biodegradable polymer matrix systems that may be engineered to release macromolecules have been described (Langer et al., 1980, 1981). This type of system
offers the major advantage that the polymer degrades and is eliminated from the body, thus avoiding the need for surgical removal.

Microspheres are an example of monolithic systems, and they have been widely used for the controlled release of chemotherapeutic agents, where the maintenance of therapeutic blood concentrations without the risk of toxicity is very important (Ghorab et al 1990, Narayani and Rao, 1994). More recent advances have shown that polymers, primarily in the form of microspheres, can release larger molecular weight drugs and hormones (Di Silvio 1994ii). The development of a controlled delivery system for water-soluble proteins and peptides is becoming increasingly important, as a large number of recombinant synthetic peptides are now available for therapeutic applications (Fryklund 1987, Edman et al., 1980, Langer et al., 1981). Proteins, however, have the disadvantage of a short in-vivo half-life; Cohen et al., (1991) described the encapsulation of polypeptides as a method of increasing their therapeutic efficacy, by releasing the drug continuously over a certain period. A similar system for the release of proteins has also been described by Rao et al. (1994i).

Models for the release of osmotically active agents have been described; osmotically driven pumps can be preprogrammed for rate-controlled delivery of solutions and osmotically active drug salts can be released from monolithic polymer matrices (Eckenhoff 1981, Wright et al., 1981). Chemically controlled systems are biodegradable and the drug is distributed in the same way as in matrix systems; the difference, however, is that in the matrix system the polymer phase remains unchanged with time and the drug is released by diffusion whereas, in the degradable system, the polymer phase decreases with time. In degradable systems where the drug is released by erosion, the release is dependent on degradation of the polymeric material at the appropriate rate, with the production of harmless degradatory products. For example, poly(ortho esters) hydrolyse very slowly at a physiological pH of 7.4, but become more labile at lower pHs. This is either brought about physically by dissolution of the polymer, or chemically, by the hydrolysis of the backbone or cross-links (Makino et al., 1985). This type of system is therefore capable of a wide range of erosion rates so that the release rate of the incorporated agent is controlled (Heller 1985, Yolles et al, 1975). The use of pH sensitive hydrogels, in the form of injectable collagen for the release of drugs has been described by Rosenblatt et al., (1994), and the ability to change the properties by pH variations of these materials could prove useful for biomedical applications.

Hopfenberg (1976) has examined factors affecting the release rates and he found that in order to obtain a zero-order release, it would be necessary for the delivery system to be designed in a form where the surface-area did not change as a function of time and he suggested a slab, neglecting the edge effects, as an ideal shape. However, the ideal
situation has rarely been observed in practice, and as the majority of the biomaterials used in controlled release systems are generally hydrophilic, bulk erosion may occur in addition to surface erosion thus making the release process difficult to predict (Heller 1980, 1985) When the drug is released by diffusion, it is usually dispersed within the polymer matrix, in which the drug is permeable. Gale et al. (1980) used ethylene-vinyl acetate (EVA) copolymers in monolithic systems with the drug particles dispersed within the polymer matrices in order to achieve nearly zero-order release kinetics. In monolithic matrix devices the release of the drug may be either by simple diffusion or through triggering by an environmental agent such as water penetrating into the matrix.

Mathematical models for drug release from controlled or sustained release polymeric systems have been described by Baker and Lonsdale (1974), Chien (1976), Gurney et al., (1982) and Lee (1985). These systems are usually designed to release the encapsulated drug at a constant or slowly declining rate by erosion of the polymer or by membrane moderated Fickian diffusion. The regulation of drug release from diffusion controlled and surface erosion controlled matrix systems, and polymer dissolution in swellable systems has been well described (Lee 1986, Lee and Peppas, 1987).

Chemically controlled systems incorporate pendant chain systems and the drug is attached to a degradable or non-degradable polymer backbone which is released by hydrolytic or enzymatic cleavage. The choice of polymer is important in this type of system to prevent immunological responses when it is coupled to the drug. Peterson et al., (1979) have used polyaminoacids with steroids as pendant side chains, where the drug is released by hydrolysis of the drug-polymer ester bonds. The design of pendant chain systems for use as drug-delivery systems has been reviewed by Kim et al., (1980).

Swelling-controlled systems are usually solvent activated and the drug is usually dispersed within the polymer solution; the solvent is then evaporated resulting in a solid polymer phase. In order for the drug to be released, the dissolution medium such as water penetrates the polymer matrix which swells and allows the drug to diffuse outward. In magnetically controlled systems, the drug is usually dispersed within the polymer matrix in combination with small magnetic beads. When placed in aqueous media, the drug is released in the same manner as in matrix systems, but the amount of drug released can be increased by exposure to an external magnetic field.

Although the various systems described are advancements in the field of drug delivery, they all have the major disadvantage of giving release rates that are either constant or are dependent upon degradation of the system with time, a problem common to the entire field of controlled release. Relatively few delivery systems have been described which allow drug release to be altered or discontinued once commenced, but no one technology will be
suitable for all indications, due to the diversity of the drugs, its dosages and routes of administration and duration of action.

Drug-delivery systems have therefore to be selected to provide an optimal release rate for the additive, and physical and chemical stability of the system. The aim is, therefore, to select the best controlled-release technology for each particular drug and indication (Yang et al., 1994). If the control of drug release were the only factor to be considered, then a non-degradable controlled release device would be the obvious choice for the implant. Drug delivery systems can take many forms and sizes; Hopfenberg (1976), and Arshady (1993) have reviewed in detail the properties of microspheres that facilitate their use for biomedical applications. They have been used in the biomedical field as diagnostic cell markers and cell separators, and they can be programmed to dissolve or degrade after their function has been completed. The use of microspheres for extracorporeal therapy and targeted drug delivery is based on their uniform and well characterised surface, their narrow size distribution and a large specific area (Davis and Illum, 1988).

Microspheres are generally monolithic and have the drug dispersed throughout the matrix particles, and in general, give a typical first-order rate of drug release. Polymeric microspheres for drug delivery can be produced from natural materials such as albumin, gelatin, starch, or from synthetic polymers e.g. polylactides, polyglycolides and polycyacrylates (Juni et al., 1987, Edman et al., 1987, Burgess et al., 1987, Oppenheim, 1987). Many techniques are available for making them and the choice depends upon the desired size and proposed function, the physicochemical properties of the drug and polymer matrix, and also tissue and drug compatibility (Jeyanthi and Rao, 1987, Di Silvio et al., 1994, Cremers et al., 1994). By choosing the appropriate formulation and process manipulation, microspheres can be used to release drugs in a controlled manner at the appropriate rates (Kwon et al., 1991, Cremers et al., 1990, Tice et al., 1989). Di Silvio et al., (1994) have shown that it is possible to enhance the release by the application of an external stimulus. One of the first peptides to be released using poly(DL-lactide-co-glycolide) microspheres was luteineizing-hormone releasing hormone (LHRH) (Sanders et al., 1984). Depot-systems have been investigated by Kissel and co-workers (1991) for the release of bromocriptine for a one-month period. To date, however, no method has been described that uses biodegradable microspheres for the release of large molecular weight polypeptide growth factors.

The application of biomaterials for drug delivery in Orthopaedics

Biomaterials have been used extensively in the field of Orthopaedics for the replacement of joints, the fixation of prostheses and, more recently, the delivery of therapeutic agents. The application of biomaterials suitable for bone replacement is restricted; in particular total hip
replacement (THR). Materials such as titanium and iron have been used for joint replacement because of their mechanical stability, although they are subject to wear and corrosion which frequently results in aseptic loosening (Charnley and Cupic, 1973, Willert 1974, Jasty 1993). The survival of these types of implants is limited and toxic products leaching from the implant may cause the formation of a fibrous capsule leading to impaired function (Oh and Harris, 1978, Salvati et al., 1981, Harris 1980).

Bioactive materials such as calcium phosphate ceramics have been shown to have good bone-bonding properties (Downes et al., 1992ii, Klein et al., 1994). In-vitro studies have shown that bone cells readily produce a calcified matrix layer on titanium surfaces (Lowenberg et al., 1991). Calcium phosphate ceramics have been widely used as bone-replacement materials in orthopaedic surgery, facial bone augmentation and dentistry. By changing the calcium/phosphorous ratio or by the addition of ions such as magnesium and fluorine or increasing the porosity; these materials are capable of achieving different properties depending on the desired implantation site (de Bruijn et al., 1992). Furthermore, they have been shown to be good delivery systems for peptides (Downes et al., 1991i, 1992ii).

Bioactive materials have been selected for their combined mechanical and biocompatibility properties for clinical use; for example, hydroxyapatite-coated hip prostheses have shown good bone-bonding properties (Geesink et al., 1987, 1988, Cook et al., 1988). These materials have been shown to stimulate adjacent bone cells, and studies based on histological and biochemical evaluations (Vrouwenvelder et al., 1993, Meyer et al., 1993). In other applications, Iyoda et al. (1993) cultured chondrocytes on hydroxyapatite, a proliferation of chondrocytes was observed two weeks post-implantation, suggesting that it may act as a scaffold for new bone formation. This type of implant using osteoblastic cells could have great potential for early fixation and bone defect supplementation (Courteney-Harris et al., 1995).

Orthopaedic surgery routinely uses implantable materials, these can be either permanent or temporary, each of which will require materials with different properties depending on the projected life time. Permanent devices are used in the total replacement of joints, whereas temporary fixation devices are used for stabilisation, until the bone is sufficiently healed and has regained its own natural strength. Degradable polymers are especially advantageous in this type of application where the implant is needed only on a temporary basis.

Most orthopaedic surgery leads to unavoidable bone cell death at the trauma site, for example, in THR, reaming of the medullary cavity results in the destruction of the vascular supply. Remodelling of bone should eventually occur but any stimulus or factor that could
improve the initial healing response would be advantageous. With this in mind, present day design of biomaterials is being aimed at creating biomaterials capable of eliciting a specific response from the appropriate cells and tissues at the implant site. In the case of orthopaedic implants, for example, it would be desirable for osteoblasts to be stimulated so that a rapid deposition of mineralized matrix on the surface or in close apposition to a newly implanted prosthesis could take place. Improved prosthesis design and cementing techniques have led to fewer prosthesis fractures and less bone resorption, achieving a better integration at the bone-cement interface (Crowninshield et al., 1980, Sutherland et al., 1982). However, all these materials have the major disadvantage of having to remain in-situ once the drug has been exhausted.

The use of degradable materials is becoming an attractive alternative as this eliminates the need to remove the implant. These materials, however, are hampered by the fact that they are too weak to be used for load-bearing implants. Alternatives have been to synthesize stronger polymers containing aromatic monomers with degradable backbones (Pulapura 1990). The most important surgical biodegradable polymers are aliphatic polyesters of hydroxy acid derivatives which have been used for sutures, porous composites and drug delivery systems.

In children where the skeleton is in a constant state of growth, the long term presence of implants may interfere with the physiological remodelling process, and biodegradable implants would therefore be a more appropriate choice. Biodegradable screws for fixation of osteotomies in children have been investigated by Illi et al. (1992). Internal fixation using biodegradable polyactic acid (PLA) screws provided an interface that aided bone healing and led to a rapid and complete ossification of the soft callus tissue. Biodegradable gelatin has been tested as a vehicle for drug and peptide delivery in microsphere form and as a coating on titanium implants (Di Silvio et al., 1994i). Paediatric surgery is another area where degradable materials may have an important role and, by incorporating growth factors (GFs) in these materials it is possible to stimulate the appropriate cellular response during the critical post-operative period. Controlled-drug delivery systems, which will deliver directly to a target site are of potential use for the release of growth factors or osteogenic promoters to improve bone-bonding and remodelling for the treatment of fractures and bone defects.

Growth factors and their effect on bone

Bone is a complex living tissue and its formation, destruction and the remodelling process that occurs throughout life is dependent on cellular activity, controlled by numerous systemic and local factors. Many naturally occurring, soluble polypeptides which have potent stimulatory effects on epithelial or mesenchymal cells have been identified in the past
ten years; the majority have been named according to their biological activity, examples being epidermal growth factor (EGF), fibroblast growth factor (FGF) and transforming growth factor (TGF), Insulin-like growth factor -I (IGF-I), platelet derived growth factor (PDGF), β-2-microglobulin and other osteoinductive factors such as bone morphogenic proteins (BMPs) (Zheng et al., 1992, Hauschka et al., 1988). The BMPs are of particular interest and were isolated from extracts of demineralised bone matrix (Bauerand Urist, 1981, Bentz et al., 1989, Sampath et al., 1990, Urist 1994, Urist et al., 1983). They are thought to play important role by inducing the formation of new cartilage and bone. Bostrum and co-workers (1995), using monoclonal antibodies against BMPs, demonstrated immunolocalization of BMP 2 and 4 in fracture healing using a rat model. Their data suggests an important regulatory role in cell differentiation during fracture repair. Considerable progress has been made recently in the characterization of bone related GFs and these have been extensively reviewed by Canalis (1985), Canalis et al., (1988), Mohan and Baylink, (1991), Hauschka (1990) and Raisz (1988). Although there are many GFs associated with bone, this study will be concerned primarily with the effects of GH and IGF-I. The general effects of GH and IGF-I on bone growth will be discussed in this chapter, but the more specific actions on the osteoblast will be discussed in detail in Chapter 3.

In the adult skeleton a continuous turnover of bone matrix and mineral takes place at discrete foci throughout life-bone remodelling. This process ensures the mechanical integrity of the skeleton and plays an important role in calcium homeostasis. It constitutes a very complex sequence of events, involving activation of several different stem cell populations and rigid regulation of two contrasting processes; bone resorption and bone formation.

Bone is a unique, highly dynamic, adaptive tissue and its function is dependent on actions at the cellular level. It has the ability to regenerate in response to mechanical injury or tissue trauma and it can modify its structure in response to both mechanical and hormonal stimuli. This regenerative property is primarily due to the presence of GFs in the bone matrix (Raisz and Kream 1983, Triffitt 1987). These GFs were initially believed to act as systemic agents, but there is now increasing evidence that they act mainly as local regulators of cell growth, and that they mediate the effects of systemic hormones, which can modify the synthesis or effects of local factors (Canalis et al., 1988, Hauschka 1990, Mohan and Baylink, 1991ii).

GFs play a role in differentiation by directly or indirectly stimulating the phenotypic transformation of cells. They also affect cell development, chemotaxis, activation of inflammatory cells, tissue repair and disease. The processes of cellular proliferation and the acquisition of a specific phenotype show a high degree of co-ordination. It is now
becoming evident that these peptides have a much wider range of biological activity than originally thought. Sporn and Roberts (1988) have shown that the actions of many GFs may be stimulatory or inhibitory depending on the presence of other factors and the cell type exposed to them. These GFs have effects on physical parameters, extracellular matrix components, cell adhesion molecules and membrane junctional complexes between adjacent cells. The response of a cell to a particular GF may not be a simple direct effect, it can also involve interference between stimuli, so that exposure of a cell to one GF compromises its ability to bind, and hence respond to, another. These peptide factors were described by Green (1989) as "multifunctional mediators of cellular growth and differentiation" as a result of their wide range of actions.

Regulation of bone development and bone mass in foetal and adult life is dependent on an interplay between bone formation by the osteoblasts and bone resorption by the osteoclasts. Recent research has indicated that GFs may act locally to modulate bone formation by stimulating osteoblast proliferation and differentiation (Stracke et al., 1984, Chen and Bates 1993). Osteoblasts play an important role in intercellular communication and in the control of bone resorption by osteoclasts. The mechanisms by which GFs produced by the bone cells exert their action can be either paracrine effect (local release by cells to modulate the activity of neighbouring cells) for example, the stimulation of osteoblast DNA synthesis by tumour necrosis factor (TNF-a), produced by active macrophages, or by autocrine modification of the activity of the cell of origin to produce a feedback regulation. The majority of GFs influence osteoblast activity in this way. GFs may also act through a juxtacrine mechanism through binding of a membrane anchored GF from one cell to its receptor on the adjacent cell. The non-diffusible mechanism of intercellular stimulation distinguishes the juxtacrine effect from the paracrine and autocrine modes of action, where GFs are mediated by diffusion to the target cell.

Growth is a complex biological phenomenon and rapid growth in childhood results from the increased production of cells and their subsequent expansion (Isaksson et al., 1987, Deuel 1987). After the epiphyses have closed following the pubertal growth spurt, effects on the skeleton must be exerted directly on bone cells and it is the local growth factors that are thought to stimulate bone formation by increasing osteoblast proliferation and matrix biosynthetic activity (Nijweide et al., 1986). Isgaard and co-workers (1986) have shown that local administration of GH and IGF-I stimulated local bone growth when administered into the epiphyseal growth plate, in contrast GH administration into the metaphysis did not cause a similar stimulation, indicating that GH stimulates unilateral bone growth and that local administration of IGF-I stimulates longitudinal bone growth. It is has been generally accepted that IGF-I production is regulated by GH (Russell & Spencer 1985, Isgaard et al., 1986). Nilsson et al., (1990i) have shown that the IGF-I gene is expressed in rat epiphyseal growth plate chondrocytes, and that its expression is dependent on GH. The
results from this study further substantiates a paracrine/autocrine role of IGF-I for the stimulatory effect of GH on bone growth.

It would seem that the bone inducing activity is not due to just one growth factor, but probably represents a combination of actions of several factors acting at specific stages and in different cells during osteogenesis (Hauschka et al., 1988). Bone cells synthesize a number of GFs and the bone matrix is a rich source of these (Canalis 1988, Mohan and Baylink, 1991). Being a heterogeneous tissue, bone is comprised of a mixed cell population and is in direct contact with cartilage and marrow cells; therefore the regulators of bone remodelling may originate from a variety of cells, and cellular interaction plays a critical role in physiological tissue remodelling (Wergedal et al., 1990, Nijweide 1986, Owen 1970). Since multiple GFs can be present at any one time within the bone matrix, interactions between GFs could be important for the regulation of proliferation and differentiation. Kasperk et al., (1990) showed that GFs singularly and in combination has both stimulatory and inhibitory affects on DNA synthesis and alkaline phosphatase activity in osteoblastic cells.

Although most of the information concerning GFs is derived from in-vitro studies, there is much evidence to support the fact that they do control growth and differentiation in vivo. Examples of this are the administration of GFs to influence mesoderm induction in Xenopus embryos and the association of developmental abnormalities with alterations in GF or GF-receptor gene function (Kimelman and Kirschner 1987, Paterno et al., 1989, Merimee et al., 1989, Goodman et al., 1968, Mullis et al., 1991). An example is Laron type dwarfism, where the major cause is a defective GH-receptor-GH interaction (Laron et al., 1971). The study of factors involved in the control of bone remodelling is complicated by the involvement of local factors (such as other GFs, prostaglandins and inorganic ions) and systemic factors (calcitropic hormones) that interact at cellular, tissue and organ levels (Kobayashi et al., 1994).

Cross and Dexter (1991) have reviewed the effect of GFs on the many parameters of cell development and behaviour. Their review indicates that, any effect elicited by a GF is determined by the responding cell type. Antoniazzi and co-workers (1993) looked at the effects of 1,25-dihydroxyvitamin D$_3$(1,25(OH)$_2$D$_3$) and GH on the serum osteocalcin levels in growth hormone deficient children. Their results showed that 1,25(OH)$_2$D$_3$ caused an increase in osteocalcin, but no additive effect was seen when GH was given. Linkhart et al. (1991) showed a differential regulation of IGF-I and II release from neonatal mouse calvaria in the presence of parathyroid hormone (PTH), TGF-$eta$ and 1,25(OH)$_2$D$_3$ indicating that they have different modulator effects.
Function and origin of bone cells

There are four morphologically distinct cell types in bone: osteoprogenitor cells, osteoblasts, the osteocytes and osteoclasts. Osteoprogenitor cells are those cells that give rise to the bone forming cells found in abundance on the endosteal and periosteal surfaces of bone; these cells resemble fibroblasts morphologically. Osteoblasts, responsible for bone matrix synthesis, appear on bone surfaces that are undergoing growth and development. Osteocytes, are found buried within mineralised bone matrix and are connected to one another and to osteoblasts on the bone surface by extensive projections or canaliculi; they are thought to be responsible for bone nutrition. Osteoclasts are the principle bone-resorbing cells; they are large (diameter ranging from 20-100\(\mu\)m) and multinucleate (2-100 nuclei). They are rich in lysosomal enzymes such as acid phosphatase and cathepsin; osteoclasts are found where bone is being resorbed. When in contact with the bone surface, their membranes form cytoplasmic projections known as 'ruffled borders' that appear to penetrate the bone surface. Osteoblasts and osteocytes are derived from mesenchymal precursors whereas osteoclasts are thought to be derived from macrophages or other vascular non-skeletal cells. In fact, macrophages have been shown to behave like osteoclasts in vitro, attaching to bone and causing the hormone-stimulated release of mineral and collagen.

Osteoblasts are highly differentiated cells responsible for laying down the inorganic matrix of bone. They are characteristically found in a layer one cell thick close to the surface of the developing bone or in Haversian systems, the sites of bone remodelling (Marie 1994, Tenenbaum 1990). Active osteoblasts are columnar in shape (diameter 20-30\(\mu\)m) and have an extensive endoplasmic reticulum often studded with ribosomes, a large Golgi complex lying near the nucleus and a high mitochondrial content. This abundance of proteinsynthesizing and processing machinery is indicative of a cell whose primary function is secretory. Osteoblasts synthesize and secrete type I collagen, proteoglycan, cytokines, GFS such as TGF-\(\beta\), and glycoproteins such as osteocalcin, osteopontin and osteonectin, into a region of unmineralised matrix (osteoid) between the cell body and the mineralised matrix. Long processes are extended into the matrix and make contact with other osteoblasts and osteocytes to a depth of up to 100\(\mu\)m. Osteoblasts are rich in alkaline phosphatase, an enzyme that may play a major role in calcification; this activity also provides a useful marker for the characterisation of osteoblast-enriched populations of cultured bone cells (Silver et al., 1994, Rodan et al., 1983, Rodan et al., 1988, Nijweide et al., 1988).

Evidence regarding the stromal origin of osteoblasts was first obtained by Friedenstein (1976), who pioneered the use of the diffusion chamber for the study of the differentiation of bone marrow stromal cells. Using a combination of in-vivo and in-vitro methodology
Owen and Friedenstein (1988) were able to demonstrate that single bone marrow cells could give rise to a variety of stromal lines. Many systemic hormones regulate osteoblast proliferation, differentiation and function; including osteotropic hormones, steroid hormones, sex hormones and GH. These hormones may affect the osteoblast directly, via special receptor events or indirectly as mediated by GFs. GH, for example, can have both direct and indirect effects on osteoblasts mediated by IGF (this will be dealt with at a later stage in more detail).

Many culture systems have been set up to study osteoblastic activity, either from primary cultures of bone-derived cells or, more frequently, using osteoblastic cell lines. Chiba et al., (1993) transformed human foetal osteoblast cells so that they can be used as a suitable model for studying bone cell metabolism. An SV40-immortalised human foetal osteoblastic cell line was similarly established by Harris et al., (1995); differentiated cells showed high levels of osteopontin, osteonectin and expressed collagen type I. Clover and Gowen (1994) investigated two human osteosarcoma cell lines (MG-63 and HOS TE85) to see if they were representative models of osteoblastic phenotype, they found that both proliferated more rapidly than osteoblast-like cells but the HOS TE85 exhibited higher levels of alkaline phosphatase under basal conditions, indicating that cell lines are not always representative of primary osteoblast cells. The majority of cell lines (rat, mouse-UMR 106) express osteoblastic phenotype by responding to osteotropic hormones, by synthesis of collagenous and non-collagenous proteins of the extracellular matrix and by positive reaction to alkaline phosphatase (Hassanger et al., 1992, Mohan and Baylink 1991i, Amarnani et al., 1993). However, although helpful in contributing significantly to the knowledge of the osteoblast’s physiology and pathology, the extent to which these cells are representative of normal human osteoblasts is not really known (Marie 1994). The culture models of primary human osteoblasts have been developed by many research workers using different techniques. However, special attention has to be paid to ensure that these cells continue to express the differentiated phenotype of interest. The biology of bone cells have been reviewed in detail by Stein et al., (1990), Stein and Lian (1993), Aufmkolk and Schwartz, (1985) and Robey and Termine, (1985).

GROWTH FACTORS - Mechanism of action

The strength of the bone is dependent on its volume; this is determined by the balance between the two opposing processes, osteoblastic bone formation and osteoclastic bone resorption. During the past twenty years there has been a remarkable change in the approach to the study of regulation of bone metabolism. Two mechanisms are thought to be responsible for the maintenance of bone volume: (i) Systemic regulation by calcium and phosphate regulating hormones e.g PTH, 1,25(OH)₂D₃ and calcitonin, (ii) local humoral factors, involving growth factors, which stimulate bone formation by increasing osteoblast
proliferation and matrix biosynthetic activity. Systemic regulation of bone also utilises GFs; there is evidence that the skeletal effects of some hormones, such as GH and PTH, are mediated by the production of local growth factors under the influence of these systemic hormones.

GFs play an important role in the development and growth of osseous tissue and bone is unique among tissues in the variety of GF which it harbours. GFs are sequestered in mineralised extracellular matrix and their biological actions are modulated through complex modes of release and presentation to responding cells. The effects of these GFs on bone can be divided into two specific areas; exogenously produced endocrine factors which act on specific bone target cells and endogenously produced local factors with possible autocrine or paracrine action.

**Interactions between Growth Factors**

The target cells for growth factors are characterised by the expression of specific transmembrane receptors that bind the factor and stimulate the cell to respond in one of a number of different ways (Klaus *et al.*, 1994, Mohan *et al.*, 1989, Mathews *et al.*, 1991). Of these responses the most accessible to study is that of proliferation, and in particular the recruitment of quiescent, serum-starved cells in ($G_0$) into the cell cycle. The cellular mechanisms responsible for the multiple actions of GFs are still unknown, but one likely explanation is the ability of a receptor for a specific peptide to alter either the cellular distribution or the binding affinity of the receptor for a second peptide GF, independent of any direct cross-reactivity of the peptides themselves, for example Insulin does not bind to the IGF-II receptor, but it does cause the cycling and redistribution of IGF-II receptors, a process known as transmodulation (Tiong and Herington 1991, Wakefield *et al.*, 1987, Carlsson *et al.*, 1990, Mullis *et al.*, 1991). Any one particular GF can elicit a variety of qualitative different responses in a concentration-dependent manner depending on the specific cell type (Slootweg *et al.*, 1990, Nilsson *et al.*, 1990). GFs are usually present in very low concentrations, and the majority are synthesized as high molecular weight precursors, which are then cleaved by proteolysis to generate active species. They usually consist of single or multiple subunits of the same or different polypeptide chains and are often associated with other proteins such as binding proteins or other GFs which may be involved in their biological action. They can generally be classified into two classes; broad spectrum - those that have an effect on different cell types, and those that are cell specific, the major ones are listed in Table 1.1. The broad spectrum bone-related growth factors can be further subdivided and are listed in Table 1.2. In this introduction only GH and IGF-I, both broad spectrum growth factors will be discussed.
GROWTH HORMONE

In this study GH was the polypeptide chosen for incorporation into the two biomaterials investigated for use as drug delivery systems. The reason GH was chosen is that, it is a well characterised therapeutic agent for actively stimulating growth. It is readily available as authentic sequence 22K GH, it is a stable and robust molecule with low antigenicity (Di Silvio et al., 1987, Pringle et al., 1989), and its effect on in-vivo bone growth have been well documented (Thorngren et al., 1973, Isgaard et al., 1986). GH is a globular polypeptide consisting of an unbranched chain of 191 amino acids, with two disulphide bridges. The disulphide bridges induce additional stability to the conformation of the molecule as determined by its primary structure, and appears to play an essential role in the maintenance of the active principle of the molecule. GH is released in a pulsatile manner, and the amount secreted changes with age (Finkelstein et al., 1972). Under normal conditions throughout the day GH levels are low, however, it is secreted in episodic bursts in response to physiological stimuli as required by the body (Drobny et al., 1983).

Table 1.1 Broad spectrum growth factors

<table>
<thead>
<tr>
<th>(1) Broad-Spectrum Growth Factors</th>
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<tbody>
<tr>
<td>Growth Hormone (GH)</td>
</tr>
<tr>
<td>Insulin-like Growth Factor (IGF, I and II)</td>
</tr>
<tr>
<td>Skeletal Growth Factor (SGF)</td>
</tr>
<tr>
<td>Epidermal Growth Factor (EGF)</td>
</tr>
<tr>
<td>Fibroblast Growth Factor (FGF) acidic and basic</td>
</tr>
<tr>
<td>Platelet derived growth factor (PDGF)</td>
</tr>
<tr>
<td>Transforming Growth Factor (TGF, acidic and basic)</td>
</tr>
<tr>
<td>Bone Derived Growth Factor (b2-microglobulin)</td>
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</tbody>
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<table>
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<tr>
<th>(2) Cell Specific Growth Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Monokines</td>
</tr>
<tr>
<td>Tumour Necrosis Factor α (TNFα)</td>
</tr>
<tr>
<td>Macrophage Growth Factor (MGF)</td>
</tr>
<tr>
<td>Interleukin-1</td>
</tr>
<tr>
<td>(b) Lymphokines</td>
</tr>
<tr>
<td>Interferon γ (IFN-γ)</td>
</tr>
<tr>
<td>Tumour Necrosis Factor β (TNF-β)</td>
</tr>
<tr>
<td>Interleukin - 1</td>
</tr>
<tr>
<td>Interleukin - 2</td>
</tr>
<tr>
<td>(c) Haematopoietic Growth Factors</td>
</tr>
<tr>
<td>Erythropoietin</td>
</tr>
<tr>
<td>Colony Stimulating Factors</td>
</tr>
</tbody>
</table>
Table 1.2 Broad spectrum bone-related growth factors can be further subdivided.

<table>
<thead>
<tr>
<th>1. Growth factors synthesized by skeletal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-b, BDGF (B2-m), IGF-I, GH and PDGF.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2. Growth factors isolated from bone matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-b1 &amp; 2, BDGF (B2-m), IGFs, PDGF, aFGF, bFGF</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3. Growth factors synthesized by cells from adjoining tissues cartilage and bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH, IGF-I, bFGF</td>
</tr>
</tbody>
</table>

Human GH in plasma is heterogenous with respect to molecular size and charge and different size variants have been characterized as an oligomeric series of GH monomers (Stolar et al., 1984). The two major variants are; 22K which constitutes approximately 95% of the circulating GH and 20K which makes up the remaining 5%. Under normal physiological conditions approximately 50% of the 22K and 29% of the 20K are found complexed to binding proteins (BPs) (Baumann et al., 1986, Herington et al., 1986). One of the functions attributed to these BPs is to prolong the biological half-life of GH by restricting their access to degradation sites. It has been speculated that GH-BPs may act as modulators of GH action; circulating GHBP has been shown to alter the distribution of GH and influence its clearance (Baumann et al., 1987).

GH action in-vivo is characterised by a multiplicity of effects. GH has characteristic intermediary effects on adipocytes and skeletal muscle in addition to its major effects on skeletal growth (Narici et al., 1993). GH is anabolic, promoting protein synthesis in muscle cells. Conversely, it has a catabolic effect on fat and carbohydrate metabolism, inducing lypolysis in adipocytes (Degerbland et al., 1992, Ogle et al., 1994). GH is known to play a major role in postnatal longitudinal bone growth due to its ability to stimulate precursor cells in the epiphyseal cartilage (Isaksson et al., 1987, Russell and Spencer, 1985, Syftestad and Urist, 1980, Maor et al., 1989). GH is a major regulator of skeletal growth and has been shown to stimulate longitudinal bone growth in a dose-dependent manner (Thomgren et al., 1973, and Isgaard 1986). Isaksson and co-workers (1982) reported that unilateral injections of human GH into tibial epiphysis stimulated unilateral bone growth, this was subsequently confirmed by Russell and Spencer (1985) who demonstrated that GH and IGF-I, have direct growth promoting effect on cartilage in vivo; this is compatible with the Green (1985) dual effector theory which states that GH acts by stimulating IGF-I synthesis. Nilsson et al., (1988) demonstrated a direct effect of GH on IGF-containing cells in the rat growth plate. It is well established that GH release is markedly pulsatile and Isgaard et al., (1988) investigated the effect of pulsatile intravenous
GH on hypophysectomized rats. Their study showed that pulsatile GH treatment induced IGF-I mRNA more effectively than continuous GH in skeletal muscle and rib growth plate, but not in the liver.

There is now mounting evidence to suggest that the relevance of GH and IGFs to skeletal physiology goes beyond the well established control of longitudinal growth, to the level of bone turnover and metabolism in the adult (Harris et al., 1972, Nijweidi et al., 1986, Ohlsson et al., 1992). Brixen et al., (1992) have shown that GH may cause an enhancement in bone mass. Recent clinical data has confirmed that GH therapy may activate osteoblasts and bone remodelling in human volunteers and elderly males (Marcus et al., 1990, Strollo et al., 1992, Sartorio et al., 1993). Brixen et al., (1990) investigated the effect of a short course of GH on collagen production in a group of normal human volunteers; the study demonstrated that GH was able to stimulate the production of collagen type I and III.

The growth stimulating effect of GH in man is best shown in GH-deficient patients who have been treated with GH for a sufficient period (Sartorio et al., 1991, Nielsen et al., 1991, Saggese et al., 1993, Zamboni et al., 1993, Sidenius et al., 1990). The mechanism of GH-dependent protein synthesis has been investigated in-vitro and has been found to be cyclic AMP-dependent. Intracellular transport of amino acids is increased, as is messenger ribonucleic acid (mRNA) and protein synthesis. GH acts as do all peptide hormones, by binding to surface membrane receptors of target cells and activating adenylate cyclase systems. The intermediate metabolic effects of GH are probably mediated directly through its own receptors. However, the growth promoting and cell differentiation effects are likely to be mediated through production of the IGF's and possibly other as yet undetermined factors (Foster et al., 1988, Tiong et al., 1989, Carlson et al., 1991, Waters et al., 1990, Strous et al., 1994).

Insulin-like Growth Factor (IGF-I)

IGF-I, a protein of 70 amino acids, plays an important role in post-natal growth and IGF-II, a 67 amino acid is involved in foetal development. IGFs do not arise from a single organ source but are secreted by most tissues in response to GH stimulation and circulate in plasma bound to high affinity binding proteins. Animal studies suggest that the liver is the greatest source of total serum IGF activity. This led to Daughaday's original hypothesis that growth promoting effects of GH were mediated via stimulation of the liver to secrete the IGFs which in turn were carried peripherally to exert their effects on cartilage and extraskeletal cell proliferation (Daughaday 1966).
Chapter 1

There is now substantial evidence however, that strongly favours an autocrine or paracrine role, rather than the classical endocrine concept. IGF-1 levels increase in extrahepatic tissues in response to GH stimulation. IGF-I availability and bioactivity are determined by IGF-binding proteins (IGFBPs), six of which have been identified in plasma and other biological fluids and in the conditioned medium of cultured cells (Baxter and Martin, 1989, Hassanger et al., 1992). The precise biological function of IGFBPs are unknown, but they are being recognized as modulators of IGF actions in both inhibitory and stimulatory ways, and their purification and characterization has been described by Baxter and Martin (1989). Determination of the physiological significance of IGF-regulated IGFBP availability will be important to our overall understanding of IGFs in bone metabolism and growth.

Specific receptors for the IGFs have been identified. The Type I' receptor is similar to the insulin receptor and binds IGF-I preferentially but will also bind IGF-II and insulin with lower affinity. The Type-II' receptor is structurally very different, binding IGF-II preferentially, IGF-I with low affinity and does not bind insulin at all. IGF-I is predominantly responsible for the growth promoting effects of GH, whereas IGF-II is a potential autocrine/paracrine regulator of local bone cell metabolism and may mediate other metabolic actions of GH (Mohan et al., 1989).

Summary

Bone formation is a complex process regulated by systemic and local growth factors. It has become increasingly apparent that all the bone-associated GFs have mitogenic activity for cells of the osteoblastic lineage. In addition, some of the GFs have stimulatory effects on bone matrix synthesis, for example IGF-I. Growth factors may therefore increase directly or indirectly bone matrix synthesis, by increasing the number of collagen secreting cells. Bone growth factors possess unique properties for the stimulation and differentiation of bone cells. The behaviour and response of primary human osteoblast cells to GH and IGF-I will be compared with a commercial human osteosarcoma cell line (TE85), this will be discussed in detail in Chapters 2 and 3. Enhancing the basic understanding of the mechanism of action of GH on bone development will allow better control of potential incorporated osteogenic promotors in orthopaedics to stimulate the regeneration and repair of damaged tissue.

Aims

The aims of this thesis were; (1) the development of a culture system for primary human osteoblasts suitable for studying the effects of GH, (2) to investigate the novel application of two biomaterials, Gelatin a degradable material in the form of microspheres and as a
coating, and (3) PEM/THFMA a non-degradable material, for use as drug delivery systems for the release of GH, (4) to optimize their ability to release GH in a biologically active form in a controlled manner, by exploiting various properties of the materials.

This chapter is intended as a general introduction and as a background to the work undertaken. Each subsequent chapter contains its own relevant introduction in more detail, materials and methods and discussion of results.
CHAPTER 2

Development of Cell Culture Model
and the
Characterization of Primary Human Osteoblasts
Local cellular activity at the implant site plays a critical role in determining the fate of the implant and the time taken for tissue and wound to repair. Knowledge of events occurring at the cellular level which induce osteoblast interactions, for example, adhesion and early expression of phenotype will aid in the design of improved orthopaedic biomaterials for use as implants or as local drug-delivery systems. Cell culture provides a useful model to investigate the interaction between cells and their environment, for example, their response to stimulatory factors and at the interface with biomaterials. The cellular events at the implant site have not been addressed specifically to date, primarily due to the lack of availability of well characterized cell lines and also some limitations with cell culture techniques.

In order to investigate the effect of human growth hormone (GH) on the proliferation and differentiation of primary human osteoblasts (HOBS), it was necessary to develop a tissue culture system for primary non-transformed human osteoblast-like cells. By using a well-characterized in-vitro osteoblast model a better understanding of the local effects of GH on osteoblast function can be attained, so that biomaterials can be designed to promote and sustain an enhanced bone repair at the implant interface (Bizios, 1994). Numerous in-vitro models using human, animal, non-transformed and transformed osteoblastic cells have been described for studying the biology of bone cells with primary cultures of osteoblast-like cells being favoured for studies of bone cell metabolism and differentiation (Harris et al., 1995, McAllister 1971, Aubin et al., 1982, Sudo et al., 1983). Osteogenic cell subpopulations derived from colonies of bone marrow stromal cells have also been described, and Friedenstein (1976) showed that cells grown from bone marrow formed fibroblastic colonies which, when implanted in diffusion chambers in-vivo formed calcified tissue that resembled bone. Owen and Friedenstein (1988) proposed a hypothesis for differentiation of the marrow stromal compartment analogous to that in the haemopoietic systems, where stromal cell lines give rise to committed progenitors for different cell lines (Fried et al., 1993, Haynesworth et al., 1992). More recently, Gundel et al., (1995) reported bone formation under specific culture conditions in diffusion chambers with cultured human bone-derived cells and cultured marrow stromal cells.

Understanding of the mineralization process in lamella bone and the factors controlling it, has developed slowly, because of the difficulty in obtaining and maintaining a sufficient number of primary cells. Furthermore, studies describing primary cells frequently use foetal bone; the nature of the foetal cell in its transformed state, the stage of maturation and responsiveness will give rise to different growth characteristics from adult bone (Chiba et al., 1993, Riccio et al., 1994, Harris et al., 1995). Most mineralization studies have used osteoblastic cells derived from human and rodent bone tissue; in the majority of cases these have been derived from embryonic or neonatal bone or marrow (Rodan et al., 1983, Nefussi et al., 1989, Robey and Termine 1985, Harris et al., 1995, ). Transformed cell
lines which express osteoblastic phenotype, have the advantage of being maintained in permanent culture and provide a good source of cells for biochemical studies (Rodan and Rodan, 1983). It has been reported that a prerequisite of osteoblast mineralisation is the formation of multi-layered cultures (Bellows et al., 1986), and various culture methods have been described which involve the growth of osteoblastic cells as compact cell colonies, and calcification in-vitro, including suspension of cells in agarose, methylcellulose and collagen type-1 (Nishimoto et al., 1987, Sudo et al., 1986). Shima et al., (1988), described cultures of osteoblast-like cells in the presence of microcarrier beads as a means of facilitating mineralization of MC3T3-E1 cells. Ahrens et al. (1977), used a micromass culture technique; the rationale for using compact cell colonies for culturing osteoblastic cells is that a three-dimensional network of collagen is required to provide a framework for the necessary spatial arrangement for the cells. Casser-Bette et al. (1990) proposed that a three-dimensional structure was a prerequisite for calcification in the osteogenic mouse calvaria cell line, MC3T3-E1. In comparison, Sudo et al., (1983) cultured these cells as monolayers and were able to show differentiation and calcification of spontaneously formed nodules and the ability to deposit hydroxyapatite in well-developed bone matrix. Kodama and co-workers (1986), who established the MC3T3-E1 osteogenic cell line, have demonstrated that culture conditions contribute significantly in the ability of these cells to differentiate and calcify; whilst β-glycerophosphate accelerated mineral deposition in MC3T3-E1 in cell cultures, it did not have any effect on their growth and differentiation into osteoblasts.

Aubin et al. (1982), in their study, isolated clones of non-transformed hormone-sensitive cells from a mixed cell population of foetal rat calvaria. Whilst they were able to show that the clones were PTH- responsive, they did not make reference to the ability of the clones to mineralize in-vitro. In other studies reported, the addition of agents such as calcium β-glycerophosphate, sodium β-glycerophosphate, calcium hexose monophosphate and dexamethasone have been used to promote mineralization of osteoblasts in culture (Koshihara et al., 1989, Casser-Bette et al., 1990, Robey and Termine 1985). Bellows and co-workers (1986,1987) showed that glucocorticoids were able to stimulate the formation of bone nodules in long term cultures in isolated rat calvaria cells. In another study, Gerstenfeld et al.,(1987) compared mineralization of chicken osteoblasts in the presence or absence of β-glycerophosphate. They reported that treated cultures showed visible calcification at day 12, when monolayers became confluent, and by day 30, a 20-fold increase in calcium content was observed. In contrast the untreated cultures had only a 3-fold increase in calcium content. However, DNA, RNA and total protein content showed little difference, indicating that β-glycerophosphate had no marked effect on either cell proliferation or transcriptional activity, this was in agreement with the findings of Kodama et al.,(1986).
The numerous studies described in the literature indicate that the cell-lines described are valuable models; however, they are not, always representative of the osteoblastic phenotype. The differences observed could be due to the developmental stage of the cell when the transformation occurred, or else it may arise from dedifferentiation in culture. Osteosarcoma cells, for example, have an inherent problem with regard to their phenotype, and any possible contribution of the transformed phenotype to a measured response must be taken into consideration. Also, clonal cell lines may express certain receptors in culture which are not normally expressed in-vivo (Clover and Gowen 1994, Ng et al., 1983). This is of particular relevance in studies investigating the effect of growth factors, since transformed cells may respond differently to normal cells and may produce different growth regulators. In addition to heterogeneity between clones, phenotypic variations can exist between progeny cells within a single clone (Wong 1990). These differences may occur as a result of the cells being exposed to different growth conditions at different stages in the cell cycle. A high or low cell density, or the presence or absence of mineralizing agents such as β-glycerophosphate, can also cause the differences observed. At a high density, for example, it is believed that osteoblastic expression is increased, whereas at low density, these cell markers are reduced (Gerstenfeld et al., 1987, Rodan and Majeska 1983). In general, however, these cells exhibit several osteoblastic properties and characteristics, such as high ALP, response to PTH and 1,25(OH)2D3, production of osteocalcin and collagen type I and in some cases in-vitro mineralization (Ecarot-Charrier et al., 1983, Lomri et al., 1988, Nefussi et al., 1989, Sudo et al., 1983).

Commonly used cell lines are usually rat, mouse or human osteosarcoma and include; UMR 106, ROS 17-2.8, SaOS-U-2OS, MC3 T3-E1, MG-63, HOS TE-85. Whilst these models have greatly contributed to the understanding of osteoblastic function, they each have their limitations with regard to studying human osteoblast biology. Differences have been noted even within species (Ng et al., 1983) and transformed and non-transformed cells (Ikeda et al., 1992). These cells are therefore not analogous to adult human bone cells and they have been shown to express different receptors in-vitro. Clover and Gowen (1994) showed that two different human osteosarcoma cell lines had different proliferation rates and expressed different levels of ALP and only the MG-63 cells increased ALP activity and osteocalcin in response to 1,25(OH)2D3. Their findings whilst interesting, indicate the diversity of clonal cell lines, and that caution should be applied when using them as experimental models to study osteoblast function.

It has been known for some time that cells will grow from explants of normal adult bone in culture (Gallagher et al., 1985, Beresford et al., 1983, Jones and Boyde, 1977). The ability of osteoblasts to migrate from bone explants from various species has been reported by many workers, and some of the methods have been adapted for human bone (Beresford et al., 1983, Ecarot Charier et al., 1983, Robey and Termine 1985). The cells appear to
be primarily osteoblastic (Aufmkolk et al., 1985) although it has proved difficult to isolate a homogenous primary cell population that is able to retain its characteristic phenotype in-vitro. The behaviour of these cells is dependent on the stage of phenotypic maturation; Majeska and Rodan (1982) examined the effects of 1,25(OH)\(_2\)D\(_3\) on alkaline phosphatase activity, cell growth and cell protein in 'pre-osteoblastic' and 'mature osteoblastic' cultures and found that the results were different depending on the stage of osteoblastic maturation. Although primary cultures derived from normal bone have an osteoblastic phenotype, they have the disadvantage that they proliferate at a very slow rate and become senescent after a short time in culture. To overcome these problems several methods have been described for transforming human osteoblasts (Marie et al., 1989, Harris et al., 1995, Chiba et al., 1993). Whilst these models offer an immortal cell population, they have some drawbacks; the methods use human foetal osteoblasts making it difficult to extrapolate the effects of osteotropic agents, firstly, because the cells possess abnormal growth characteristic in view of their transformed state and secondly, because the state of maturation and responsiveness of the foetal cells is not comparable to adult human bone cells.

An ideal system should enable the use of long term culture of cells in-vitro without 'dedifferentiation' and under the correct conditions it is possible to develop cell culture system which favours the expression of differentiated phenotypes (Wong 1990). The progressive loss of expression of phenotype is a major problem with clonal lines; 'dedifferentiation' in-vitro has caused much discussion with regard to stability of the differentiated cells. There are numerous factors that may contribute to the development of osteoblast phenotype and one of these may be the response to cell shape. Cell shape leads to intracellular remodelling of nuclear matrix, influencing changes in gene expression. The acquisition of normal osteogenic cell morphology may be a prerequisite for their development and maturation (Jones and Boyde, 1976, Newman and Watt, 1988).

The objective of the study described in this chapter was to consider some of the limitations of the systems described in the literature and develop an optimized well-defined primary human osteoblast (HOB) cell model. The aim was to produce enough cells, which retained their osteoblast phenotype, to investigate the effect of growth hormone and other growth factors on bone cell function. The behaviour of the primary cells was compared to a human osteosarcoma clonal cell line (McAllister et al., 1971), in order to eliminate species differences. In addition, in view of the numerous and diverse culture conditions described in the literature for mineralization to occur, it was necessary to determine which of the culture conditions mentioned above best favoured proliferation and differentiation for the cells described in this study. Both cell types were maintained in micromass and monolayer cultures to establish which method gave a more predictable pattern of differentiation. Cell growth and matrix production were assessed using biochemical, light and electron microscopy techniques for the two models used.
Chapter 2

Materials and Method

The methods described in this chapter have been used in work described in subsequent chapters and reference will be made to these; other, more specific methods, will be described in the appropriate chapter.

Isolation of primary human osteoblasts (HOBs)

Human bone cells were isolated from femoral heads obtained from patients undergoing surgery for total joint replacement (Figure 2.1). Trabecular bone fragments were dissected under sterile conditions, cut into very small pieces (2-3mm), thoroughly washed in calcium and magnesium-free phosphate buffered saline solution (PBS) and then given a final wash in culture medium. The washing steps are important for the removal of any blood or bone debris and the surface layer of cells, and also to expose the trabecular surfaces of the bone fragments. These were cultured in complete Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% foetal calf serum, non-essential amino acids (1%), ascorbic acid (150\mu g/ml), L-glutamine (0.02M), HEPES (0.01M), penicillin (100units/ml) and streptomycin (100\mu g/ml), at 37°C and 5% carbon dioxide in a humidified atmosphere. The addition of \( \beta \)-glycerophosphate to the culture medium was tested and shown to have no significant effect on cell mineralization and was therefore omitted from the culture medium in all subsequent experiments.

A schematic representation of the method used to isolate the HOB cells can be seen in Figure 2.2. The bone fragments were left in culture medium for a period of 4-5 days, with one or two medium changes during this time. This pre-digestion explant incubation period is important as it allows the migration and removal of marrow cells, blood cells and other non-adherent cells. During this period cells were seen to migrate from the bone and form 'seams' around the bone fragments. This method appears to select for cells with the highest proliferation capacity, and cell survival may be enhanced by close contact between the migrating cells. The bone fragments were then digested using a mixture of 0.01M HEPES, collagenase (100U/ml), and trypsin (0.02%), (Sigma, England) in PBS; fragments were incubated for precisely 20 minutes at 37°C on a rotating mixer in this solution in order to allow the release of bone cells from within the bone fragments. Following digestion the 'osteoblast-rich' supernatant was transferred to a sterile universal and centrifuged at 2,000 rpm (700g) for 10 minutes at 4°C to pellet the cells. The cells were washed several times and re-centrifuged in complete DMEM to remove all traces of the digestion medium. The cell pellet was resuspended in a small volume of medium and a cell count was performed using trypan blue exclusion dye with a haemocytometer, the cells were then seeded in the appropriate density for the various experiments.
Normal femoral head demonstrating the source of bone explant taken for the isolation of osteoblast-like cells. Trabecular bone fragments (as indicated by arrows) are removed and cut into small pieces and are thoroughly washed prior to culture in DMEM (10% FCS).
Chapter 2

Preparation of Primary Bone Cells

Femoral head

Trabecular - bone chips
x 3 wash in PBS
x 3 wash in DMEM

Bone chips cultured in DMEM (between 4 - 5 days) until osteoid seams are visible and cells are seen migrating out

Osteoblast-like cells in culture
DMEM (10% FCS)

Figure 2.2 A schematic representation of the steps involved in the preparation of primary osteoblasts from the femoral head.
Human osteosarcoma cell line (HOS)

The human osteosarcoma cell line TE85 (HOS, ECACC No 87070202) was used as a comparison. This cell line exhibits specific osteoblastic characteristics (McAllister et al., 1971). These cells were cultured under the same conditions as the HOB cells. For the biochemical analysis, the HOB and HOS cells were plated out onto culture dishes (35 x 10mm) at a cell density of 2.5 x 10^5 cells/dish in 1 ml complete DMEM containing 10% FCS. For monolayer culture (m), the cells were plated with a 10μl aliquot and flooded immediately with 1 ml DMEM. For micromass culture (mm), the cells were plated with a 10μl droplet; the dishes were then placed at 37°C for a minimum period of 2 hours, after which they were very carefully flooded with 1 ml DMEM. The culture medium was changed every 3 days. At each of the sample collection point, the medium was retained for biochemical analysis and to each culture dish 1ml of sterile distilled water was added. The cells underwent three cycles of freezing at -70°C for 20 minutes followed by thawing at 37°C for 15 minutes, and the cell lysate was then retained and frozen at -20°C for biochemical analyses.

The following analyses were performed: cell viability counts with Trypan blue exclusion dye, DNA, alkaline phosphatase (histochemical staining and biochemical measurement), osteocalcin and procollagen type 1 (PICP) and response to PTH by measuring cyclic adenosine monophosphate (cAMP) production.

Tritiated Thymidine labelling[^3H]-TdR

DNA synthesis was assessed using[^3H]-TdR, which is selectively incorporated into the cell nucleus during the S-phase of the cell cycle. There are a number of potential artifacts in relating thymidine uptake to deoxyribonucleic acid (DNA) synthesis (Maurer 1981). Although most DNA synthesis occurs during cell replication, not all the DNA detected by[^3H]-TdR incorporation reflects proliferation. DNA is constantly being restored to maintain its integrity in cases where it has been damaged.[^3H]-TdR incorporation into HOB and HOS DNA was assessed as follows; cell cultures were incubated in 24 well plates (30,000 cells per well) in complete DMEM until they had reached confluency. The cells were then cultured in serum-free DMEM for 16 hours before the addition of fresh control or test medium in order to arrest cell growth. The cells were incubated in the presence of 1μCi/ml of[^3H]-TdR (Amersham, International plc, England) for 24 hours. The cells cultures were washed 4 times in DMEM containing cold thymidine (5.0μg/ml) in order to reduce non-specific binding. Following this, the cells were digested using a papain digest solution, containing 1ml papain suspension (type III, Sigma, Poole, England) in 1ml PBS supplemented with 5 mM cysteine hydrochloride (Merck, UK) and 5 mM ethylenediaminetetraacetic acid (EDTA, Merck, UK) at pH 5.7 for 24 hours at 60°C.
overnight. 100µl of the digest was transferred to a scintillation vial and the amount of radiolabel incorporated was measured on a scintillation counter.

**Measurement of total DNA**

The DNA content of the cells was measured using a modification of the method of Kapuscinski and Skoczylas (1977). A 20ml aliquot of papain digested cells was added to 480ml 10mM NaCl, followed by 300ml distilled water, 100ml Tris (40mM, pH 7.4) and 100ml of a 2mM 4',6-Diaminodino-2-phenylindole.2HCl (DAPI; Sigma, England) solution. After thorough mixing the samples were read in a LS2B fluorimeter (Perkin-Elmer, UK) at a wavelength of 460nm. The standards contained 12.5, 25, 50, 250 ng/ml DNA (Calf thymus, Type 1 from Sigma, UK).

**Methods used for HOB Characterization**

**Biochemical measurement of Alkaline Phosphatase**

The alkaline phosphatase activity in the cell lysate and the medium was determined using a COBAS-BIO (Roche, UK) centrifugal analyser. The assay measures the release of p-nitrophenol from p-nitrophenol phosphate (PNPP) at 37°C in buffer containing 1M Diethanolamine (DEA), 10mM PNPP and 0.5mM MgCl₂ and 0.22M NaCl, pH 9.8 (Merck, UK).

**Histochemical detection of Alkaline Phosphatase using Light Microscopy**

Histochemical detection of alkaline phosphatase was performed using a modification of the method described by Stutte H.J (1967). The incubation mixture consisted of 4% New Fuchsin in 2M HCl plus an equal volume of 4% sodium nitrite which were shaken together and then added to 40ml 20mM Tris, pH 9.0. To this was added 10mg of the substrate Napthol AS-BI-phosphate, sodium salt (Sigma, England): the solution was mixed and added to the dishes or coated onto the slides as appropriate and they were incubated for 30 minutes at 37°C. The medium was discarded from the culture dishes and the cell layer was washed with PBS, and then counterstained with methyl green for 2 minutes followed by a further wash in water; the dish was then drained and mounted in an aqueous mountant Aquamount (Merck, UK).

Sections were incubated for 15 minutes at 37°C, then the incubation mixture was washed off with distilled water, the sections dehydrated through alcohols to xylene and mounted in DPX (distrene/tricresyl phosphate plasticizer and xylene, Merck, UK) and examined under the light microscope.
Histochemical detection of Alkaline Phosphatase activity using Electron Microscopy

An in-house modification (Rees and Ali, 1988) of the lead nitrate method (Lewinson et al., 1982) was used to localize ALP activity. Cells were fixed in their culture dishes with 1.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) at 4°C for 1 hour, followed by a further 30 minutes in 0.1M sodium cacodylate buffer only. The incubation medium was made up from the stock solutions: 0.045M sodium β-glycerophosphate, 0.025M magnesium chloride, and 0.018M lead nitrate. These were aliquoted in 2ml volumes and stored separately at -20°C. Tris(hydroxymethyl) Methylamine/HCl buffer at pH 9 was prepared fresh each time (all the chemicals were from Merck, UK). The working solution of 10ml was made up from the stock as follows: 2ml distilled water, 2ml 0.2M Tris/HCl (pH 9.0), 2ml 0.045M sodium β-glycerophosphate, 2ml 0.025M magnesium chloride and 2ml 0.018M lead nitrate was added in this order. To test for false non-enzymatic deposition of reaction product the control sample was incubated in a 2mM levamisole hydrochloride (Aldrich, UK) solution as an inhibitor of alkaline phosphatase activity. The incubation medium of 40mM Tris/HCl, 9mM sodium β-glycerophosphate, 5mM magnesium chloride and 3.6mM lead nitrate was added to the culture dishes, incubated for 30 minutes at 37°C, washed briefly in 0.1M sodium cacodylate buffer (pH 7.4), post-fixed for 30 minutes in 1% osmium tetroxide in 0.1M sodium cacodylate buffer and washed in several changes of buffer. The cells in the culture dish were dehydrated through a graded series of ethyl alcohol starting from 70% to 100% and then released from the tissue culture plastic by a brief soaking in propylene oxide. They were then placed into fresh propylene oxide for 5 minutes and infiltrated with 1:1 Spurrs' resin/propylene mixture for 1 hour followed by 2 changes in pure resin. The cell sheet was chopped finely, placed into an eppendorf tube with fresh resin, centrifuged and cured for 18 hours at 70°C. Sections for electron microscopy were cut on an LKB Ultratome III, picked up onto copper grids and viewed unstained on a Philips CM12 electron microscope.

Measurement of Procollagen Type 1 (PICP)

PICP was measured using a radioimmunoassay (Orion Diagnostica, Pharmacia UK) for the in-vitro measurement of carboxyterminal propeptide of type I procollagen (PICP). Cross-reactivity with the carboxyterminal propeptide of type III procollagen has been minimized in this kit by purification of the antigen and selection of antiserum. Assay conditions were modified for the purpose of this study by increasing the first antibody incubation period to 24 hours at 4°C in order to increase assay sensitivity.
Measurement of Osteocalcin

Osteocalcin was determined by a competitive radioimmunoassay (OSCAtest Henning, Berlin) using an antibody coated-tube technique and \(^{125}\)I-labelled osteocalcin. Osteocalcin release into the medium was determined, with basal medium as a control. The antibody used was raised against intact human osteocalcin. The assay was validated for tissue culture medium prior to use.

Measurement of cyclic AMP (cAMP)

cAMP production was measured in HOBS treated with PTH \((10^{-8} \text{ M})\) for 24 hours on days 3, 7, 14 of culture. (PTH- bovine 77/533 from NIBSC). Intracellular cAMP was measured using the BIOTRAK kit (Amersham International, Amersham, UK) and adopting the non-acetylation method, suitable for culture medium.

All statistics were performed using Statview software package (Apple Computer, USA). Paired Student's t-test was used to compare the biochemical findings for the monolayer versus micromass culture methods.

Results

Primary cells

Cell migration was observed in over 80% of the bone fragments after 4-5 days of the initial incubation period, with seams formed around the fragments. The morphology of the cells in culture was that exhibited by mature osteoblasts, and, five days post-seeding, the primary culture of enzymatically isolated cells had a polygonal morphology, with the cells becoming cuboidal on reaching confluency between 12-14 days (Figures 2.3a-f). Once confluent, the cells formed multilayers and, by approximately day 21, nodular-type structures were observed (Figures 2.3g- 2.3l). By day 14 calcium deposits were detected using the Alizarin red stain. The biochemical results reported in this chapter are up to day 14 of culture, though the cells have been successfully maintained in culture for over 30 days. The HOB cells grew well with confluency being achieved usually within 4 days. Light and Electron microscopy indicated fine healthy ultrastructural features even after numerous passages. The cells were rounded with large nuclei, numerous golgi bodies and endoplasmic reticulum (Figure 2.4a). The cells showed obvious signs of proliferation and various stages of mitosis can be seen in Figures 2.4b and 2.4c.
Osteoblast characterization

The method described in this chapter using human bone explants resulted in a more selective separation of adherent lining bone cells from the non-adherent cells. The preparation procedure and the inclusion of a pre-digestion incubation period reduced contamination with marrow cells and allowed the cultivation of a predominantly osteoblastic cell population. Following subsequent digestion, the seeded HOBS rapidly attained cuboidal morphological appearance characteristic of osteoblasts. The cells migrating from the bone fragment showed a very low rate of cell proliferation in comparison to the 'osteoblast-rich' cells that were obtained following digestion. Under normal conditions the HOBS displayed a logarithmic growth pattern, which is dependent on cell number. They had a doubling time of approximately 36 hours. The isolation procedure described resulted in the isolation of a mature osteoblast primary cell population. If digestion was stopped at 10-15 minutes, mainly fibroblastic cells were obtained. If the digestion time was increased the cells were damaged and grew very slowly did not respond as well when stimulated. It was found that a digestion period of 20 minutes yielded a reproducible cell population highly rich in osteoblasts. These cells have been tested by measuring cell viability, doubling time and phenotypic markers at various passage stages after storage in liquid nitrogen and have maintained their osteoblastic phenotype up to passage 19.

Optimization of culture method

The effect of different culture methods on the relationship between cell growth and differentiation was examined and significant differences were observed in both cell growth regulation and differentiation. The results of these findings are given below.

Cell Counts

A plot of viable cell counts for HOB cells, determined by trypan blue exclusion can be seen in Figure 2.5; the results are expressed as percentage increase compared to the control cell number at time 0. It is evident that a greater number of cells were present in the monolayer compared to the micromass culture system. Results obtained for the HOS cell counts were comparable to the HOBS (Figure 2.6); the HOS cells however, showed a greater proliferation capacity than the HOB cells in monolayer culture. Absolute cell counts for the time points studied are given in Table 2.1. These results indicate that there is no advantage in using a micromass culture method, and that the higher cell numbers seen in the HOS cells are a result of the transformed state of the cells.
Figure 2.3

Figure 2.3a Light micrograph of HOB cells (arrow) migrating from bone fragment (B) after 3 days in culture prior to enzymatic digestion. Magnification x 218

Figure 2.3b Light micrograph of HOB cells migrating from bone fragment (B) after 5 days in culture prior to enzymatic digestion. Note the 'osteoid seam' beginning to form (OS). Magnification x 218

Figure 2.3c Light micrograph of HOB cells 5 days post enzymatic treatment of bone fragments; cells are already beginning to attain a cuboidal morphology. Magnification x 218

Figure 2.3d Light micrograph of HOB cells at 12-days in culture; the cells have formed a confluent monolayer and have a cuboidal morphology. Magnification x 218

Figure 2.3e Light micrograph of HOB cells at 14-days in culture. The cells are confluent and densely packed with a cuboidal morphology. Magnification x 218

Figure 2.3f Light micrograph of HOB cells at 21-days in culture. Note the densely packed cells beginning to form multilayers (arrow). Magnification x 218
Chapter 2

Figure 2.3

Figure 2.3g Light micrograph of HOB cells in monolayer at 24-days in culture. Note the densely packed cells and the appearance of 'nodular-like' structures.
Magnification x 218

Figure 2.3h Light micrograph of HOB cells after 14-days in culture stained with Mayers's Haematoxylin and Eosin. The cells have attained a characteristic cuboidal morphology.
Magnification x 218

Figure 2.3i Light micrograph of HOB cells in monolayer culture at 24 days. The cells have formed layers, with large nodular formation.
Magnification x 218

Figure 2.3j Light micrograph of HOB cells after 14-days in culture stained with Toluidine Blue. The cells had a cuboidal morphology with distinct nuclei visible.
Magnification x 218

Figure 2.3k Light micrograph of HOB cells in monolayer after 28 days in culture. Note the densely packed cells, with areas beginning to mineralize.
Magnification x 218

Figure 2.3l Light micrograph of HOB cells after 14 days in culture stained with Alizarin Red to show localization of calcium deposits
Magnification x 218
Figure 2.4

Figure 2.4a Electron micrograph to show fine ultrastructure of HOB cell: these cells are characteristic of those cells examined from various passage levels. The cells had a rounded contour, with a large nucleus with two prominent condensed nucleoli (N) and endoplasmic reticulum (ER) were also present in the cytoplasm.

Figure 2.4b - Electron micrograph to show ultrastructure of HOB cell in the anaphase stage of mitosis, the sister chromatids (arrow) have separated, and the two groups of chromosomes are moving towards opposite poles of the spindle (arrow-heads).
Figure 2.4

Figure 2.4c Electron micrograph to show ultrastructure of HOB cell in the telophase stage of mitosis, new membranes are forming around the daughter nuclei (arrow), and the chromosomes (Cr) have uncoiled and become less distinct, formation of the cell wall can be seen as the cells complete their separation.
Figure 2.3: Graph representing the percentage increase in cell number as compared to the control cell number at time 0 (2.3 x 10^5 cells/mL) for cells cultured in medium and 115 monocytes. The cells were maintained in complete DMEM (115 FCS), with a medium change every three days. The vector plotted represents the mean of two cultures ± SD for each time point. Cox regression was employed using output data that indicates the following: statistical analysis of the cell data showed a significant difference in cell number for the 115 monocytes compared to the untreated culture system. "Amorat (*)" denotes significance, p < 0.05 = NS for day 6,
p < 0.05 = NS for day 8,
p < 0.05 = NS for day 10,
p < 0.05 = NS for day 12,
p < 0.02 = NS for day 14.
Figure 2.5 Graph representing the percentage increase in HOB cell number as compared to the control cell number at time 0 (2.5 x 10^5 cells/well) for cells cultured in micromass and in monolayer. The cells were maintained in complete DMEM (10% FCS), with a medium change every three days. The values plotted represent the mean of two cultures ± SD for each time point. Cell viability was determined using trypan blue exclusion dye, following trypsinization of the cells. Paired Student's t-test values indicate a significant difference in cell number for the HOB monolayer compared to the micromass culture system. Asterisk (*) denotes significance.

- p > 0.05 = NS for day 4
- p > 0.05 = NS for day 6
- p < 0.05 = day 8
- p < 0.025 = day 10
- p < 0.025 = day 12
- P<0.025 = day 14
Figure 2.6 Graph representing the percentage increase in HOS cell number as compared to the control cell number at time 0, \((2.5 \times 10^5 \text{ cells/well})\) for cells cultured in micromass and in monolayer. The cells were maintained in complete DMEM (10% FCS), with a medium change every three days. The values plotted represent the mean of two cultures \(\pm\) SD for each time point. Cell viability was determined using trypan blue exclusion dye, following trypsinization of the cells. Paired Student's t-test values indicate a significant difference in cell number for the HOS monolayer and the micromass culture system. Asterisk (*) denotes significance.

\[P > 0.05 = \text{NS days 4}\]
\[P > 0.05 = \text{NS days 6}\]
\[P < 0.025 = \text{days 8}\]
\[P < 0.025 = \text{days 10}\]
\[P < 0.05 = \text{days 12, 14}\]
Table 2.1 Comparison of cell counts for the HOB and HOS cell monolayer (m) and micromass (mm) culture system.

<table>
<thead>
<tr>
<th></th>
<th>HOB m</th>
<th>HOB mm</th>
<th>HOS m</th>
<th>HOS mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deep4</td>
<td>3.3 x 10^5 ± 10^3</td>
<td>2.5 x 10^5 ± 10^3</td>
<td>5.1 x 10^5 ± 1.1 x 10^4</td>
<td>2.8 x 10^5 ± 2 x 10^4</td>
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<tr>
<td>Day 6</td>
<td>5.4 x 10^5 ± 4 x 10^4</td>
<td>2.8 x 10^5 ± 0</td>
<td>6.4 x 10^5 ± 2 x 10^4</td>
<td>3.4 x 10^5 ± 1.1 x 10^4</td>
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<td>Day 8</td>
<td>9.1 x 10^5 ± 10^3</td>
<td>3.1 x 10^5 ± 3 x 10^3</td>
<td>1.3 x 10^6 ± 9 x 10^3</td>
<td>3 x 10^5 ± 6 x 10^4</td>
</tr>
<tr>
<td>Day 10</td>
<td>1.2 x 10^6 ± 5 x 10^3</td>
<td>3.1 x 10^5 ± 10^3</td>
<td>1.8 x 10^6 ± 8 x 10^3</td>
<td>3.2 x 10^5 ± 4 x 10^4</td>
</tr>
<tr>
<td>Day 12</td>
<td>1.3 x 10^6 ± 1.3 x 10^4</td>
<td>2.8 x 10^5 ± 0</td>
<td>1.24 x 10^6 ± 1.8 x 10^4</td>
<td>3.2 x 10^5 ± 2 x 10^4</td>
</tr>
<tr>
<td>Day 14</td>
<td>9.3 x 10^5 ± 3 x 10^3</td>
<td>3.9 x 10^5 ± 5 x 10^3</td>
<td>1.6 x 10^6 ± 1.1 x 10^4</td>
<td>5.3 x 10^5 ± 2 x 10^3</td>
</tr>
<tr>
<td>Day 18</td>
<td>1.3 x 10^6 ± 7 x 10^4</td>
<td>1.2 x 10^6 ± 8 x 10^3</td>
<td>1.2 x 10^6 ± 2 x 10^4</td>
<td>1 x 10^6 ± 1.1 x 10^3</td>
</tr>
<tr>
<td>Day 21</td>
<td>1.4 x 10^6 ± 10^3</td>
<td>1.6 x 10^6 ± 0</td>
<td>2.1 x 10^6 ± 7 x 10^4</td>
<td>8.3 x 10^5 ± 3 x 10^4</td>
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<tr>
<td>Day 25</td>
<td>1.8 x 10^6 ± 2 x 10^4</td>
<td>5.6 x 10^5 ± 2 x 10^3</td>
<td>1.4 x 10^6 ± 1.5 x 10^3</td>
<td>2.5 x 10^5 ± 1.1 x 10^3</td>
</tr>
<tr>
<td>Day 28</td>
<td>1.9 x 10^6 ± 3.8 x 10^3</td>
<td>1 x 10^6 ± 0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Measurement of DNA**

DNA levels were comparable for both HOB and HOS in the two culture systems. Higher levels were detected in the monolayer compared to the micromass; they were significant on days 6, 8, 10 and 12 (P<0.05). A rapid increase was observed between days 8 and 12 for the HOB cells indicating a faster replication rate (Figure 2.7). Similar results were obtained for the HOS cells (Figure 2.8). Proliferation, as indicated by ^3H-thymidine incorporation, can be seen in Figure 2.9 for HOB and HOS cells in monolayer. A sharp rise was observed on day 4, indicating increased cell proliferation, this was followed by a rapid fall as proliferation was down-regulated and superseded by differentiation of the cells (as indicated by an increase in ALP, osteocalcin release and PICP)
Figure 2.7 Graph indicating DNA content for HOB cultures grown in monolayer and micromass. All cells were maintained in complete DMEM (10% FCS), with a medium change every three days. Cells were papain digested at the appropriate time point and assayed for DNA. The values plotted represent the mean of two cultures ± SD for each time point. Paired Student's t-test values indicate a significant difference on some days in HOB DNA content between cells grown in monolayer and in micromass as indicated below. Asterisk (*) denotes significance.

- p > 0.05 = NS  day 4
- p > 0.05 = NS  day 6
- p < 0.05 = day 8
- p < 0.025 = day 10
- p > 0.05 = NS  days 12
- P < 0.05 = day 14
Figure 2.8 Graph indicating DNA content for HOS cultures grown in monolayer and micromass. All cells were maintained in complete DMEM (10% FCS), with a medium change every three days. Cells were papain digested at the appropriate time point and assayed for DNA. The values plotted represent the mean of two cultures ± SD for each time point. Paired Student's t-test values indicate a significant difference in HOS DNA content on some days between cells grown in monolayer and in micromass as indicated below. Asterisk (*) denotes significance.

- $p > 0.05 = \text{NS day 4}$
- $p < 0.05 = \text{NS day 6}$
- $p < 0.05 = \text{day 8}$
- $p < 0.02 = \text{day 10}$
- $p < 0.05 = \text{NS day 12}$
- $p < 0.05 = \text{NS day 14}$
Figure 2.9 Proliferation of HOB and HOS cells as indicated by $^3$H-thymidine incorporation per μg DNA synthesized in the monolayer culture. The values plotted represent the mean of two cultures ± SD for each time point.

Alkaline Phosphatase

In order to identify the point at which proliferation ceases and differentiation commences ALP activity was also monitored histochemically using light and electron microscopy. The expression of a differentiated phenotype can be seen in Figure 2.10a for a 14 day HOB monolayer culture, ALP positive, red-brown staining can be seen in most of the cells. By day 21 all the HOB cells in monolayer are expressing alkaline phosphatase activity (Figure 2.10b). In contrast, in the HOS cell line, whilst red-brown positive ALP staining was observed at day 14, it was confined to some and not all the cells (figure 2.10c). By 21 days the ALP staining had become more widespread and was present in more cells, but not as diffuse as in the HOB cells (Figure 2.10d). Alizarin red positive staining was seen in 21 day culture of HOB cells, indicating deposition of calcium (Figure 2.10e). Figure 2.10f is a rabbit kidney positive control for ALP histochemical detection. dense red-brown areas were observed, indicative of strong ALP activity.
Electron microscopy was used to compare the ultrastructural localization of ALP activity for both the monolayer and micromass culture system in the HOB and HOS cells. Figure 2.11a shows the ultrastructural localization of membrane-bound ALP activity in a 12 day monolayer HOB culture. Electron dense areas of ALP activity was localized along the cell membrane. In contrast, the control section treated with levamisole hydrochloride (an inhibitor of ALP activity) showed no reactivity for ALP (Figure 2.11b). Similar findings were observed for the HOB 12 day micromass cultures, with positive activity for the test section (Figure 2.11c) and no reactivity for the control (Figure 2.11d). A positive distribution of ALP activity was seen along the cell membrane in the HOS 12 day monolayer culture system (Figure 2.11e). Sections of the control for the HOS 12 day monolayer cultures however, showed some reaction products for ALP, indicating that the levamisole had not completely inhibited ALP activity (Figure 2.11f). The HOS micromass 12 day culture cells also showed reaction product for alkaline phosphatase (Figure 2.11g) compared to the levamisole inhibited controls (Figure 2.11h), however this was not as intense as in the monolayer culture.

A qualitative increase in the distribution of localization of ALP activity in HOB monolayer cultures was seen in relation to the age of the culture. Dense electron positive ALP localization was observed as early as day 4 (Figure 2.11i), with the control showing negligible reactivity (Figure 2.11j). As the age of the culture increased, more cells were observed having positive ALP reactivity, day 7 results can be seen in Figure 2.11k, with the corresponding control seen in Figure 2.11l. By day 14, very dense positive localization of ALP reactivity was observed along the cell membrane (Figure 2.11m), with only background reactivity observed in the control (Figure 2.11n). At day 28 the distribution of ALP reactivity was intensified along the cell membrane (Figure 2.11o) and negligible reaction in the control (Figure 2.11p).
Figure 2.10

Figure 2.10a Histochemical staining for ALP in a 14-day monolayer culture of HOB cells. Magnification x 218

Figure 2.10b Histochemical staining for ALP in a 21-day monolayer culture of HOB cells. Magnification x 218

Figure 2.10c Histochemical staining for ALP in a 14-day monolayer culture of the osteosarcoma cell line HOS TE85. Magnification x 218

Figure 2.10d Histochemical staining for ALP in a 21-day monolayer culture of the osteosarcoma cell line HOS TE85. Magnification x 218

Figure 2.10e Alizarin red staining in a 21-day monolayer culture of HOB cells. Large areas stained red indicating calcium deposition. Magnification x 218

Figure 2.10f Histochemical staining for ALP in control rabbit kidney, with large areas stained dark red indicating ALP activity. Magnification x 218
Chapter 2

Figure 2.11

Figure 2.11a Electron micrograph of a 12 day monolayer culture of HOB cells. Note the electron dense positive localization of ALP along the cell membrane (arrow-heads). Section viewed unstained. Bar= 2 |μm

Figure 2.11b Electron micrograph of a 12 day monolayer culture of HOB cells, negative control. Note the absence of any positive reaction. ALP activity was blocked using levamisole hydrochloride. Section viewed unstained. Bar= 2 |μm
Figure 2.11c Electron micrograph of a 12 day micromass culture of HOB cells. Note the electron dense positive localization of ALP along the cell membrane (arrow-heads). Section viewed unstained. Bar= 1μm

Figure 2.11d Electron micrograph of a 12 day micromass culture of HOB cells, negative control. Note the absence of any positive reaction. ALP activity was blocked using levamisole hydrochloride. Section viewed unstained. Bar= 1μm
Figure 2.11

**Figure 2.11e** Electron micrograph of a 12 day monolayer culture of HOS cells, showing a positive localization for ALP activity (arrow-heads). Section viewed unstained. Bar= 2μm

**Figure 2.11f** Electron micrograph of a 12 day monolayer culture of HOS cells, negative control. Note the presence of some electron dense positive ALP activity, indicating incomplete blocking with levamisole. Section viewed unstained. Bar= 2μm
Chapter 2

Figure 2.11

Figure 2.11g Electron micrograph of a 12 day micromass culture of HOS cells, showing a positive reaction for ALP (arrow-heads)
Section viewed unstained. Bar= 2μm

Figure 2.11h Electron micrograph of a 12 day micromass culture of HOS cells, negative control following blocking with levamisole hydrochloride.
Section viewed unstained. Bar= 2μm
Chapter 2

2.11 g

2.11 h
Figure 2.11

Figure 2.11i Electron micrograph of a 4 day monolayer culture of HOB cells, showing a positive ALP activity (arrow-heads).
Section viewed unstained. Bar= 0.5 μm

Figure 2.11j Electron micrograph of a 4 day monolayer culture of HOB cells, negative control following blocking with levamisole hydrochloride.
Section viewed unstained. Bar= 0.5 μm
Figure 2.11

**Figure 2.11k** Electron micrograph of a 7 day monolayer HOB cells, showing positive ALP activity (arrow-heads). Note the more dense localization. Section viewed unstained. Bar= 2µm

**Figure 2.11l** Electron micrograph of a 7 day monolayer HOB cells, negative control following blocking with levamisole hydrochloride. Section viewed unstained. Bar= 2µm
Figure 2.11

Figure 2.11m Electron micrograph of a 14 day monolayer HOB cell culture, showing a positive ALP activity (arrow-heads). Section viewed unstained. Bar= 2µm.

Figure 2.11n Electron micrograph of a 14 day monolayer HOB cell culture, negative control following blocking with levamisole hydrochloride. Section viewed unstained. Bar= 1µm.
**Figure 2.11**

**Figure 2.11o** Electron micrograph of a 28 day monolayer HOB cells culture showing a positive reaction for ALP (arrow-heads).
Section viewed unstained. Bar= 1μm

**Figure 2.11p** Electron micrograph of a 28 day monolayer HOB cell culture, negative control following blocking with levamisole hydrochloride.
Section viewed unstained. Bar= 1μm
Biochemical

Alkaline phosphatase as a predictive marker of osteoblast phenotype and of differentiation was best confirmed by the monolayer culture system for both HOB and HOS cells. The biochemical detection of ALP was a more sensitive method for detecting its activity at the earlier time points. Table 2.2 shows that the differences observed between the monolayer and the micromass were significant from as early as day 4. The rise in biochemically detected ALP activity parallels the increase seen histochemically, at the later stages. These findings are further corroborated by the EM localization of ALP reactivity. The increase in ALP indicates that the cells had begun to differentiate; this was observed earlier in the HOB than in the HOS cells. ALP is predominantly membrane-bound and this can be seen by the high levels produced by the HOBS compared to the amount released in the medium (Figure 2.12a).

Table 2.2 Comparison of Alkaline Phosphatase production in monolayer (m) and micromass (mm) for HOB and HOS cells (IU/l)

<table>
<thead>
<tr>
<th>Days in culture</th>
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<th>HOB ALP mm</th>
<th>HOS ALP m</th>
<th>HOS ALP mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>85.6 ± 3.6</td>
<td>18.7 ± 1.6</td>
<td>48.2 ± 1.3</td>
<td>9.2 ± 2.6</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.02</td>
<td>p-value &lt;0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>151 ± 28.1</td>
<td>22.1 ± 0.1</td>
<td>48.2 ± 1.6</td>
<td>12.3 ± 0.5</td>
</tr>
<tr>
<td>p-value</td>
<td>NS</td>
<td>p-value &lt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>406.3 ± 11.5</td>
<td>9.5 ± 1.6</td>
<td>96.8 ± 7.2</td>
<td>28.5 ± 1.3</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.025</td>
<td>p-value NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>503.5 ± 2.1</td>
<td>32.7 ± 1.2</td>
<td>83.8 ± 0.8</td>
<td>28.9 ± 2.3</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.005</td>
<td>p-value &lt;0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>436 ± 20.3</td>
<td>51.7 ± 5.7</td>
<td>107.1 ± 11.7</td>
<td>37.5 ± 1.9</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.025</td>
<td>p-value NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>368.4 ± 0</td>
<td>87.3 ± 10.4</td>
<td>88.3 ± 3.9</td>
<td>72.2 ± 5.8</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.025</td>
<td>p-value NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The production of ALP by the HOBS in monolayer occurs much earlier, from day 4 onwards, with approximately a five-fold increase in ALP production on day 10; in contrast, ALP levels in the micromass cultures did not start to increase until much later (Figure 2.12b). Similar results were observed for the HOS in monolayer, but ALP levels were much lower than those observed in the HOBS (Figure 2.13a). In the HOS micromass culture the production of ALP occurred later and the levels were lower (Figure 2.13b).
In monolayer culture a much higher level of ALP production per cell was seen in the HOB than in the HOS, indicating that the HOB cells are able to express ALP at a better rate per cell as a result of a higher percentage of committed osteoblast cells being present. A plot of ALP per μg DNA for the monolayer model, indicates a rapid fall in cell proliferation coincident with a rapid increase in ALP production (Figure 2.14). ALP production by HOB cells in monolayer at different cell densities, showed similar patterns, but the amount of ALP produced was directly related to the number of cells present. These results indicate that cell number, rather than age of the culture, influences the expression of ALP in culture.

Figure 2.12a ALP activity in HOB cells cultured in monolayer, this was measured in the cell lysate and its release into the medium. For each time point the medium was retained and frozen, and the cells were subjected to a freeze-thaw cycle in order to release the cell contents, the cell lysate was frozen until assayed. Each time point represents the mean of two cultures ± SD
Figure 2.12b  ALP activity in HOB cells cultured in micromass; this was measured in the cell lysate and its release into the medium. At each time point the medium was retained and frozen, and the cells were subjected to a freeze-thaw cycle in order to release the cell contents, these were frozen until assayed. Each time point represents the mean ± SD
Figure 2.13a ALP activity in HOS cell cultured in monolayer; this was measured in the cell lysate and its release into the medium. For each time point the medium was retained and frozen, and the cells were subjected to freeze-thaw cycle in order to release the cell contents, these were frozen until assayed. Each time point represents the mean of two cultures ± SD.
Figure 2.13b ALP activity in HOS cell cultured in micromass; this was measured in the cell lysate and its release into the medium. At each time point the medium was retained and frozen, and the cells were subjected to a freeze-thaw cycle in order to release the cell contents, these were frozen until assayed. Each time point represents the mean of two cultures ± SD.
Figure 2.14 ALP production by HOB and HOS cells in monolayer culture expressed per μg of DNA. Cells were maintained in complete DMEM (10% FCS), with a medium change every 3 days. Each point is the mean ± SD.

Osteocalcin

Osteocalcin, a vitamin K-dependent, calcium-binding protein is solely synthesized by the osteoblasts and is a sensitive marker of osteoblast differentiation and mineralization. Its production seems to be dependent on the extent of mineralization, however, low levels were detectable as early as day 4 for both HOB and HOS cells in monolayer culture. A rapid release of osteocalcin into the medium was observed from the HOB cells, with a peak at day 10, reflected by much lower levels in the cell lysate (Figure 2.15).

Approximately 30% more osteocalcin, was produced and retained by the HOS cells compared to the HOBs, resulting in lower levels being released into the medium (Figure 2.16).
Figure 2.15 The production of osteocalcin by HOB cells in monolayer and its release into the medium. At each time point the medium was retained and frozen, and the cells were subjected to a freeze-thaw cycle in order to release the cell contents, these were frozen until assayed. Each time point represents the mean ± SD.

An interesting sequence of events was observed with ALP and osteocalcin, the down-regulation of proliferation was followed by a surge of ALP and osteocalcin. A plot of ALP and osteocalcin production by the HOB monolayer culture showed that the production of osteocalcin was concomitant with that of ALP and, at day 10 when ALP had peaked, the production of osteocalcin was increased (Figure 2.17).
Figure 2.16 The production of osteocalcin by HOS cells in monolayer and its release into the medium. At each time point the medium was retained and frozen, and the cells were subjected to a freeze-thaw cycle in order to release the cell contents, these were frozen until assayed. Each time point represents the mean ± SD.

The increase observed in osteocalcin production was followed by a fall in the amount released into the medium, and corresponded to the same time point, 10 days, at which ALP production peaked, indicating that this may be a signal to 'switch' on osteocalcin production (Figure 2.18).
Figure 2.17 The production of ALP and osteocalcin by HOB cells in monolayer. Cells were maintained in complete DMEM (10% FCS), with a media change every 3 days. At each time point the cells were subjected to a freeze-thaw cycle in order to release the cell contents, these were frozen until assayed. Each time point represents the mean ± SD.

Figure 2.18 Graph representing the production of osteocalcin and ALP expressed per DNA synthesized. At each time point the cells were subjected to a freeze-thaw cycle in order to release the cell contents, these were frozen until assayed. Each time point represents the mean of two cultures ± SD.
Procollagen Type I (PICP)

Collagen is the most abundant of the bone matrix proteins. Type I collagen derived from the larger protein- Type I procollagen , is an indicator of matrix synthesis. The carboxyterminal propeptide of type I procollagen (PICP) was measured as the biochemical indicator of type I collagen production, and this was found to be much higher in both the HOB and HOS cells in monolayer culture. A functional relationship appears to exist between the down-regulation of proliferation and the initiation of extracellular matrix (ECM) maturation as indicated by a sharp rise on day \(8\) in the production of PICP by HOB cells in monolayer and micromass culture (Figure 2.19).

![Graph indicating the production of PICP by HOB cells in monolayer and micromass culture. A sharp rise in the production was seen at day 8, but levels were much higher for the monolayer. Each time point represents the mean of two cultures ± SD.](image)

**Figure 2.19** Graph indicating the production of PICP by HOB cells in monolayer and micromass culture. A sharp rise in the production was seen at day 8, but levels were much higher for the monolayer. Each time point represents the mean of two cultures ± SD.

**cAMP Production**

Responsiveness to PTH has been regarded as specific to osteoblastic cells. The HOBS responded to PTH by increasing intracellular cAMP production. The amplitude of the response to PTH was variable depending on the age of the cultures. Under basal conditions cAMP production was undetectable on day 3 for the HOB cell cultures, but became detectable and was seen to increase with age of the culture.
Following stimulation with PTH an increase was observed for all days studied with a tenfold increase in cAMP production at day 7 (Figure 2.20).

![Graph representing HOB cell responsiveness to PTH stimulation as estimated by the increase in intracellular cAMP for three different time points. Each point is the mean of two replicates.](image)

**Figure 2.20** Graph representing HOB cell responsiveness to PTH stimulation as estimated by the increase in intracellular cAMP for three different time points. Each point is the mean of two replicates.

The results have conclusively shown that the monolayer culture model favours both proliferation and differentiation of HOB and HOS cells. The expression of biochemical markers of differentiation can be detected much earlier in monolayer than in micromass culture. Table 2.3 summarizes the different markers for proliferation and differentiation for the 10 day cultures for both cell types using the two different models. It is evident from these observations that higher cell numbers, higher DNA levels and increased ALP activity was observed in the monolayer model. An increased production in PICP and osteocalcin was also seen.
Table 2.3 Levels of osteoblast markers for 10-day culture in monolayer (m) and in micromass (mm).

<table>
<thead>
<tr>
<th>DAY 10 CULTURE</th>
<th>HOB m</th>
<th>HOB mm</th>
<th>HOS m</th>
<th>HOS mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell No</td>
<td>1.2 x 10^6 ± 5 x 10^4</td>
<td>3.1 x 10^5 ± 10^4</td>
<td>1.8 x 10^6 ± 8 x 10^4</td>
<td>3.2 x 10^5 ± 4 x 10^4</td>
</tr>
<tr>
<td>DNA (µg/ml)</td>
<td>174 ± 0.9</td>
<td>20.1 ± 0</td>
<td>244 ± 6.5</td>
<td>29.7 ± 1.2</td>
</tr>
<tr>
<td>ALP (IU/l)</td>
<td>Production</td>
<td>503.5 ± 2.1</td>
<td>32.7 ± 1.2</td>
<td>83.8 ± 0.8</td>
</tr>
<tr>
<td>ALP Release</td>
<td>16.2 ± 0.3</td>
<td>7.9 ± 0.07</td>
<td>8.1 ± 0.04</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>Oc (ng/ml)</td>
<td>Production</td>
<td>3.68 ± 0.04</td>
<td>Undetectable</td>
<td>9.85 ± 0.56</td>
</tr>
<tr>
<td>Oc Release</td>
<td>4.90 ± 2.89</td>
<td>5.11 ± 0.128</td>
<td>3.91 ± 0.149</td>
<td>4.98 ± 0.154</td>
</tr>
<tr>
<td>PICP (µg/l)</td>
<td>Production</td>
<td>176.6 ± 8.5</td>
<td>15.5 ± 0.04</td>
<td>151.8 ± 31</td>
</tr>
<tr>
<td>PICP Release</td>
<td>1857.4 ± 100</td>
<td>586.4 ± 50</td>
<td>2399.5 ± 189</td>
<td>579.8 ± 25</td>
</tr>
</tbody>
</table>

Discussion

In view of the numerous methods to induce bone cell mineralization, described in the literature, it was necessary for the purpose of this study, to establish the most favourable conditions for a primary bone cell culture model that would differentiate and mineralize in-vitro under controlled conditions. This chapter describes the observations obtained from two different cell culture models; monolayer versus micromass, for primary human osteoblast cells and an osteosarcoma cell line.

Primary non-transformed cultures are favoured for studying bone cell function, in particular for investigating the effect of bone growth factors. Primary cells potentially contain all classes of bone forming cells, however, this study has shown that by adopting a suitable preparation method and controlled culture conditions, it is possible to achieve a separation of these cells and select for mature osteoblasts. The cell population migrating from digested bone fragments generally show a lower rate of proliferation, indicating that the osteoblastic cells from the bone surface are most likely to be immature precursors of
osteoblasts (Owen 1985, Auf’molk et al., 1985, McDonald et al., 1986). However, by introducing the pre-digestion incubation period in the culture model described in this study, any immature precursors are removed from the bone surfaces, and subsequent digestion results in committed stem cell osteoblasts with a rapid proliferation and maturation of cells.

This study has shown that culture method has a significant effect on the expression of phenotypic markers of primary HOB cells, with monolayer being superior for detecting changes in particular during the earlier time periods. The lower expression of markers in the micromass cells may be attributable to variations in oxygen tension or nutrient supply in the micromass culture model, as indicated by the lower cell viability, or additionally some may have died during the initial 2 hour incubation period prior to flooding. These findings agree with those of Tang et al., (1994), who demonstrated no obvious advantage using a micromass method. As expected, the osteosarcoma cell line HOS TE85 showed a greater proliferation capacity, as they represent a clonal populations derived from specific stages of the osteoblast lineage (Wong 1990, Rodan and Rodan 1984). Differences have been observed and it is not known whether the differences between clones are due to the presence of cells at different stages of maturation, or arise from dedifferentiation in culture (Rodan and Majeska, 1983, Clover and Gowen 1994, Evans et al., 1995). Grigoriadis and co-workers (1985), showed that in a rat cell line, it is possible for a cell population to alter its expressed phenotype and generate a number of subpopulations each of which may change at any time.

Variations in phenotype even between progeny of cells within a single clone can occur; these changes are not limited to transformed cells (Rodan and Majeska, 1982), but have also been reported in normal osteoblasts (Gerstenfeld et al, 1987). This present study has indicated a stability in the HOB phenotype; high ALP activity, a good predictor of the differentiative state of the cells was detected biochemically and confirmed by EM ultrastructural localization as early as day 4. Specificity of ALP activity was confirmed by blocking with levamisole hydrochloride. In the case of the 12 day HOS monolayer culture, incomplete inhibition was observed, a possible explanation could be that whilst levamisole may specifically inhibit ALP activity, the presence of other phosphatases; as potassium or sodium ATPases may give rise to 'false positive' reaction products.

Alkaline phosphatase has been implicated for the initiation of mineralization, but histochemically in cultured cells only a fraction of the cells stain positively even in clonal lines. Matsuyama et al., (1990) investigated monolayer cultures of normal human bone cells and reported subpopulations of alkaline phosphatase positive cells. The isolation method they employed involved the immediate digestion of the bone fragments, from our experience, this approach results in a highly mixed-cell population with predominantly immature precursors of osteoblasts and it is probable that their finding was a direct result of
this. In addition, their study was reliant on cytochemical evaluation of ALP-positive stained cells, which is subjective and prone to a high interobserver variation and is therefore not a reliable method for identifying different cell populations. Only a proportion of cells undergo maturation in culture, and this proportion may differ amongst different clonal lines. Furthermore, ALP levels may vary with cell cycle, Fedarko et al., (1989) have shown that ALP activity in human bone cell cultures is dependent on cell cycle distribution, cell density or length of time in culture. Also, the timing of the different phases that accompany differentiation in-vitro differs depending on the method used resulting in a lag between the different phases of expression of the individual markers (Stringa et al., 1995). Therefore the differences reported by different groups may be a result of the different techniques applied to isolate the bone cells and could explain the differences seen in terms of cell confluence, differentiation, matrix formation and mineralization.

On reaching confluency down-regulation of proliferation an increased production in ALP followed by osteocalcin and procollagen type I was also observed, thus rendering the cells competent for mineralization. The timing of these differentiating events appears to be very important; as the cultures began to mineralize, cellular levels of ALP were reduced.

Numerous reports have described bone cell culture methods, but few have shown spontaneous mineralization (Bellows et al., 1986, Ecarot-Charrier et al., 1983, Sudo et al., 1983). In all reported cases, and in contrast to this particular study, certain factors or conditions were a necessary prerequisite for terminal cell differentiation. Examples include; (i) a three-dimensional cell culture model for the induction of mineralization (Casser-Bette et al., 1990), (ii) the use of Na-β-glycerophosphate or other organic phosphates as stimulators of mineralization (Tenenbaum and Heersche, 1982, Ecarot-Charrier et al., 1983, Nishimoto et al., 1987, Bellows et al., 1986) and (iii) the time period in culture and enriched nutrient medium have also been reported (Sudo et al., 1983, Gerstenfeld et al., 1987, Casser-Bette et al., 1990).

HOB cells in monolayer spontaneously formed multilayers nodules at approximately 21 days with calcium deposition in the absence of β- glycerophosphate. These findings contrast with those of Casser-Bette et al., (1990) who reported that 56-day cultures of MC3T3 cells had to form a three-dimensional network, prior to the formation of bone-like tissue in the presence of β- glycerophosphate. Other workers have observed mineralization in conventional monolayer cultures, but only after extended periods in culture were dense multilayered nodules observed (Gerstenfeld 1987, Bellows et al., 1986, Whitson et al., 1984). Harris et al., (1995) in their study, showed that primary transfected foetal bone cells were able to form nodules with calcium deposits in the absence of β- glycerophosphate. Their method, however, has the disadvantage that viral proteins may also be expressed and that variations in phenotype within cell subpopulations or phenotype change may be
induced by viral infection. Sudo et al., (1983) observed calcification in multilayered cell nodules in the absence of exogenous phosphate. They demonstrated the ability of MC3T3-E1 cells to differentiate into osteoblasts and deposit hydroxyapatite once they became confluent and formed multilayers.

Both the HOB and HOS cells produced continuous low levels of osteocalcin, but the binding of the osteocalcin within the extracellular matrix only occurred after ALP levels had peaked. It appears that the production of ALP is a necessary transition signal to switch on the protein expression of osteocalcin, the majority of which is bound within the ECM, and hence very low levels were detected in the medium. Lian and Stein (1992) showed, in their detailed review that peak levels of expressed genes are indicative of the sequence of bone development and include proliferation, extracellular maturation and mineralization. In order for the cells to pass from one stage to another specific signals are necessary. One of these is the production of osteocalcin post-proliferatively with the onset of nodule formation. The findings in this study agree with this; an increase in ALP was observed prior to the significant expression of osteocalcin. They also reported that not all osteoblastic cell types were able to express osteocalcin an example being the cell line UMR 106 they concluded that any differences observed within the different cell lines may represent the presence of less differentiated cells (Stein et al., 1990, Owen et al., 1990).

In contrast to the findings of other workers who have claimed that osteoblastic characteristics decrease rapidly with number of passages and time in culture (Schmidt and Kulbe 1993), this study has shown that it is possible to selectively separate predominantly osteoblastic cells and achieve sufficiently high yields of HOB cells to study human bone cell function. The HOBS have successfully retained their phenotype up to passage 19 without the need for transformation. The cells have shown high levels of ALP activity, the production of osteocalcin and procollagen Type I, and a responsiveness to PTH, as demonstrated by increased cAMP and furthermore, the spontaneous formation of mineralized nodules. These cells provide a good model to study the biological response of bone. They have demonstrated the ability to be cultured through numerous passages and still retain metabolic, phenotypic and morphological characteristics, and have the ability to initiate mineral deposition. This technique provides a useful system for the study of osteoblast metabolism in-vitro.
CHAPTER 3

Introduction

Salmon and Daughaday (1957) first hypothesized that the growth promoting effects of GH were indirect and mediated systemically by somatomedins, also known as Insulin-like growth factors (IGFs), synthesized in the liver. Studies by Harris and Heaney (1969) demonstrated a marked increase in bone formation in adult dogs following GH administration. However, a local effect of GH \textit{in-vivo} was demonstrated by Russell and Spencer, (1985), who showed that a continuous infusion of a low dose of GH into hypophysectomized rats was able to maintain tibial cartilage when delivered to the appropriate target cells.

By understanding the mechanism of action of GH and related growth factors on cell proliferation and differentiation, one can use the approach of targeting the growth factor to the site where it has to exert its action. GH will be incorporated into a degradable and a non-degradable carrier system with a view to stimulating osteogenesis \textit{in-vivo}. The incorporation and subsequent release of GH from biomaterials could prove to be an important contributory factor in enhancing the healing process of many tissues, and improving osseointegration at the tissue and implant site. The release of GH from drug-delivery systems will be discussed in the subsequent chapters 4 and 5.

Normal skeletal growth and repair is dependent upon adequate levels of circulating vitamins, minerals, hormones and a complex interaction of numerous growth factors which direct the behaviour of osteoblastic cells (Bouillon 1991, Inzucchi and Robbins 1994). One of the most important of these growth factors in supporting osseous tissue development is GH. Administration of GH to human volunteers, has been shown to increase the biochemical markers of bone formation, suggesting increased activity of osteoblasts and induction of the proliferation of their precursors (Brixen \textit{et al.}, 1990). The availability of recombinant authentic sequence 22K human GH has shed new light on its possible therapeutic use in specific bone disease and tissue repair.

GH has been shown to have a mitogenic effect on osteoblast-like cells (Slootweg \textit{et al.}, 1988, Stracke \textit{et al.}, 1984, Morel \textit{et al.}, 1993), mediated by the paracrine or autocrine action of IGF-I (Chenu \textit{et al.}, 1990, Ernst and Froesch, 1988). Schmid \textit{et al.}, (1989) showed that IGF-I was able to support the differentiation of cultured osteoblast-like cells and increase alkaline phosphatase activity. In another study, it was reported that the stimulatory actions of GH were blocked in the presence of IGF-I antibodies. This demonstrates that IGF-I needs to be present in a biologically active form for GH to exert its action on osteoblast proliferation (Schmid \textit{et al.}, 1991). Chenu and co-workers, (1990) found that the markers of osteoblast differentiation, namely osteocalcin and alkaline phosphatase, were produced in response to GH and IGF-I, only in the presence of 1,25
(OH)₂D₃. Similar findings were observed by Beresford et al., (1984) who demonstrated that human bone cell cultures produced osteocalcin in response to 1,25 (OH)₂D₃.

Although GH has been shown to have an effect on osteoblast-like cells *in-vitro*, it has not been determined however, whether this is due to an increase in osteoblast proliferating precursors, or an increase in the number of preosteoblasts that differentiate, resulting in an increase in the synthesis and secretion of extracellular matrix proteins. The study described in this chapter was designed to determine which of these occurs *in-vitro*. Using the primary human osteoblast model described in chapter 2, the direct effect of GH, and the intermediate role of IGF-I and IGF-BP3 on the biochemical markers of bone turnover was investigated.

In normal bone growth factor production and availability play a key role in bone formation and remodelling, and it is likely that locally produced growth factors are intimately involved in the balance between resorption and accretion (Canalis *et al.*, 1988, Mohan and Baylink, 1991i). Cell culture provides a useful means for studying the cellular responsiveness of these factors, however, a major requirement for this type of study is the purity of the cell population and its ability to express the necessary phenotypic characteristics. The HOB cell model described in the previous chapter fulfils these criteria and will be used to study the effects of human GH, IGF-I and IGFBP-3.

Although osteotropic factors such as insulin and thyroid hormones may influence bone growth, GH is the only recognized hormone able to stimulate longitudinal bone growth in a dose-dependent manner; its importance for normal skeletal growth being well recognised (Isaksson *et al.*, 1987, Nilsson *et al.*, 1987, Isgaard *et al.*, 1986). Schlechter *et al.* (1986) were able to show that the direct growth-promoting effect of GH on cartilage *in-vivo* was mediated by the local production of IGF-I, however a direct role for IGF-I is not excluded. Thus IGF-I may act as a regulator of cellular proliferation via autocrine or paracrine as well as endocrine mechanisms. Isaksson *et al.*, (1982) showed that a low dose of GH injected directly into a tibial cartilage plate unilaterally stimulated epiphyseal growth, whilst a similar systemic dose had no effect. The direct action of GH was the promotion of the differentiation of precursor cells, and although IGF-I is not able to act at this level, its mitogenic action is selective in promoting cell proliferation in differentiated cells.

In adults both cortical and trabecular bone are in a constant state of remodelling, involving a close interplay between osteoblasts and osteoclasts (Parfitt, 1991). Several recent studies in GH-deficient adults have shown that GH replacement therapy increased bone turnover in terms of the circulating biochemical markers osteocalcin, (Johansen *et al.*, 1990), procollagen (Bengtsson *et al.*, 1993) and total ALP, although data on the bone isoenzyme
is limited. Less is known in adults about the effect and interaction of GH and IGF-I on mature bone. It has been reported that in order to sustain an appropriate level of bone turnover and local repair, a specific 'hormonal milieu' is required to support calcium homeostasis, and to be able to respond to biomechanical demands (Canalis et al., 1988, Mohan and Baylink, 1991). Bone is rich in growth factors and both GH and IGF-I have been implicated in the normal physiology of adult bone even after the cessation of longitudinal growth (Inzucchi and Robbins 1994, Brixen et al., 1993).

The responsiveness of osteoblast-like cells to GH, IGF-I and IGFBP-3 in-vitro has, to date, been studied mostly in rodent foetal cell cultures (Stracke et al., 1984, Ernst and Froesch, 1988, Slootweg et al., 1988). For example, the in-vitro effect of GH on chondrogenesis and osteogenesis in mouse cartilage progenitor cells in promoting de novo bone formation has been demonstrated by Maor et al., (1989). In a study by Hassanger et al., (1992), various rodent and human cell lines were examined and compared for their basal and regulated release of IGFBPs. Their study indicated variations among the osteoblastic cell lines, and no direct effect of GH on the release of the IGFBPs was observed. As a result, it has been difficult to compare and correlate the data. More recent studies have shown that GH can effect both the proliferation (Scheven et al., 1991) and differentiation (Kassem et al., 1993, Chenu et al, 1990) of normal human osteoblasts.

IGFs have been implicated as mediating GH effects on a number of target tissues, but their role in mediating the effects of GH actions on osteoblasts are still controversial (Ernst and Froesch, 1988, Scheven et al., 1991). Investigations by Scheven et al., (1991) indicated that GH and IGF-I and IGF-II were able to stimulate sub-confluent cultures of osteoblasts in a dose-dependent manner. Whilst IGF-I stimulation could be blocked by using a neutralising antibody, the actions of GH remained unaffected, suggesting an independent action of IGF-I and IGF-II. The IGF-I effect on an osteosarcoma cell line could however, be blocked completely indicating that GH in these cells was acting via endogenously produced IGF-I, in order to locally regulate primary and transformed human bone cells. Ernst and Froesch (1988) showed that GH elicited a dose-dependent stimulation of osteoblast-like cells in serum-free cultures. This could be inhibited in a dose-dependent manner by the addition of an antiserum IGF-I; again showing that direct stimulation of osteoblasts by GH is in part mediated by IGF-I.

The bioactivity of GH and IGF-I is regulated by BPs which are regulated by both systemic hormones and local factors, and are expressed in a tissue-specific manner. At least two different binding proteins (80 kDa and 100kDa) have been identified for GH with varying degrees of affinity for GH (Baumann et al., 1986, Herington et al., 1986).
Approximately 98% of IGFs are bound to specific binding proteins, the major binding proteins in bone being IGFBP-3 and 4 (Mohan et al., 1989, Schmid et al., 1989). Osteoblasts have been shown to produce a variety of IGFBPs, some having stimulatory effects and others having inhibitory effects. For example, IGFBP-4 has been shown to have an inhibitory effect on IGF-I activity in bone cultures (Mohan et al., 1989), whereas IGFBP-3 in certain culture conditions has been shown to have stimulatory effects (Ernst and Rodan 1990).

In this study, various parameters will be investigated to assess whether GH and IGF-I exert their effect by increasing cell number or by enhancing the differentiating capacity of osteoblasts or both. Cellular proliferation will be assessed using cell counts, by measuring the incorporation of bromodeoxyuridine (BrdUrd) in S-phase cells and also by the incorporation of \(^{3}\text{H}\)-thymidine following stimulation with GH and IGF-I. The effect of GH on osteoblast differentiation will be assessed by measuring ALP activity in the cell layer and the production and release of procollagen type I (PICP) and osteocalcin into the medium. The responsiveness of HOB cells to the osteotropic systemic hormone 1,25(OH)_2D_3 and IGF-BP3 will also be investigated to see if they play a contributory role in the actions of GH and IGF-I.

Materials and Methods

All the methods used in this Chapter for both the cell culture conditions and biochemical assessments have been described in chapter 2, unless otherwise stated.

\[ ^{3}\text{H}\] -TdR uptake

Experiments were performed twenty-four hours after plating the HOBS in 24 well plates (30,000 cells per well), the medium was changed to serum free to arrest cell growth. The incubation for control or test (GH or IGF-I, IGFBP-3 dose range 0-50 ng/ml, 1,25 (OH)_2D_3 10^{-9} \text{M}, in the presence or absence of respective antibodies for the IGF-I and GH stimulated cells, was for 48 hours in total, but, for the final 16 hr of the culture, the cells were incubated in the presence of 1\mu\text{Ci/ml of} \[ ^{3}\text{H}\] -TdR (Amersham International plc., England, SA 5mCi/mmol). The cell cultures were washed four times in DMEM media containing unlabelled thymidine (5.0\mu\text{g/ml}) in order to reduce non-specific binding. This was followed by digestion using a papain mixture (see chapter 2). 100\mu\text{l of the digest was transferred to a scintillation vial and the amount of radiolabel incorporated was measured on a scintillation counter. The antibodies used were a polyclonal IgG-fraction of a rabbit anti-human IGF-I, (a gift from Ms J.Jones, Institute of Child Health, London) and a polyclonal IgG-fraction of guinea pig anti-human GH, (075, a gift from Novo Nordisk A/S, Denmark).
Cell Proliferation

Dose-dependent stimulation of HOBS by GH (dose range 0-100ng/ml) was assessed using a proliferation assay (RPN 210, Amersham, UK). This assay determines the amount of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdUrd), incorporated into cellular DNA. The cells were seeded (40,000 cells/well) in a 96-well plate in DMEM (10% FCS) for 24 hours, after which the media was replaced with serum-free medium containing the GH. The cultures were labelled with BrdUrd (200ml) and incubated for a period of 2 hours, and were then fixed in 70% ethanol. After washing in PBS, a further incubation with a specific mouse anti-BrdUrd monoclonal antibody followed. The incorporation of BrdUrd by the cells was detected using a peroxidase labelled goat anti-mouse IgG substrate. The absorbance of the reaction was quantified spectrophotometrically at 410nm and the results were expressed as a percentage of control values not stimulated with GH.

MTT assay

A 5mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, (Sigma Chemical Co., Poole, UK) was prepared in PBS and filtered prior to use. 10μl was added to all wells to give a final concentration of 0.5mg/ml and the plates were incubated at 37°C, 5% CO₂ for 4 hours. Excess medium containing the MTT was removed by inversion of the plate and gentle tapping to remove any excess. 100μl dimethyl sulphoxide (DMSO) was added to all wells. The plates were mixed for 10 minutes to ensure complete crystal dissolution and the absorbance measured on a Dynatech MR700 plate reader, using a test wavelength of 570nm and a reference wavelength of 630nm.

Neutral Red Cell Viability Assay

Adherent cells were incubated in DMEM containing Neutral Red (NR, 50μg/ml) for three hours at 37°C, 5% CO₂. Excess medium was removed and the cells washed in 0.5% (v/v) formaldehyde/1% (v/v) calcium chloride. The incorporated dye was extracted by the addition of a mixture of 1% (v/v) acetic acid/ 1% (v/v) ethanol (50%) and the cells were incubated at room temperature for 30 minutes on a TiterTek plate shaker. The absorbance was measured at test wavelength 540 nm and reference 630 nm on a Biorad Model 3550m plate reader.

Coomassie Blue (CB) Assay

The CB assay was performed on cells which had previously been tested using the NR assay. Following the aspiration of the NR stain, 150μl of CB stain (60μg/ml) was added to each well, and the plate agitated for 20 minutes.
The stain was removed and the plate washed twice with 0.5% formaldehyde/ 1% Calcium Chloride, followed by 150µl of desorbing solution (a mixture of 1M potassium acetate/ 70% (v/v) ethanol). The plate was agitated for 20 minutes (until a homogenous solution was observed). The plate was read immediately at test wavelength 570nm and reference 404nm.

**Insulin-like growth factor -I (IGF-I)**

IGF-I was measured using an 'in-house' radioimmunoassay (RIA) with an acid/ethanol extraction. This ensures the removal of any binding proteins that may interfere with the assay. The assay uses a rabbit anti-human polyclonal anti-IGF-I.

**Insulin-like growth factor binding protein-3 (IGFBP-3)**

This was measured using a coated-tube immunoradiometric (IRMA) kit (Diagnostic Systems Laboratories, Webster, Texas)

[125I] - GH binding experiments

In order to establish the optimal conditions for the binding of GH, the cells were subcultured in four replicates per time point in small culture dishes (35x15cm²); two for maximal binding estimation (in the absence of unlabelled GH) and two for non-specific binding (determined by incubation with excess, 10µg/ml of unlabelled GH). Binding at different temperatures (4°C, 22°C and 37°C) and various incubation times was tested. For the actual binding experiment the cells were cultured in 6 well plates (100,000 cells/well). On reaching confluency, the cells were washed twice with PBS containing 0.25% (w/v) BSA. The wash medium was removed and the cells incubated for 2 hours at 4°C in 1ml of fresh DMEM (0.25% BSA) containing 125I-GH 150,000 cpm./well (Specific activity 69µCi/mg; kindly provided by Novo Nordisk A/S, Denmark) and increasing amount of cold GH (range 1-10,000 ng/ml, kindly provided by Novo Nordisk A/S, Denmark). At the end of the incubation period, the cell monolayers were washed with PBS, trypsinized, harvested with a syringe and counted on a gamma counter (NEN, 76% Efficiency). Non-specific binding was calculated as the amount of 125I-GH bound to the cells in the presence of excess (10 µg/ml) of unlabelled GH. The binding affinity was assessed by Scatchard analysis of 125I-GH bound to cell monolayers in the presence of increasing concentrations of unlabelled GH. All data have been plotted as a percentage of specific binding, i.e non-specific binding was subtracted. Curves were fitted using the Rodbard and Munson (1980) Scatchit program.
Statistics

All statistics were performed using the statistical software programme, Statview (Apple Computer, USA). The Student's t-test was used to perform the statistics, which were also tested long hand.

Results

Cell Proliferation

GH stimulated HOB cell proliferation in a dose-dependent manner under serum-free conditions (Figure 3.1a). A significant increase in BrUrd incorporation was observed across a range of GH concentrations ( \( p=0.001 \)). These results were corroborated with a dose-dependent increase in cell numbers counted on a haemocytometer using trypan blue exclusion dye to determine the ratio of viable to dead cells. Light micrographs of the HOB cells stimulated with a low (25ng/ml), a medium (50ng/ml) and a high (100ng/ml) dose of GH can be seen in Figures 3.1b,c,d respectively. A significant effect was observed for all GH doses compared to the control, with maximal stimulation occurring at 50 ng/ml (Figure 3.2). This dose was selected and used in all stimulation experiments over various time courses. A further confirmation of the effect of GH on HOB proliferation was given by the results of the MTT (Figure 3.3) and the NR assays, both of which measure cell viability. The increase in cell number was reflected in an increase in total cellular protein as indicated by the CB assay (Figure 3.4).

Stimulation of cell growth was confirmed by the incorporation of \(^3\)H-thymidine in the DNA of HOB cells stimulated by both GH and IGF-I. To test the role of endogenously produced IGF-I and the possibility that GH might be acting through the IGF-I produced by the HOBS, an antibody to IGF-I was added to the cultures. A 30% reduction was observed in the response to IGF-I in the presence of an antibody dilution of 1:500, and this inhibition was significant for the dose range 12.5-50 ng/ml (Figure 3.5). The specificity of the GH stimulation effect on the HOB cells was also tested using an antibody directed against human GH. A 50% reduction in response was observed in the presence of the antibody, which was significant for the dose range 6-25 ng/ml (Figure 3.6).
Figure 3.1a Effect of GH on HOB cell proliferation as measured by the incorporation of the nucleoside analogue 5-bromo-2-deoxyuridine (BrdUrd) into replicating nucleic acids and localised using a specific monoclonal antibody in order to determine the number of S-phase cells. Twenty-four hours after seeding, the medium was replaced with serum-free medium containing GH; BrdUrd was then added and incubated for a further 2 hours, followed by fixation of the cells. The results represent the mean of two experiments (n=8) ± SD. Paired Student's t-test indicates differences from the control. Asterisks denote significant statistical difference ** P=0.001
Figure 3.1b Light micrograph of HOB cells stained with Coomassie Blue following stimulation with a low dose of GH (25ng/ml) for 24 hours. Magnification x 218

Figure 3.1c Light micrograph of HOB cells stained with Coomassie Blue following stimulation with a medium dose of GH (50ng/ml) for 24 hours. Magnification x 218

Figure 3.1d Light micrograph of HOB cells stained with Coomassie Blue following stimulation with a high dose of GH (100ng/ml) for 24 hours. Magnification x 218
Figure 3.2 Dose response for the effect of GH on cell growth of primary HOB cells. Cells were cultured as described in chapter 2, twenty-four hours prior to stimulation the medium was changed to serum-free to induce growth arrest. This was followed by a further 24 hours in medium containing GH at the concentrations shown. Cell viability count was performed using the trypan blue exclusion dye. The results represent the mean of two experiments (n=8) ± SD. Paired Student's t-test indicates differences from the control. Values in the presence of GH are significantly different from those in the absence. *P< 0.05, **P=0.001
Figure 3.3 Effect of GH on HOB cell measured using the MTT assay which measures the action of intracellular enzymes on tetrazolium salts. A dose-dependent increase in cell viability was observed. The results represent the mean of three experiments (n=24) ± SD. Paired Student's t-test indicates differences from the control. Values in the presence of GH are significantly different from those in the absence. *P<0.05, **P=0.001

Figure 3.4 Effect of GH on HOB cell measured using the vital dye NR (test wavelength 540 nm) for the assessment of cell viability and the CB assay (test wavelength 570 nm) which measure total protein content of the cells. The results represent the mean of three experiments (n=40) ± SD. Paired Student's t-test indicates differences from the control. Values in the presence of GH are significantly different from those in the absence. **P=0.001 *p<0.05
Figure 3.5 Effect of anti-IGF-I antibody on the proliferation of HOB cells stimulated with various concentration of IGF-I. The IgG-fraction of the antiserum (dilution 1:500) was added at the same time as the IGF-I of the culture period. A mean 30% reduction was observed in the presence of the antibody. The results represent the mean expressed as a percentage of the control of two experiments (n=4) ± SD. Paired Student's t-test indicates differences from the control. Values in the presence of Ab are significantly different from those in the absence *P< 0.05 versus controls. Note that basal growth is significantly inhibited in the presence of the antibody (*P< 0.05)
Figure 3.6 Effect of anti-GH antibody on the proliferation of HOB cells stimulated with various concentration of GH. The IgG-fraction of the antiserum (dilution 1:800) was added at the same time as the GH of the culture period. A reduction in excess of 50% was observed in the presence of the antibody. The results represent the mean expressed as a percentage of the control of two experiments (n=4) ± SD. Paired Student's t-test indicates differences from the control. Values in the presence of GH are significantly different from those in the absence. *P< 0.05 versus controls. The basal growth was only slightly inhibited in the presence of the antibody, but this was not significant.

Effect of GH and IGF-I on the biochemical markers of HOB activity and differentiation

The pattern of osteoblast growth was investigated by measuring the variation of culture DNA with time, this was investigated for a 21-day period in order to establish the relationship of cell growth to the expression of bone cell differentiation markers. The results indicate that from day 3 to day 7, the cells were rapidly proliferating, this was followed by a decrease indicating a down-regulation of proliferation prior to the expression of alkaline phosphatase and other markers of differentiation (Figure 3.7). The effect of GH singly, and in combination with 1,25(OH)\textsubscript{2}D\textsubscript{3} on cell number and production of ALP on day 14 of culture can be seen in Table 3.1.
Figure 3.7 The relationship between cell number and the onset of expression of ALP— the biochemical marker of differentiation. Incorporation of $^3$H-thymidine in DNA of HOB cells at different time points of culture. The results of the experiment show that a rapid period of proliferation from day 2 to day 5. The down-regulation of proliferation at day 10, following inhibition of DNA synthesis a rapid increase in the release of ALP was observed.

Table 3.1 The effect of GH and 1,25(OH)$_2$D$_3$ (Vit D$_3$) on HOB cell number and the production of ALP

<table>
<thead>
<tr>
<th>Day 14 culture</th>
<th>Control GH vitD$_3$</th>
<th>GH 50ng/ml</th>
<th>VitD$_3$ 10$^{-9}$M</th>
<th>VitD$_3$ + GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell No ± SD</td>
<td>3.4 x 10$^5$ ± 1.2 x 10$^5$</td>
<td>1.8 x 10$^6$ ± 2.5 x 10$^5$</td>
<td>4.8 x 10$^5$ ± 1.3 x 10$^5$</td>
<td>1.4 x 10$^6$ ± 5 x 10$^4$</td>
</tr>
<tr>
<td>ALP/DNA ± SD</td>
<td>5.3 ± 2.2</td>
<td>12.8 ± 1.5</td>
<td>10.5 ± 1.3</td>
<td>4.4 ± 0.75</td>
</tr>
</tbody>
</table>

A significant increase of 141.5% (compared to the control) in the production of ALP was observed in the presence of GH and an increase of 98% was seen in cells treated with 1,25(OH)$_2$D$_3$. However, the addition of GH and 1,25(OH)$_2$D$_3$ in combination resulted in a 17% reduction in the production of ALP compared to the control. A comparison of ALP production in GH and GH in combination with 1,25(OH)$_2$D$_3$ stimulated cells, indicates a
reduction of 66% in the presence of 1,25(OH)_2D_3. In the absence of 1,25(OH)_2D_3, GH induced a significant (p< 0.05) increase in ALP production by the HOB cells at all time points studied, from as early as day 5 of culture, where an increase of 50% in the production of ALP in the presence of GH was seen (Figure 3.8). When the cells were exposed simultaneously to 10^{-9} M 1,25(OH)_2D_3 and GH (10ng/ml), no significant increase was observed, 1,25(OH)_2D_3 alone did not increase ALP significantly compared to the control (Figure 3.9).

**Figure 3.8** Effect of GH on the biochemical marker of differentiation -ALP on different age cultures over a 21 day period. ALP activity was measured in the cell lysate. HOB cells were cultured for the time period indicated, cells were incubated for 24 hours prior to harvesting in the presence of GH (50ng/ml). The results represent the mean of two experiments (n=4) ± SD. Paired Student's t-test indicates differences from the control. Values in the presence of GH are significantly different from those in the absence. *P< 0.05
Figure 3.9 Effect of 1,25(OH)\(_2\)D\(_3\) stimulation and in simultaneous combination with GH on ALP activity. HOB cells were cultured for the time period shown, and cells were incubated for 24 hours prior harvesting in the presence of test agent (10\(^{-9}\) M, 1,25(OH)\(_2\)D\(_3\) and 50ng/ml GH). The results represent the mean of two experiments (n=4) ± SD. An increase in ALP activity was observed on days 12 and 21, but this was not significant.

The production of osteocalcin was not affected by 1,25(OH)\(_2\)D\(_3\) or PTH (data not shown), whilst GH at 100 ng/ml increased the release of osteocalcin, but not to a statistically significant degree. IGF-I, however, significantly stimulated the production of osteocalcin at 50 and 100 ng/ml compared to the basal control (Figure 3.10). Treatment of HOB cultures with GH increased the production of PICP for the time period studied, but this was significant only on day 14 (Figure 3.11).

The effect of GH on the induction and accumulation of IGFBP-3 was tested at two concentrations of GH, 100 and 200 ng/ml. IGFBP-3 was released by the HOBs under basal conditions, but a significant increase was observed in its release in the presence of GH (Figure 3.12). In another experiment GH was tested to see whether the induction effect of IGFBP-3 altered the release of IGF-I and the results indicated that GH stimulated both IGF-I and IGFBP-3 in a dose-dependent manner (Figure 3.13). The possible mitogenic effect of IGFBP-3 was tested at two concentrations 100ng/ml and 200ng/ml on HOB cells and a significant increase (p < 0.05) in viable cell number was observed for both doses studied indicating that the cells were responsive to stimulation by IGFBP-3 (Figure 3.14). Addition of GH to cultures at various time points resulted in an increase in IGF-I levels (Figure 3.15).
**Figure 3.10** Effects of GH and IGF-I on the release of the protein osteocalcin, a marker of osteoblast differentiation. Osteocalcin release into the medium was measured following a 24 hour incubation with GH and IGF-I at the stated concentrations. The results represent the mean ± SD (n=2). A significant increase in osteocalcin activity was observed in the presence of IGF-I (* P<0.05). An increase in osteocalcin release was seen following stimulation with GH (100ng/ml) but this was not significant.

**Figure 3.11** Effects of GH (50ng/ml) on the release of the biochemical marker of osteoblast differentiation PICP. HOB cells were cultured for the time period shown, and cells were incubated for 24 hours in the presence of GH prior to harvesting. The results represent the mean ± SD (n=2). A significant increase in PICP release was seen at day 14 in the presence of GH. The results represent the mean ± SD (n=2). *P<0.05
**Figure 3.12** Effect of two different doses of GH on the production of IGFBP-3. HOB cells under basal conditions produce IGFBP-3, but its release can be significantly increased following stimulation with GH. The results represent the mean ± SD of 3 assays (n=24) **P=0.001**

**Figure 3.13** The Effect of GH on IGF-I and IGFBP-3 release from HOB cells. The cells were incubated for 24 hours in the presence of GH. A dose-dependent increase in IGF-I and IGFBP3 was observed. The results represent the mean ± SD (n=2). Paired Student's t-test indicates differences from the control *P<0.05
**Figure 3.14** The effect of various concentrations of IGFBP-3 on HOB cell proliferation, measured by the MTT assay which measures the action of intracellular enzymes on tetrazolium salts. A significant increase in cell proliferation was observed in the presence of IGFBP-3 at both doses, indicating that it has a mitogenic effect. The results represent the mean of three experiments (n=48) ± SD **P=0.001**

**Figure 3.15** The effect of GH (50ng/ml) on IGF-I release. HOB cells were cultured as described, the media was removed at the indicated times and replaced by GH for a period of 24 hours prior to harvesting. The results represent the mean ± SD (n=2). An increase in IGF-I release was observed for all the time points, and was significant for all days studied except day 14. *P<0.05
Specificity of binding $^{125}$I-GH

The maximal binding in initial experiments was very low and various conditions were tested to see whether binding could be increased. The binding of an antigen to its receptor is temperature dependent, and it is possible that at 37°C GH binding to the receptor may have occurred but was subsequently followed by an equally rapid dissociation from the receptor giving rise to the low binding detected. From the results of an experiment to test optimal conditions, an incubation time of 2 hours at 4°C gave the most reproducible binding and these conditions were used for subsequent experiments.

Primary HOBs showed low, but highly reproducible specific binding (for three experiments), with non-specific binding representing less than 50% of total binding. Competitive displacement by increasing doses of unlabelled GH showed a typical curve for a receptor, with half maximal displacement at approximately 25ng/ml. Scatchard analysis of the data in Figure 3.16, showed the presence of only one class of receptors with an affinity constant of $1.03 \times 10^9$ M$^{-1}$. However, on closer examination of the plot of the ratio of bound (B) to the free (F) counts (B/F), the graph appears to curve and to consist of two components (Figure 3.17) indicating that there may possibly be two classes of receptors; a high and a low affinity. However, this is only speculative at the present time and requires further investigation. The receptor binding assay requires further optimization in order to increase maximal binding by the HOB cells and the NSB needs to be reduced further, as at present it constitutes approximately 40% of the total binding.
Figure 3.16 (a) A competition binding curve to show specificity of binding of [¹²⁵I] labelled GH to HOB cells in the presence of competing unlabelled GH. Binding was for 2 hours at 4°C in DMEM (0.25% HSA). The points represent the mean of n=4 determinations. (b) Inset; shows the earlier portion of the displacement curve for the % maximum displacement. Results are for specific binding, corrected for non-specific binding. Half-maximal displacement was approximately 25 ng/ml.
Figure 3.17 Scatchard analysis of the data shown in Figure 3.18 (b) with GH as the competing unlabelled ligand. Curve was fitted with the Rodbard-Munson (1980) SCAFIT program. B/F, bound/free ratio. Specific activity of $^{[125]}$I-GH was 69 μCi/μg. Association constant (Ka) for the data was $1.3 \times 10^9$ M$^{-1}$

Discussion

An understanding of the regulation of cellular differentiation in bone formation at the cellular level can only be achieved by examining the cells predominantly responsible for this, that is, the osteoblasts. Most studies of bone metabolism and hormonal regulation have to date been confined to transformed rodent cells, or isolated primary human foetal cells (Harris et al., 1995, Aubin et al., 1982, Sudo et al., 1983). Species differences and maturity of the cells makes it very difficult to extrapolate and compare responses.

This present study was undertaken to examine the direct effect of GH and its intermediary action via IGF-I and possibly IGFBP-3 on adult human primary osteoblasts under defined conditions using the model described in chapter 2. The aim was to establish the cell-specific metabolic responses to GH on the time and production of biochemical markers of bone formation in-vitro.

In this study GH stimulated HOB cell proliferation in a dose-dependent manner; significant effects were observed with maximal stimulation at 50 ng/ml, within physiological range of
the hormone in humans. The increase in $^3$H-thymidine incorporation and BrdUrd uptake indicated a real increase in cell number which was confirmed by actual cell counts and cell viability as indicated by the MTT and NR results.

Neutralization experiments showed incomplete inhibition of the GH and IGF-I stimulation response. The antibody to IGF-I was only partially able to block the IGF-I induced stimulation of HOB cells, but did affect the basal growth rate. In the case of the GH-stimulated HOB cells, a reduction greater than 50% was observed for all doses in the presence of an anti-GH antibody. The inability to completely block the stimulatory effect of IGF-I and GH in this study was probably due to the lack of accessibility and effectiveness of the antibodies chosen; the antibody may not have been specific enough and the titre inappropriate to completely neutralize the response. In the case of the GH stimulated HOB cells, it would appear that GH is directly responsible for the growth stimulatory action in the bone cell system. Interestingly, in the IGF-I stimulated cells, the reduction in the basal response indicates the involvement of another mechanism.

Ernst and Froesch (1988) showed that whilst GH-induced proliferation of rat osteoblasts involved the local synthesis of IGF-I, the presence of antibody to IGF-I did not affect the basal growth rate of the osteoblasts indicating that another autocrine or paracrine mechanism was involved, different from IGF-I. Scheven et al. (1991) in another study, showed that a specific monoclonal antibody to IGF-I, was able to neutralize IGF-I stimulated growth, but did not interfere with GH action.

Whilst it is generally accepted that the actions of GH are mediated via IGF-I; IGF-I per se has been shown to be a potent growth promoter of chondrocytes in-vivo (Nilsson et al., 1986, Schlechter et al., 1986), and osteoblasts in-vitro. IGF-I has been shown to increase cell proliferation and matrix formation of differentiated osteoblasts (Canalis 1980, Hock et al., 1988). Similar findings were observed in this study, with a dose-dependent increase in $^3$H-thymidine incorporation in the presence of IGF-I.

The effect of GH on the biochemical markers of HOB differentiation in-vitro indicated a significant dose-dependent increase in absolute viable cell counts and also an increase in ALP activity particularly evident in the earlier time periods on days 5 and 7 for the cells in culture. The surge in ALP release in the controls was seen much later at day 14, which agrees with the normal reported time periods for mineralization to commence (Lian and Stein 1992). The osteotropic hormone 1,25(OH)$_2$D$_3$ alone was able to stimulate ALP activity, resulting in a twofold increase in the ALP by the HOB cells. Lajeunesse et al., (1990) reported a similar increase in ALP activity after 4 hours post treatment with 1,25(OH)$_2$D$_3$. A significant GH induced increase in ALP activity was observed. Similar findings have been reported by other workers, and in some cases a differential effect on
skeletal cells was observed depending on their state of growth and maturation (Majeska and Rodan, 1982, Chenu et al, 1990). The stimulatory effect of 1,25(OH)2D3 and GH in combination on ALP activity whilst increasing cell number did not result in an increase in ALP activity, instead, a decrease in ALP activity was seen. It is known that 1,25(OH)2D3 plays a major role in the control of skeletal growth, and it has been shown to regulate various aspects of cellular activity. However, it appears to have pleiotropic effects on bone, in particular on osteoblastic cells where it has differential actions depending on the stages of phenotypic maturation (Majeska and Rodan, 1982). Similar findings have been reported by Beresford et al., (1984), who showed that 1,25(OH)2D3 alone was able to stimulate ALP activity in a dose-dependent manner.

In this present study 1,25(OH)2D3 alone was able to stimulate ALP activity, yet in combination with GH the opposite effect was observed, this indicates that 1,25(OH)2D3 is able to exert multiple effects, and that it may be an important modulator of growth and differentiation of the HOB cells in-vitro. Although an increase in PICP was observed in the presence of GH, it was, however, only significant on 14-day old cultures. An increase in osteocalcin release was observed in the presence of GH but this was also not significant. IGF-I, however, significantly increased osteocalcin release, this has not been shown previously in adult primary HOBS in-vitro.

Osteocalcin production in response to 1,25(OH)2D3 has been described in primary human bone cultures (Beresford et al., 1984), in foetal rat calvarial cells (Lian et al., 1985), and in rat clonal osteosarcoma cells ROS 17/12.8 (Price and Baukol, 1980). Lajeunesse et al. (1990) showed that an increase in osteocalcin secretion in a human osteosarcoma cell line was induced, but this was dependent on cell density. Chenu and co-workers (1990) reported an increase in osteocalcin in short-term cultures treated with 1,25(OH)2D3. Furthermore, they showed that GH had no effect on osteocalcin release. In contrast to these reports, 1,25(OH)2D3 in this study had no effect on osteocalcin production (data not shown).

There have been conflicting and inconsistent reports on the effect of 1,25(OH)2D3 and these are probably related to the cell origin that is the species and cell density. Majeska and Rodan, (1982) showed that 1,25(OH)2D3 was able to exert differential effects on a rat osteosarcoma cell line, and these were dependent on their state of growth and maturation. In contrast to the observations of Kassem et al., (1993) who were unable to detect an increase in osteocalcin or, the release of IGF-I in either the basal or the GH stimulated cultures; a significant dose-dependent increase in IGF-I release was observed in the HOBs following exposure to GH in this study. Furthermore, this effect was seen at all time points studied indicating that different age cultures were responsive to GH stimulation. Chenu et al. (1990) on the otherhand, were able to detect an increase in IGF-I after GH exposure,
but no reference was made of the possible interference of IGFBPs in their assay, which might give rise to overestimation of the measurable IGF-I resulting in 'false positive' results. The possible interference of IGFBPs has been taken into account in this present study and samples were treated prior to assay, indicating 'de novo' synthesis of IGF-I.

In a detailed analysis of the relationship between proliferation and expression of differentiation Lian and Stein (1992) have shown that peak levels of expressed genes principally reflect a developmental sequence of bone cell differentiation, proliferation, extracellular maturation and mineralization. The stage of maturation or differentiation of the osteoblast and their responsiveness to physiological modulators of bone cell function such as growth factors is dependent on the expression of these genes. Agents affecting mineralization elicit their effects at different times of the culture period, indicating that they are able to influence cells at different stages of the proliferation-differentiation sequence. In the present study, GH has been shown to modulate the behaviour of the HOBs by affecting the rate at which proliferation takes place, and also by advancing the timing of differentiation of these cells. The increased proliferation rate resulted in an earlier induction of ALP, with levels 50% greater than the corresponding control for the same time point. This increase in ALP was followed by an increase in osteocalcin release and a sharp rise in pro-collagen type I (PICP) peaking at day 12. These findings are in agreement with Lian and Stein (1992) who report that down-regulation of proliferation, as reflected by a fall in DNA synthesis, is followed by an increase in the expression of ALP the major protein associated with bone cell phenotype. The expression of ALP mRNA and enzyme activity suggests its involvement with the maturation of the extracellular matrix in order that mineral deposition can take place, and that its presence is a pre-requisite for initiating osteocalcin release and their subsequent co-expression as shown biochemically in chapter 2, results in the progression of mineralization.

Taken together, the pattern of release of these markers, as determined biochemically, demonstrates that a time-dependent sequence of events occur during the 21-day culture period associated with the development of an extracellular matrix and reflects maturation of the osteoblast in-vitro. In the presence of GH, this process is activated much earlier than normal resulting in expression of markers that would normally be seen much later, thereby indicating that GH has differentiating-promoting properties on HOBs.

The results obtained from this study for primary HOBs have shown biochemically that GH acts directly at the osteoblast level. This, of course, can only be confirmed by examination of the mRNA for the individual markers, so that any changes in their expression in the presence of GH can be seen by an alteration in the functional roles of bone matrix proteins in relation to the sequence of events in bone formation. This type of control has been demonstrated in experiments in which inhibition of DNA synthesis in actively proliferating
osteoblasts by hydroxyurea results in a rapid and selective down-regulation of cell growth genes (Owen et al., 1990a). Similarly an increase in ALP expression following a decrease in proliferation activity has been reported in ROS 17/2.8 cells by Majeska et al., (1985).

The concomitant release of IGF-I resulted in the stimulation of the differentiated cells, giving rise to the induction of an alteration in the rate of production of bone mineralizing proteins. Furthermore, the release of IGFBP-3, post GH stimulation gives rise to a further independent stimulation of HOB cell proliferation. This combined release indicates that IGFBP-3 may be acting as a paracrine regulator by modifying the biological activity of IGF-I. A positive correlation was observed between the IGF-I and IGFBP-3 release, indicating a physiological significance of IGF-I regulated IGFBP for bone metabolism and growth. This positive feedback mechanism may possibly explain the simultaneous GH-induced release of both IGF-I and IGFBP-3.

An increase in IGF-I post GH treatment has been observed by Chenu et al (1990), however, they make no reference to IGFBPs which might give falsely elevated levels of IGF-I. Their results suggest that the two markers ALP and osteocalcin reflect different roles in bone formation.

The results reported in this chapter are indicative that GH receptors should be present on these cells. Morel et al. (1993) were able to show a GH-like immunoreactivity in rat calvaria and a mouse clonal cell line (MC3T3-E1). They too showed that this was not the result of local synthesis of GH, as in-situ hybridization using a GH oligonucleotide did not reveal the presence of GH mRNA, thus indicating that the actions of GH are a result of binding with its receptor.

Leung et al. (1987) reported the purification of the GH receptor from rabbit liver and the identification of a 130K protein as the putative GH receptor, this had an apparent association constant (Ka) for human GH binding to the purified receptor of 10-30 x 10^9 M^-1.

The presence of GH receptors on osteoblasts in a rat osteosarcoma cell line has been reported by Barnard et al., (1991). In contrast to the liver, the density of these receptors was much lower and had a Ka of 1.9 x 10^9 M^-1. Werther et al. (1993) have reported the expression of GH receptors on human foetal mesenchymal tissues, they showed that these receptors were capable of binding GH and postulated a functional role in the human foetus by 15 weeks gestation. To the knowledge of the author, the presence of GH receptors on primary human osteoblasts has not to date been reported.

The data in this study shows specific binding of radiolabeled GH to primary HOB cells, which suggests the presence of functional receptors. Scatchard analysis indicates that there are possibly two classes of receptors on HOB cells; higher affinity receptors which dissociate very rapidly and also lower affinity receptors. Detection of human GH receptor
mRNA on the HOB cells would confirm their presence and nature, since it may be that these receptors are not expressed spontaneously in culture and being a cell surface receptor its expression is transient and or it may be dependent on culture conditions and time.

The effect of GH at the nuclear level was reported by Slootweg et al. (1990) who reported that the addition of GH to primary mouse osteoblasts, resulted in a rapid and transient induction of the c-fos and c-myc proto-oncogenes and preceeded hGH induced mitogenesis. The induction of c-fos gene by hGH was dose-dependent, and mediated by a somatotropic (GH) receptor as opposed to lactogenic receptor on the primary mouse osteoblast. Merriman et al., (1990) in their study showed that IGF-I and IGF-II caused a rapid and transient induction of c-fos mRNA in murine osteoblast.

In this study GH has been shown to increase biochemical markers of osteoblast activity. This has been shown to be a result of both the direct stimulation of proliferation and an increase in the expression of the proteins associated with differentiation. Furthermore, GH has a paracrine action which is mediated by IGF-I and affected by IGFBP-3. It can be concluded that IGF-I, GH, and 1,25(OH)2D3 have synergistic effects on cellular activities related to bone formation. IGF-I could be the mediator of GH activity, which in turn regulates the local production of IGFBP3.

Incorporation of GH into biomaterials can provide a method for eliciting a specific cell response by delivering the agent directly to the cells concerned. The information gained from this study has helped to confirm and substantiate that there is an obvious advantage in using GH to stimulate a local effect. This knowledge will be important in devising new therapeutic strategies to control bone formation based on novel regulatory mechanisms. There are numerous orthopaedic clinical applications where this may be of value.
CHAPTER 4

The Use of Biodegradable Gelatin Microspheres for the Delivery of Growth Hormone
Introduction

Site-specific drug delivery offers the advantage of localizing a therapeutic concentration of a drug directly at the target organ, thus avoiding systemic effects. The choice of polymer matrix for this type of system is a key factor in the design of a controlled drug release system, as it influences the release kinetics of the incorporated drug (Kim et al., 1980, Kissel et al., 1992). Another factor to be considered is the ability to incorporate and release optimal concentrations of the drug in the bioactive form. The system should be stable after synthesis and have an acceptable clinical shelf-life, and its preparation should be simple and reproducible. The toxicity, biocompatibility and immunogenicity of the polymer system once placed within its biological environment should also be considered.

Biodegradable materials are becoming increasingly important for use as drug delivery systems as they have many advantages over non-degradable ones. The major advantage is that once the drug has been exhausted the material readily degrades and does not have to remain in-situ (Schakenraad et al., 1988, Rao et al., 1994). The design of a drug delivery system that fulfils both the pharmaceutical and biological criteria is not easy, and many microsphere preparations do not fulfil all the requirements mentioned.

Microspheres are well suited as drug delivery systems, as they can be made in various sizes, ranging from 50 nm to 2 mm diameter. They have a uniform well-characterised surface, with narrow size distribution and a large specific area. According to the literature the morphologies of the microspheres can vary between homogenous, heterogenous, or encapsulated forms (Arshady 1993). Drug release is governed by diffusion and/or degradation of the polymer; the release kinetics are usually more complicated since drug diffusion is influenced by the degradation of the matrix (Tice et al., 1989, Langer 1990). Erodable matrices are controlled by both chemical reactions and diffusion, and release drugs at constant rates. Monolithic systems, where the drug is dispersed within the polymer matrix, are diffusion controlled; they usually exhibit first-order release behaviour (Lee 1986, Lee and Peppas 1987).

Numerous techniques have been described for making microspheres, and the choice depends on their proposed function and size and the physicochemical properties of the drug and polymer matrix. Their use as delivery systems has been described by other workers: for example, albumin microspheres most commonly used are prepared by the formation of a water-in-oil emulsion, and are stabilized either by chemical crosslinking or by thermal denaturation of the protein. In most cases the drug loading is done during the preparation process, and it is probable that chemical reactions occur with the drug, or it may undergo thermal decomposition. Burgess et al., 1987 used thermal denaturation for the preparation
of albumin microspheres for the release of steroids, whilst Cremers et al., 1994 used a chemical crosslinking agent for the stabilization of albumin-heparin microspheres.

Other natural materials such as starch (Edman et al., 1987), and synthetic polymers including polylactides, polyglycolides and polyalkylcyano-acrylates have also been used for the preparation of microspheres; the majority are stabilized by chemical crosslinkers (Juni and Nakano 1987, Okada et al 1989). More recently Rao et al., (1994) and Jeyanthi and Rao (1987) have described the preparation of gelatin microspheres for the release of proteins. In both cases the microspheres were prepared without thermal denaturation with the crosslinking agent added in an organic medium to the aqueous gelatin solution for microsphere preparation. All the methods described in the literature use either thermal denaturation at elevated temperatures (95-165°C) for time periods between 10-60 minutes, or chemical crosslinking (Tomlinson and Burger 1987).

Gelatin, a naturally occurring polymeric protein, was chosen for preparing the microspheres in this study. It is non-toxic and non-immunogenic; with diverse uses such as food stuffs and medical products such as plasma substitutes. In addition it is widely used commercially for gel formation, emulsion and foam stabilisation, film forming, adhesives and as protective colloids. The hydrophilic nature of gelatin has advantages and disadvantages; the reactions in water are very rapid and, when placed in an aqueous solution, the chemical reaction that occurs often depends upon selecting the correct conditions so that the speed of the reaction is faster than the rate of hydrolysis (Clark and Courts 1977). The aqueous properties of hydrophilic polymers such as gelatin play an important role in solute permeability, in determining the diffusibility of the incorporated drug and the biodegradation rates of the polymer based drug delivery system.

Commercially available gelatin is obtained by the partial hydrolysis of animal collagenous tissues such as skin, tendon, ligament and bone. The hydrolysis procedure is controlled to ensure that the correct water soluble polypeptide chain is obtained (Oppenheim 1987). The hydrolysis can be conducted either under acidic conditions, producing Type A, gelatin or under alkaline conditions, which produce Type B. In this study Type A swine gelatin was used; acid prepared swine gelatin is usually isoionic with a pH range between 8.5 - 9.4. Type B swine gelatins are isoionic at about pH 5 with a minimum pH of 4.8.

Gelatin has different gelling capacities depending on the chains, mol wt, structure, crosslinking which in turn depend on the preparation procedure; this is characterised and standardized by a Bloom number and the viscosity of a 6.67% solution at 40°C (Oppenheim 1987). The Bloom number is an indication of the strength of the gels produced, the higher the Bloom number the stronger the gel. A 300 Bloom gelatin was used to make the biodegradable microspheres for this study; this has a strong gelling
capacity. Gelatin has the advantage of "gelling" on cooling, whereas albumin is "gelled" by heating or chemical cross-linking. The preparation of gelatin is less likely to cause drug damage or degradation as a result of the preparation technique.

Whilst the use of gelatin microspheres for drug release has been reported (Jeyanthi and Rao 1987), few methods have been described for the release of macromolecules (Rao et al., 1994, Di Silvio et al., 1994), and to date no method has described the use of gelatin for the controlled and modulated release of growth hormone. The aim of this present study was to develop a natural, biodegradable, biocompatible membrane-moderated, monolithic microsphere system capable of controlling the rate of drug released, able to sustain the duration of therapeutic efficacy and able to deliver the drug to specific cells in a target organ. This chapter describes the preparation of gelatin microspheres, their validation for the release of GH and optimization for controlled and modulated release.

Materials and Methods

Preparation of gelatin microspheres

The microspheres were prepared using a 20% solution of 300 Bloom gelatin (Swine skin Type A, Sigma) dissolved in sterile water. This was mixed and the gelatin left to dissolve at 40°C, the solution was then divided into two aliquots, one of which was left plain as a control. Human GH (Novo Nordisk A/S Denmark) was added to the second aliquot at a concentration of 8 IU in 10 ml of sterile water. The microspheres were formed by forcing the gelatin mixture through a pre-heated syringe with a 21G needle into chilled paraffin oil in a 30 cm long cooled column (Figure 4.1). The microspheres solidified as they passed through the column and were collected at the bottom. The paraffin oil was removed and the microspheres were collected and washed three times in chloroform to remove all traces of the oil.

Stabilization of the microspheres

Various methods for cross-linking the microspheres were investigated prior to choosing the method described below in order to achieve optimal cross-linking. The gelatin was prepared as described and the following cross-linking agents were tested: glycidyl acrylate 4%, (Aldrich), acyl azide (Sigma) and carbodiimide (Sigma) at concentrations of 0.5 and 1mg/ml respectively. In each case the cross-linking agent was added to the gelatin, thoroughly mixed at room temperature for two hours and gently heated until the gelatin was dissolved. The different mixtures were then divided into two aliquots and GH was added to one half of each gelatin batch containing the various cross-linkers. The microspheres were then made by the method described above.
Elution studies were set up in phosphate buffered saline (PBS) and horse serum (HS, Gibco Life Technologies, Paisley): the microspheres however, had completely dissolved within 24 hours in all cases, indicating that stabilization by addition of the crosslinking agents had been unsuccessful.

The method chosen to achieve optimum cross-linking without the addition of a cross-linking agent was glutaraldehyde vapour. The microspheres were placed on a wire mesh platform, in a dessicating chamber with 25 ml of 25% glutaraldehyde solution in the base, for 48 hours. After cross-linking, they became a deep yellow colour and were washed several times in 200 ml of PBS in order to remove any surface adsorbed drug. They were then dried in a stream of cool air overnight, to allow the evaporation of any chloroform that may have still been present and also any glutaraldehyde that may have got onto the microspheres.

**Elution of growth hormone from the microspheres**

The release of growth hormone was monitored in phosphate buffered saline (PBS) and horse serum (HS, Gibco), by adding 2.5 ml of solution to each 0.1 g of microspheres (mean = 28 microspheres). The release of GH was carried out at 37°C on a continuous rolling mixer. The PBS and HS was removed and replaced with 2.5 ml of fresh PBS or HS after one hour and then daily; the elution fluid was frozen at -20°C prior to assay for GH.

**Growth Hormone Assay (Immunoreactive GH)**

Immunoreactive GH was measured using an "in-house" enzyme-linked immunosorbant assay (ELISA). It uses two polyclonal antibodies; the coating antibody is a guinea-pig IgG fraction and the conjugate is a peroxidase-labelled Fab'-fragment of guinea-pig anti-human GH, with 3,3'-5,5'-tetramethylbenzidine (TMB) as the substrate for the enzymatic reaction. Optical density was measured at 490 nm with a reference wavelength of 650 nm. The standard used was 22K-recombinant human GH (antibodies and standard GH were generous gifts from Novo Nordisk A/S, Gentofte, Denmark).

**Assay validation and cross-reactivity**

Accurate dilutions of the GH-standard were performed in both PBS and HS to ensure that adequate recovery was observed in parallel dilution and to confirm the absence of cross-reactivity within the GH assay. Further GH recovery studies were performed in "binding protein-free" horse serum (GH-BP free HS) to determine whether the presence of binding proteins affected the amount of measurable GH in those studies where the elutions were done in serum. Growth hormone binding proteins were removed from the horse serum.
using an acid/ethanol extraction procedure. Briefly, the serum was mixed in the ratio 1:4 with a mixture of acid/ethanol (87% ethanol, 3% concentrated hydrochloric acid). This was mixed thoroughly and spun at 3000rpm for one hour. The serum supernatant was carefully removed and its volume recorded, it was then neutralized (2.5 x the supernatant volume) with 0.85M Tris base (Sigma) by mixing thoroughly, and spinning as above for a further one hour.

**Growth Hormone Bioassay**

The bioactivity of the GH was measured using an ESTA-Eluted Stain Assay according to the protocol of Ealey et al (1988). This cytochemical assay is based upon NB2 rat lymphoma cells and the assay signal depends solely upon the increase in cell number in response to GH, and utilizes the reduction of a tetrazolium salt to a formazan by intracellular dehydrogenase. Direct elution of the cytochemical stain from the cell microculture allows direct quantification using a microtitre plate reader (Dynatech), with a test wavelength of 570nm and a reference wavelength of 630nm. The determinations for all experiments were made on quadruplicate microcultures, and results expressed as means ± SD.

**Effect of ultrasound**

The microspheres were divided into 2 groups for elution into PBS and horse serum; these groups were then subdivided into control and test group. Those in the test group were exposed to a constant ultrasonic frequency of 40 kHz, using a DAWE 6441 ultrasonication bath, for two minutes prior to sampling of the PBS or HS. The temperature of the microspheres was monitored and maintained below 37°C, the PBS and HS were replaced as above at each time point. The elution media were retained and frozen at -20°C for GH assay. Another group was exposed for varying time periods at 50kHz, and a further group to frequencies 50kHz and 60kHz in Kerry ultrasonication bath for the same time period.

**Effect of pH**

The effect of changing the pH of the dissolution medium was tested for both plain and GH-loaded microspheres, by examining the swelling kinetics of the microspheres in PBS at pH 2.4, pH 7.2 and pH 10.5. The microspheres were weighed dry and 0.1g (mean = 28 microspheres) placed in 2.5ml PBS at the different pHs. At different time points the mass of the microspheres were recorded and the change in mass recorded. The swelling ratios (SR) were calculated by comparing the mass of the swollen microspheres to the their dry weights with time.
Figure 4.1 Schematic representation of the method used for the preparation of the gelatin microspheres. Droplets of warm gelatin solution were allowed to pass down a cooled column of paraffin oil. As the microspheres passed down the long column they solidified. The paraffin oil was poured away and the microspheres were collected into a volumetric flask with a tap. The microspheres were washed several times in chloroform to remove all traces of the oil, and were then placed on a mesh platform above a 25% glutaraldehyde solution for 48 hours. The mean diameter of the microspheres produced by this method was 0.4 mm (range between 0.3 and 0.5 mm). The GH-loaded microspheres were made using separate apparatus to prevent any cross-contamination with the plain microspheres.
Chapter 4

Syringe containing warm gelatin (gelatin solution kept warm in water bath)

Ice-cooled jacket

Cooled paraffin oil

Solid microspheres

Microspheres cool and solidify as they descend

Figure 4.1
Enzymatic degradation of gelatin

The effect of pepsin on gelatin was investigated on both plain and GH-loaded microspheres. In order to establish the optimal pepsin concentration a range from 20-1000 IU/ml was tested. 2.5 ml aliquots of PBS containing varying pepsin concentrations were added to groups of microspheres (0.1g of dry weight) and incubated 37°C; the change in weight for each sample was recorded at hourly intervals. A final concentration of 660 IU/ml of pepsin was chosen to test for degradation, this was added to the control and test group of microspheres; the change in weight recorded at hourly intervals for both.

Enzymatic degradation of GH

In order to investigate enzymes present in serum had any effect on either the amount of GH detected in serum or on the gelatin itself, different elution fluids were spiked with a known amount of GH. A final concentration of 30mU of GH was added to 5ml of each of the following; PBS, HS, GH-BP free HS, PBS containing 700 IU of pepsin, PBS containing 1000IU. This was incubated at 37°C and at regular intervals, aliquots were removed at 2, 5, 10, 30, 45, 60, 120 minutes and at 24 hours and immediately frozen, to prevent further degradation, until assayed for GH. In a separate experiment, 0.1g of GH-loaded microspheres (300mU) were placed in 5ml of the same elution fluids as above. These were incubated at 37°C and aliquots were removed at 2 minutes, then at 2, 3, 24 hours and the samples were immediately frozen until assayed.

Biocompatibility

The culture medium DMEM and the HOB cells were prepared as described in Chapter 2. The gelatin microspheres were washed several times in complete DMEM; 6 microspheres were placed into each well of a 24 well plate and seeded with 10^6 cells/well in DMEM and cultured at 37°C, in a humidified atmosphere of 5% CO₂. The gelatin microspheres were examined on a Joel JSM 35 Scanning Electron Microscope five days post-seeding.

Preparation of gelatin coated tissue culture dishes

Gelatin was prepared as described previously and poured into six 35 x 10mm tissue culture dishes to form an even layer just covering the surface. The dishes were cross-linked in 25% glutaraldehyde vapour for 48hrs. Following washing with sterile PBS and culture medium, they were seeded with 10^6 HOB cells per dish. These were then left in culture at 37°C, in a humidified atmosphere of 5% CO₂ for a duration of two weeks and were examined using a light microscope.
Preparation of gelatin coated implants

Commercially pure titanium screw implants, 2mm x 4mm, were supplied by Nobelpharma Ltd. and were coated by dipping the screws into a 20% gelatin solution containing 1 IU/ml of GH. The screws were then immediately plunged into cold water to allow the coating to solidify. The gelatin coating was cross-linked in 25% glutaraldehyde vapour, under vacuum, for 6 hours, and then the screws were placed for 12 hrs under an ultraviolet lamp (UV). The UV light had the dual effect of improving sterility and drying the coating. The gelatin coated titanium implants were placed in 35 x 10mm tissue culture dishes and seeded with 10^6 cells and cultured up to 14 days, and were examined using a light microscope.

Cytotoxicity Testing

The elution fluid from the gelatin microspheres was used to test the direct effect of any toxic degradation products on the cells that would be exposed to them. This was done using the Neutral Red uptake (NR) cytotoxicity assay which measures cell survival, based on the ability of viable cells to accumulate NR in lysosomes after the dye has diffused through the cell membrane. Any changes in the cell surface or the lysosomal membrane lead to changes resulting in decreased uptake and accumulation of the dye.

HOB cells were seeded at a density of 6,000 cells/well in complete DMEM in a 96-well plate. These were allowed to grow for a period of 24 hours, which usually resulted in approximately 60% confluency, after which the medium was replaced with elution fluids from both plain and growth hormone loaded microspheres (100ul/well). The plate was incubated for a further 24 hours at 37°C, and then the elution fluids were replaced with the dye solution (50µg/ml) for a further 3 hours, to allow dye uptake by the cells. The cells were washed with 0.5% formaldehyde/1% calcium chloride solution and the dye extracted using a solution of 1% acetic acid/50% ethanol. The absorbance was measured at 540nm on a Dynatech plate reader.

The Coomassie Blue assay (CB) is based upon the change in total cell protein arising from the inhibition of cell proliferation, and is a good indicator of cell number in the presence of a toxic agent, it was performed on cells which had already been assayed with NR. The NR stain was aspirated and replaced with CB dye (150µl/well), the plate was left to shake for 20 minutes, followed by two washes as described above. After the final wash, 150µl/well of desorbing solution (1M potassium acetate, 70% ethanol) was added and the plate left to shake for a further 20 minutes to allow the dye to go into solution and give a homogeneous solution. The absorbance was then read at 570nm test and 440nm reference wavelength.
Statistical analyses of the data presented were performed using the Students t-test (paired) to determine whether any significant differences existed between the test groups and the control.

Results

Gelatin microspheres

The method described produced homogeneous, microspheres of glassy appearance with a mean diameter of 0.4mm, (range between 0.3 and 0.5mm). The microspheres possessed a uniform, spherical shape with a smooth surface (Figures 4.2, 4.3). Although various methods were tested in order to achieve suitably cross-linked microspheres, the addition of crosslinkers such as glycidyl acrylate, acyl azide and carbodiimide proved unsuccessful with complete degradation of the microspheres at 37°C within a few hours of commencing the elution study. This was possibly due to the amount of crosslinking agent that was used, as other workers (Cremers et al 1994, Burgess et al 1994) have successfully used these agents for crosslinking. As the crosslinking agents investigated were toxic, the aim was to produce a natural degradable polymer with minimal toxicity, the amount of crosslinking agent used was kept to a minimum. For the microsphere system described, optimal cross-linking was achieved in the presence of 25% glutaraldehyde vapour for 48 hours; a colour change from pale yellow to deep yellow indicated when crosslinking was complete. Drying of the microspheres, in a stream of cool air in a fume cupboard, allowed evaporation of any residual chloroform or glutaraldehyde. The microsphere's performance and their stability was highly dependent on optimal cross-linking. The method described provided microspheres which lasted for over two months in-vitro in the absence of cells and over two weeks in the presence of cells in culture.

GH release

As Figure 4.4 indicates, the microspheres released high concentrations of GH into both PBS and HS; although more GH was detected in PBS compared to the HS it was not significant. However, following ultrasonication at a frequency of 40kHz for 2 minutes, a significant increase was observed in the amount of GH released in both PBS and HS, as indicated in Figures 4.5 and 4.6. The level of significance was tested using the paired Student’s t-test and the results can be seen in Table 4.1.
Table 4.1 Comparison of the effect of ultrasound on the release of GH in PBS and HS

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>p-Value</td>
<td>0.001</td>
<td>p &gt; 0.05</td>
<td>0.003</td>
<td>P = 0.002</td>
</tr>
</tbody>
</table>

A plot of the rate of release versus the square root of time was linear during the early stages of GH release for the period studied in both PBS and HS, for both the control group and the test group, indicating that the release of the GH from the microspheres was diffusion controlled for that time period. It was noted however, that in both cases the plot line did not go through the origin, but had a positive intercept, suggesting that there was also some immediate surface release of GH. The mean percentage increase in the amount of GH released for the test microspheres exposed to ultrasound compared to the control group was 70.9% for PBS and 58.7% for horse serum.
Figure 4.2 Optimal crosslinking of the microspheres was confirmed by a distinctive colour change of the microspheres from a straw yellow colour to a deep yellow. Once the microspheres have been crosslinked, washed and dried they can be stored for long periods of time either at RT or at 4°C.

Figure 4.3 Scanning Electron Micrograph (Joel JSM 35) of gelatin microspheres in culture, seeded with HOB cells (arrows). The cells readily attached themselves and grew well on the gelatin microspheres. After 5 days in culture there was some evidence of degradation of the microsphere.
Figure 4.4 Total amount of GH released in PBS and in HS. Each column represents the mean value of four replicates. Error bars indicate the mean ± SD. Paired Student’s t-test indicated that more GH was detected in PBS compared to HS, but this was not significant (p > 0.05).

Figure 4.5 Total amount of GH released from control and test microspheres in PBS. The test microspheres were subjected to 40 kHz (Dawe 6441 ultrasonication bath) for two minutes prior to sampling. Each point represents the mean value of four replicates. Error bars indicate the mean ± SD. Paired Student’s t-test indicated that significantly more GH was detected in the microspheres when subjected to ultrasound (p= 0.001) US = Ultrasound, NUS = No ultrasound.
Figure 4.6 Total amount of GH released from control and test microspheres in HS. The test microspheres were subjected to 40 kHz (Dawe 6441 ultrasonication bath) for two minutes prior to sampling. Each point represents the mean of four replicates. Error bars indicate the mean ± SD. Paired Student’s t-test indicated that significantly more GH was detected in the microspheres subjected to ultrasound (p = 0.003).

The diffusion coefficients (D) for both the control and the test groups, in both the PBS and HS were determined and are shown in Table 4.2. Exposure to ultrasound increased the diffusion coefficients by a factor of 6.2 in PBS and 3.7 in HS. Altering the frequency of the ultrasound had no significant effect on the amount of GH released as indicated in Figure 4.7. However, increasing the exposure time significantly affected the amount of GH released as shown in Figure 4.8.

Table 4.2 Diffusion coefficients (D) for the control and test group in PBS and HS.

<table>
<thead>
<tr>
<th>SYSTEM</th>
<th>SLOPE (units/day)$^{1/2}$</th>
<th>SLOPE (units/sec)$^{1/2}$</th>
<th>Mt/M∞ slope</th>
<th>D (cm² sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS control</td>
<td>0.98</td>
<td>$3.33 \times 10^{-3}$</td>
<td>$8.3 \times 10^{-6}$</td>
<td>$2.4 \times 10^{-15}$</td>
</tr>
<tr>
<td>PBS U/S</td>
<td>2.43</td>
<td>$8.27 \times 10^{-3}$</td>
<td>$2.1 \times 10^{-5}$</td>
<td>$14.9 \times 10^{-15}$</td>
</tr>
<tr>
<td>Serum control</td>
<td>0.73</td>
<td>$2.48 \times 10^{-3}$</td>
<td>$6.2 \times 10^{-6}$</td>
<td>$1.35 \times 10^{-15}$</td>
</tr>
<tr>
<td>Serum U/S</td>
<td>1.40</td>
<td>$4.70 \times 10^{-3}$</td>
<td>$1.18 \times 10^{-5}$</td>
<td>$4.86 \times 10^{-15}$</td>
</tr>
</tbody>
</table>
The derivation of the formula for calculating $D$ is given in detail in the discussion, it was calculated using the theory for diffusion from a sphere (Cranks, 1992). $M_t = \text{Total amount of drug released at time } t$, $M_\infty = \text{Total amount released at infinite time}$, $C_0 = \text{Initial drug loading}$, $a = \text{radius of microsphere}$, $s = \text{slope}$

Formula used:

$$D = \frac{S^2}{64\ \frac{C_0 \pi n^2 a^4}{}}$$

Parallelism was observed between the PBS, HS and assay buffer; indicating no cross-reactivity or matrix effect (Figure 4.9). Studies using 'neat' HS and GH-BP free HS spiked with GH, gave mean recoveries of 73% and 88% respectively, which were not significant, thus indicating that the difference in the amount of GH detected in the HS compared to that detected in the HS-BP free was not due to the presence of GH-binding proteins which might have bound the GH released, causing lower levels to be detected (Figure 4.10). The release of total bioactive GH can be seen in Figure 4.11, confirming that it had not been damaged during the preparation of the microspheres.
Figure 4.7 Total amount of GH released from the test microspheres following exposure to different ultrasound frequencies; 50kHz and 60kHz for two minutes; each point represents the mean value of four replicates. Error bars indicate the mean ± SD. Paired Students t-test indicated that there was no significant difference between the two groups (p> 0.05)

Figure 4.8 Total amount of GH released from control and test microspheres following exposure to a constant frequency of 50kHz for varying time periods. Each point represents the mean value of four replicates. Error bars indicate the mean ± SD. Paired Students t-test indicated that there was a significant difference in the amount of GH released for the time periods 2 minutes (p = 0.05), 6 minutes (p = 0.008) and at 8 minutes (p=0.008)
Figure 4.9 The assay for the measurement of immunoreactive GH was tested for cross-reactivity and matrix effect for the different biological fluids used. Varying dilutions of GH in the different fluids was tested. Parallelism was observed in all cases with no significant differences observed in the recovery of GH from the various biological fluids. Each point is the mean of four replicates. Error bars indicate the mean ± SD.

Figure 4.10 Recovery of GH at concentrations likely to be encountered were tested in normal HS and binding protein free HS (HS-BP free). No significant differences were observed between the two, indicating that GH does not bind to GH-binding proteins in HS. Each point is the mean of four replicates. Error bars indicate the mean ± SD.
Figure 4.11 Total bioactive GH released in PBS was measured using the ESTA bioassay. This cytochemical assay is dependent upon an increase in cell number in response to biologically active GH. The GH was able to stimulate cellular activity as shown by an increase in intracellular dehydrogenase activity, indicating that no damage had occurred to the GH during the microsphere preparation procedure and was still bioactive at day 13. Each point is the mean of two replicates. Error bars indicate the mean ± SD.

Effect of external pH change

The swelling ratio (SR) for the microspheres was calculated by comparing the weight of the swollen microspheres with the dry weight. Figure 4.12 and Figure 4.13 indicate that the swelling of the microspheres is pH sensitive for both plain and GH-loaded microspheres. The pH of the media (2.4, 7.2, 10.5) did not affect the microspheres during the first 3 hours of the experiment, and a rapid increase in SR was seen in all cases; following the initial increase however, the SR fell rapidly at pH 2.4 and 10.5.

These results indicated that water uptake was a function of the pH of the dissolution media. In neutral pH conditions the SR of the plain microspheres appeared very stable with only a slight fall observed even after 48 hours. At pH 2.4, the sharp fall could be due to water being removed from the microspheres and hence a loss in weight. In alkaline conditions (pH 10.5) an even sharper fall was observed in the SR; the gelatin structure may become unstable under these conditions, resulting in an even more rapid dissolution of the microspheres and hence a fall in SR. However, one would have expected the opposite to be true so that, as the structure broke down, they would swell even further, but this was not the case.
The SR of a material in any solvent ultimately depends upon (1) the chemical structure of the material, (2) the degree of cross-linking and (3) the chemical structure of the solvent. In the case of GH-loaded microspheres the trend was similar to the unloaded and no significant difference was observed. The fall in the SR was, however, more gradual and, at pH 7.2, a very slow fall was observed in the swelling kinetics with a much lower diffusivity of GH. The percentage swelling difference observed in 24 hours from the time of maximal swelling (7 hours) was 29% at pH 2.4 for GH-loaded compared to 67% for the plain microspheres. At pH 10.5 however, the percentage difference for the same time period was 61.9% and 77.6% respectively.

Figure 4.12 The effect of external pH on the swelling kinetics of the plain microspheres. The swelling ratio (SR) was calculated by comparing the weights of the swollen microspheres with their dry weight. Each point is the mean of four replicates. Error bars indicate the mean ± SD.
Figure 4.13 The effect of external pH on the swelling kinetics of GH-loaded microspheres, the results are expressed as SRs. A rapid rise in the SR was seen during the first 3 hours, the rate of hydrolysis was much faster at pH 2.4 and 10.5. Each point is the mean of four replicates. Error bars indicate the mean ± SD.

Enzymatic degradation

Pepsin had a marked effect on the swelling kinetics of the gelatin for both plain and GH-loaded microspheres. An increase in SR was observed in both the plain and GH-loaded microspheres during the first 2-3 hours which was followed by a rapid fall in microsphere mass in the presence of pepsin. The plain microspheres in PBS showed a swelling ratio of approximately 7.9 after 7 hours compared to 6.6 in the presence of pepsin (Figure 4.14). In contrast, a lower SR of 5.7 in the absence of pepsin compared to 3.9 in the presence of pepsin was observed for the GH-loaded microspheres (Figure 4.15). Gelatin is a protein which can be readily degraded by proteolytic enzymes such as pepsin; the degradation rate is highly dependent on the extent of cross-linking.

GH itself is susceptible to enzymatic degradation, and significant differences were observed in the levels of GH detected in serum compared to PBS. The most rapid degradation occurred in HS, with less than 50% of the GH being detectable after one hour. After three hours, 94% of the total GH in serum had been degraded, compared to 86% in PBS, 89.2% in GH-BP free HS, and 86% in both PBS containing 700IU of pepsin and PBS containing 1000IU of pepsin respectively.
When elutions from GH-loaded microspheres were performed in the same media, the GH level was lowest in HS, confirming that enzymatic degradation of the GH was the most likely explanation for the lower levels of GH detected in serum compared to PBS. Table 4.3 shows the p values for the levels of significance for the different groups.

**Table 4.3 p-values for the differences observed in the amount of GH detected in phosphate buffered saline PBS, horse serum (HS), growth hormone binding protein-free HS (GHBP-free HS) and different concentrations of pepsin.**

<table>
<thead>
<tr>
<th>System</th>
<th>HS vs GHBP-free HS</th>
<th>HS vs PBS</th>
<th>HS vs PBS pepsin 700IU</th>
<th>HS vs PBS pepsin 1000IU</th>
<th>GHBP-free HS vs PBS pepsin 700IU</th>
<th>700IU pepsin vs 1000IU pepsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Value</td>
<td>NS</td>
<td>NS</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td>0.019</td>
<td>0.043</td>
</tr>
</tbody>
</table>

**Figure 4.14** The effect of pepsin, a proteolytic enzyme on the swelling kinetics of plain microspheres. The swelling ratio (SR) was calculated by comparing the weights of the swollen microspheres with their dry weight. A rapid rise was seen in the SR during the first 24 hours, followed by a sharp fall in the presence of pepsin. Each point is the mean of four replicates. Error bars indicate the mean ± SD.
Figure 4.15 The effect of pepsin on the swelling kinetics of GH-loaded microspheres. A rapid rise was seen in the SR during the first 24 hours, followed by a sharp fall in the presence of pepsin. Lower SRs were observed in the GH-loaded compared to the plain microspheres. Each point is the mean of four replicates. Error bars indicate the mean ± SD.

**Biocompatibility**

Figure 4.16a shows a scanning electron micrograph of numerous osteoblasts on the gelatin microsphere indicating the biocompatibility of gelatin. The control osteoblasts growing on thermanox can be seen in Figure 4.16b. The cells grew well and appeared to embed themselves within the gelatin matrix, and, although some degree of degradation was visible, the microspheres were still not fully degraded even after two weeks in culture in the presence of cells (Figure 4.17a). At higher magnification the osteoblast were seen 'burrowed' within the gelatin matrix, some degradation of the gelatin matrix was seen (Figure 4.17b).

The cells grew to confluency on the gelatin coated tissue culture plate within a short period of time, indicating biocompatibility of the gelatin (Figure 4.18a), the cells formed a very dense covering on the gelatin surface (Figure 4.18b). Similar biocompatibility was seen with HOB cells rapidly covering the entire gelatin coated titanium implant (Figure 4.19a), osteoblasts were also observed growing down the screw thread (Figure 4.19b).
Figure 4.16

**Figure 16a** Scanning electron micrograph showing numerous HOB cells (C) after 5 days in culture growing on the gelatin microspheres (G). The cells were seen to embed within the gelatin matrix (arrows).

**Figure 16b** Scanning electron micrograph of 5 day control HOB cells (C) growing on Thermanox. Cells can be seen covering the entire surface.
Figure 4.17

Figure 4.17a Scanning electron micrograph of HOB cell (arrow) penetrating the gelatin microsphere (G).

Figure 4.17b Scanning electron micrograph at a higher magnification showing an osteoblast (C) embedded within the gelatin matrix (G). Areas of degradation can be seen around the cell.
Figure 4.18

Figure 4.18a Light micrograph of HOB cells growing on a gelatin coated plate. The cells grew on the gelatin surface becoming rapidly confluent. Magnification x 218

Figure 4.18b Light micrograph of densely packed HOB cells growing on a gelatin coated dish. After 14 days the cells began to form layers. Magnification x 218

Figure 4.19

Figure 4.19a Light micrograph of a gelatin coated titanium screw fixture (S) with HOB cells. The cells rapidly covered the screw (arrows). Magnification x 218

Figure 4.19b Light micrograph of HOB cells (C) growing on the gelatin coated screw (S), the cells were also seen growing between the screw threads (arrow). Magnification x 218
Gelatin produced no toxic degradation products; Figure 4.20 shows the results of the NR assay, where the cells were incubated in elution fluid from the microspheres. Cell viability appeared to be reduced significantly (P<0.05) only in the plain PBS solution. Cell viability in the presence of PBS containing GH was not significantly reduced. This reduction observed was probably a consequence of nutrient deprivation of the cells and not a sign of cytotoxicity. A similar reduction was observed in total protein as indicated by the CB assay(Figure 4.21). Interestingly, the GH-loaded microsphere elution fluid had a higher absorbance reading than the plain; as the NR assay measures cell survival, it appears that more viable cells were present in the wells containing the GH-eluted fluid. From the conclusion drawn in chapter 3 on the effect of GH on cell proliferation, the increase in cell number observed was due to the direct effect of GH.

![Figure 4.20 Graph of NR cytoxicity assay for gelatin elution fluids. HOB cells were incubated in control culture medium (DMEM, 10% FCS), elution fluid from plain microspheres (PBS) and elution fluid from GH-loaded microspheres (PBS). A slight, but not significant, reduction in cell viability was observed in the elution fluid from GH-loaded microspheres. However, a significant reduction was seen in the cell viability in the plain microsphere elution fluid (P<0.05). This was possibly a consequence of the cells being deprived of nutrients and not a direct effect of toxic leachables from the gelatin. The difference observed between the plain and the GH-loaded microspheres was due to the direct effect of GH on cell proliferation. Each point is the mean of 12 replicates. Error bars indicate the mean ± SD](image-url)
Figure 4.21 Graph of CB assay which measures total cell protein. HOB cells were incubated in control culture medium (DMEM, 10% FCS), elution fluid from plain microspheres (PBS) and elution fluid from GH-loaded microspheres (PBS). A significant reduction was seen in the absorbance of those cells in plain gelatin elution fluid, indicating a fall in cell proliferation (P<0.05). This was possibly a consequence of the cells being deprived of nutrients and not a direct effect of toxic leachables from the gelatin inhibiting the cells. This was not the case in the elution fluid containing GH. Each point is the mean of 12 replicates. Error bars indicate the mean ± SD

Discussion

The concept of sustained release of therapeutic agents has been in existence for many years and many systems have been described including encapsulated beads or pellets, coatings and porous materials containing the dispersed drug (Davies and Illum 1988, Cohen et al., 1991, Conte et al., 1993). Many of the systems are dependent on the polymer swelling and releasing its contents into the surrounding fluid, which can result in problems with solute stability (Lee 1985, 1986). The release of agents from monolithic systems usually displays zero-order kinetics, with release rates diminishing with time (Lee and Peppas 1987). Mathematical models for sustained release of water soluble drugs have been described which indicate that the release is dependent upon the amount of drug loaded and upon dissolution and diffusion (Baker and Lonsdale 1974, Lee 1985, Gurney et al., 1982). In part these obey Ficks Law where the diffusion coefficient is constant with time and the dimensions of the system remain the same (Lee 1986, Lee and Peppas 1987).
This study has shown that microspheres can be used as a delivery system for GH, with the drug uniformly dispersed within the gelatin matrix. The release of GH from the microspheres is partially due to diffusion. If the release process is diffusion controlled, then the theory for diffusion from a sphere should apply (Cranks 1992).

\[ \frac{M_t}{M_\infty} = C_0 \left( \frac{D_t}{a^2} \right)^{1/2} \left\{ \frac{1}{\Pi^{1/2}} + 2 \sum_{n=1}^{\infty} \text{ierfc} \frac{na}{\sqrt{D}t} \right\} - 3D_t/a^2 \]

\( M_t = \) Total amount of drug released at time \( t \), \( M_\infty = \) Total amount released at infinite time, \( C_0 = \) Initial drug loading, \( t = \) time, \( a = \) radius of microsphere, \( D = \) diffusion coefficient.
\( \text{ierfc} = \) the integrated error function.

However, the data presented demonstrates a loss of approximately 2.5% of the total drug incorporated. This represents only a small percentage of the incorporated drug, and may well be due to a partition coefficient between the gelatin and the water. The permeability of drugs through polymeric membranes is influenced by the chemical structure of the polymer itself, which determines its hydrophilicity. Kim et al., (1980) have described two basic mechanisms for explaining solute transport through a polymer membrane. The first is a "pore" mechanism where solute transport occurs via diffusion through microchannels or pores that exist within the polymer network. The second is the partition mechanism, where solute transport is thought to occur by dissolution of the solute within the polymer, followed by its diffusion through the polymer. In general the release of the solute through the "pore" is dependent on the molecular weight, which is not thought to be the case in the "partition" membrane system.

When considering solubility, one has to take into account the solubility of GH in water and in the gelatin solution. GH is very soluble in water, but it may be that it is not so readily soluble once it is incorporated within the gelatin, where it becomes chemically bound to the polymer protein.

In view of the fact that the plots in Figures 4.1 are linear, it is reasonable to assume that,

\[ \text{ierfc} \frac{na}{\sqrt{D}t} \text{ is } < \frac{1}{\Pi^{1/2}} \]

Equation (1) then reduces to:

\[ \frac{M_t}{M_\infty} = 6 \left( \frac{D_t}{\Pi a^2} \right)^{1/2} - 3D_t/a^2 \]
This equation is a general quadratic, but because of the linearity of the \( t^{1/2} \) plot, it is further assumed that the second term is much less than the first. This proves to be the case. For the highest diffusion coefficient taken and for a time of 4 days,

\[
3Dt / a^2 = 0.6, \quad \text{and} \quad 6 \left( Dt / \Pi a^2 \right)^{1/2} = 1.51
\]

Also noting that:

\[ M_{\infty} = \frac{4}{3} \Pi a^3 C_0, \]

where \( C_0 \) is the concentration of the drug incorporated. Equation (2) then reduces to:

\[
(3) \quad M_t = 8 C_0 a^2 \left( \Pi D t \right)^{1/2}
\]

However, a number of microspheres are used experimentally, hence if there are \( n_i \) microspheres of radius \( a_i \), then equation (3) becomes:

\[
(4) \quad M_t = 8 C_0 \left( \Pi D t \right)^{1/2} \sum_i n a_i^2
\]

Because the range of microsphere sizes is 0.3-0.5 mm, equation (4) can sensibly be written

\[
(5) \quad M_t = 8 C_0 \left( \Pi D t \right)^{1/2} n a^2
\]

where \( n \) = total number of microspheres, and \( a \) = mean radius (mm)

The slope \( (s) \) of the \( t^{1/2} \) plot is:

\[
S = 8 C_0 \left( \Pi D \right)^{1/2} na^2
\]

\[
(6) \quad D = \frac{S^2}{64 C_0 \Pi n^2 a^4}
\]

The outward diffusion of the drug is, dependent upon inward diffusion of water, so the process is more complex than accounted for by the above relatively simple theory. The diffusion of the water into the gelatin microsphere is concentration dependent and possibly time dependent as well. When immersed in water gelatin swells and softens, absorbing 5-10 times its weight of water. As a polyelectrolyte both the solubility and the conformation of the gelatin chain is a function of the type and concentration of other solutes present. In
view of the linear $t^{1/2}$ plots for the release of the GH in this study, this was not time dependent over the period studied, but it may have been concentration dependent.

The release of drugs from monolithic systems has been found to be dependent on numerous factors, and could be due to the drug, the nature of the microsphere and the environment it is placed in. Several mechanisms have been suggested for drug release, including surface erosion, disintegration, microsphere swelling, drug diffusion and desorption (Tomlinson 1983).

The significant effect of ultrasound on the amount of GH released in both PBS and HS suggests that the amount of drug released was dependent on polymer degradation. Kost and co-workers (1988, 1990) have shown that it is possible to increase the degradation rate of polymers significantly by using ultrasound, resulting in an increase in the release rate of incorporated agents. Ultrasonication of the microspheres was used in this system as a method for enhancing the amount of GH released; this was achieved with more GH being detected in both PBS and HS following exposure to ultrasound. The increase in degradation observed in the test group was probably caused by a breakdown in the gelatin structure, which in turn caused a change in the kinetics, resulting in further degradation. The more degradation occurs the more water penetrates into the matrix resulting in a raised diffusion coefficient and increased release of incorporated GH.

One explanation for this increase is the breakdown of covalent bonds that form the 'backbone' of the gelatin structure, and/or cavitation within the gelatin matrix induced by the ultrasonic waves. It is also possible that ultrasound may cause a 'temporary' change in the gelatin structure by non-enzymatic hydrolysis, causing more GH to be released, and followed by a "reassembling" of the structure once the ultrasound has been stopped.

The biological environment in which the delivery system is placed will have an effect on both the rate and the amount of drug released. This was found to be the case when the microspheres were placed in different media; more GH was detected in PBS than in HS. It is possible that proteins in the serum could become adsorbed onto the gelatin microspheres resulting in a protective proteinaceous coating around them preventing GH release. It has been shown that coatings on drug delivery vehicles can affect the release rates and dissolution of the incorporated drug (Eglal 1976). However, this seems unlikely because very different diffusion coefficients would be expected depending upon the medium, and this was not found to be the case.

An alternative explanation for the difference observed could be that GH was released in the same quantities in both cases, but, in the serum, GH may have become bound to, and formed a complex with the GH-binding proteins present in serum preventing detection of
all the immunoreactive GH (Baumann et al., 1988, Herington et al., 1986). This hypothesis was tested and recovery studies showed this not to be the case as the differences observed were not significant.

The most probable explanation for the difference observed is that degradatory enzymes in the serum had an effect on the released GH, so that lower levels were detected than in PBS, although similar amounts were released into both media. This was confirmed to be the case; the rate of degradation of GH was found to be much faster in serum and in buffer containing proteolytic enzymes, when tested both in a gelatin matrix and neat.

Differences were observed in the SR in the different pH dissolution media. Lee et al. (1985) have shown that glassy polymers swell until they are at thermodynamic equilibrium with the liquid penetrant or solvent. The polymer matrix did not completely dissolve due to crosslinks and entanglements found within the gelatin structure; this probably explains why the microspheres in this study did not rapidly dissolve. They showed that, within a swelling glassy polymer, there are two phases: swelling followed by true dissolution as a result of the gelatin breaking down. This theory suggests that initially a polymer solvent interface moves outwards from the centre of the material. This is due to the rapid entry of the solvent into the polymer whilst a swelling interface moves outwards, as the amount of solvent present at the centre of the polymer increases. Once the concentration of the penetrant at the polymer/solvent interface reaches a certain level it allows the molecules to disentangle. This then allows the dissolution proper of the gelatin to occur, resulting in a loss in mass and a fall in SR. If a linear polymer (no crosslinks) is placed in a liquid, there are two possibilities: (a) it dissolves or (b) it just swells, and the degree of the swelling varies with the liquid. If the polymer is crosslinked and placed in what would otherwise be a solvent it swells, and the swelling ratio is a function of the molecular weight.

Both the control and the GH-loaded microspheres swelled up to a maximum value before losing to a lower mass and beginning to degrade. The presence of GH within the microspheres had no significant effect on the SR, although the plain microspheres were more stable in neutral pH conditions than the GH-loaded ones. In the case of the GH-loaded microspheres, the GH dissolved into the penetrant and began to pass out of the gelatin as it became swollen in the surrounding media. The swelling in this case was probably controlled by a combination of both drug diffusion and the rate at which the penetrant entered the gelatin matrix. Swelling in albumin-heparin microspheres has been explained by the ionization of functional groups of the albumin and heparin (Cremers et al., 1994). The degree of swelling is influenced by the degree of stabilization achieved by the crosslinking; if this is high the swelling is reduced.
If any linear polymer is put in a liquid, there are two possible occurrences: either it dissolves or it swells. If a polymer is crosslinked, as in the case of the gelatin microspheres, it can only swell and will not dissolve irrespective of the solvent it is placed in. However, the fall in SR of the gelatin microspheres could be a direct effect of gelatin pieces "falling-off" and causing a weight loss however, this would have resulted in spurious weight losses which was not the case. The weight loss in the microspheres was consistent, so it is most likely that the change in weight was due to changes in the chemical structure of the gelatin itself.

Forni et al., (1992) showed that drug loading had a greater influence on drug diffusion than on dynamic swelling. If drug diffusion predominates then the microspheres proceed towards a lower equilibrium value, but if water diffusion predominates then it will proceed towards a higher equilibrium value. The release patterns in this study indicate that the drug followed simple diffusion for the initial period of release. The percentage increase in maximal swelling at pH 2.4 and 10.5 was higher in the plain gelatin, but overall the presence of the drug did not alter the osmolarity enough to significantly alter the SR. It has been suggested that the presence of drugs within a polymer leads to interconnecting channels, as the drug diffuses out the porosity of the polymer, increases and hence reduces the integrity of the polymer crosslinking resulting in a higher SR (Gale et al., 1980, Brook and Van Noort 1985). However, this was not found to be the case with the GH-loaded microspheres; introducing GH into the gelatin probably caused an increase in the density of the gelatin resulting in a lower swelling capacity.

Both Forni's (1992) and Lee's (1986) work suggests that water absorption into microspheres increases the diameter, whilst drug diffusion will decrease it, due to a fall in osmolarity, and that the swelling of the microspheres is a balance between the two. The differences in SR observed at the various pH's could be explained by changes in the actual gelatin structure; in both acid and alkaline conditions water is being removed from the system rather than being taken up, hence the drop in SR.

In the GH-loaded microspheres, the ionic nature of the GH dispersed within the gelatin has to be taken into account, as it gives rise to an "ionic polymer" containing ionizable groups; in the case of GH these are cationic -NH2 groups and anionic COOH-groups. The dissociation of these ions may cause changes in osmotic pressure giving rise to changes in SR. Any water soluble additive will increase water uptake due to osmosis, in which case the osmotic pressure is inversely proportional to the molecular weight of the additive. The ionic strength of the dissolution media can cause modification of the gelatin and can therefore affect the swelling of the microspheres.
In view of the fact that only a small amount of GH is actually released during the initial period, it is debatable whether the binding of GH to the gelatin is chemical or physical; drugs are known to interact or bind with the carrier polymer resulting in reduced drug release. The fact that GH was able to retain both its immunoreactivity and bioactivity suggests structural integrity of the gelatin and indicates that the GH becomes physically bound within the gelatin and is not damaged; it also confirms that the method of preparation is suitable for peptides. The methods of preparation of microspheres in other studies usually incorporates the bioactive agent either before polymerization or before crosslinking or thermal treatment. These methods are not suitable, therefore, for the incorporation of bioactive agents which are thermally unstable or can react with the monomers or crosslinking agent.

The study described in this chapter has shown that gelatin appears to be an ideal polymeric matrix for use in the preparation of microspheres to be used as drug delivery systems. The microspheres described have major advantages compared to those described by other workers; their preparation does not involve thermal reactions, there is no addition of crosslinking agent to the aqueous gelatin solution, and any residual glutaraldehyde that may be present is evaporated and removed during the extensive washing procedure. This assures physical and chemical stability of the incorporated drug. Being a natural polymer, gelatin does not pose toxicity problems; Marty (1977) found that weekly injections of drug-free gelatin microspheres into mice over a 12-week period did not induce an adverse effect.

The majority of incorporated drugs are covalently bound to the matrix carrier, which means that they will be presented to the immune system as a hapten, although separately neither the drug nor the carrier is immunogenic. However, when combined, the drug-carrier conjugate may stimulate an antigenic response. It is therefore necessary to consider the in-vivo immunological response to the drug-carrier complex when developing drug-delivery systems.

This gelatin based system offers the possibility of releasing macromolecules, providing a simple and more efficient way of delivering drugs to a target site. The setting temperature of gelatin (35°C) and its pH limits (8.5-9.4) make it a suitable matrix for the incorporation of peptides and other factors that may be susceptible to damage by excessive changes in temperature and pH. Furthermore, the system is stable, and has the advantage that once the drug has been exhausted, it is readily degraded, at a rate which can be controlled by the extent of crosslinking.

Other workers have described the addition of magnetite to the carrier matrix, so that it can be externally activated by magnetic forces, to release more drug (Kost et al., 1987,
Although these types of systems offer a mode of increasing the release of the drug, they also involve the addition of another component to the drug-delivery polymer matrix system, hence increasing the possibility of antigenicity. In comparison, the method described here offers a non-invasive method of alteration of drug release depending on clinical requirement. This may improve the release pattern and efficacy of peptides and hormones such as GH, which are usually released in a pulsatile manner.

Gelatin has been shown to be biocompatible *in-vitro* both as a coating and in the form of microspheres. Human osteoblasts grew to confluency around the coated implants and also on the gelatin microspheres. The cells retained their morphology and their phenotype, and appeared unaffected by any degradation products, making gelatin a suitable polymer for *in-vivo* use. The microspheres could be applied directly at the bone implant site, for example, for filling bone defects, or they could be incorporated into allograft material to stimulate tissue regeneration. An increase in local bioavailability of drugs such as GH, could be of potential use in disease states, for example, where there has been loss of bone stock or where tissue regeneration and wound healing is desired, by stimulating the appropriate cells directly, the repair process would be more rapid. Gelatin could also be used as a coating on metals and other materials as a delivery system to improve bonding at bone and soft tissue interfaces.

The gelatin microspheres described in this chapter have several advantages over existing systems and fulfil the majority of requirements needed for a drug-delivery matrix for peptide and protein release.
CHAPTER 5

The Use of Non-Degradable Poly(ethylmethacrylate) Tetrahydrofurfuryl Methacrylate for the Delivery of Growth Hormone
Introduction

The polymer system used in this study comprises poly(ethylmethacrylate) (PEM) powder and the heterocyclic monomer tetrahydrofurfuryl methacrylate (THFMA) containing N,N-dimethyl-p-toluidine (2.5% v/v) (developed at The London Hospital Medical College by Dr M. Patel and Professor M. Braden). It was originally developed for aural and dental applications (Bhusate and Braden, 1985, Patel and Braden 1991). Its use in orthopaedics is new, and it has many useful properties that make it desirable for use as an orthopaedic material. Its validation as a potential drug delivery system for use in orthopaedics will be described in this chapter.

As a consequence of the high molar volume of THFMA, this polymer has a lower polymerization shrinkage than poly (methyl methacrylate), PMMA. Another advantage of this polymer system is its hydrophilic nature, and as water plays an important part in the permeability and biodegradation rate of polymer-based drug-delivery systems, it is ideal for the absorption of tissue fluids and proteins from the surrounding bone matrix and marrow. The water absorption of polymers in clinical applications is important, the PEM/THFMA system studied exhibits a high water uptake up to 34% in 2 years (Patel and Braden 1991). The mechanism of this is not clear, but is believed to be related to a clustering mechanism. The hydrophilic nature of this polymer makes it suitable for the incorporation and release of water soluble agents such as growth hormone, which diffuses out of the polymer system into the surrounding tissue and so encourages repair.

The clinical success of orthopaedic biomaterials requires bony ingrowth for integration and fixation. An ideal bone implant is one that is readily incorporated into host bone to produce an osseointegrated bone-implant that is structurally and mechanically comparable to the host bone. The success of this type of interaction involves mechanical, physical and biological factors.

Bioactive materials have been described which enhance bony ingrowth, for example hydroxyapatite has been used as a coating for titanium implants (Geesink et al., 1992, Klien et al., 1994). Another approach is the controlled release of incorporated growth factors or osteogenic promoters to improve bone-bonding at the interface. Downes et al., (1991) have shown that the in-vivo response to GH released from bone cement was an increase in osteoid formation which resulted in an improved remodelling of the tissue components. In another study, GH was incorporated in calcium phosphate ceramics; an increase in osteoid formation was observed with collagen fibres extending between and in direct contact with the GH-loaded ceramics (Downes et al 1991, 1992).
An improvement in cementing techniques and prosthesis design have led to fewer fractures of prostheses and less bone resorption, achieving a better interlock at the bone-cement interface (Harris 1980, Crowinshield et al., 1980 and Huiskes 1980). However, the design of a bioactive material for use in orthopaedic surgery which has a secondary function as a controlled drug-delivery system could prove beneficial.

In situations where osteogenesis is required it would be advantageous to have an increased number of osteogenic cells in the vicinity of the implant site, this would increase the rate of repair and the osteogenic response. Bone has unique self-regenerating properties in response to mechanical injury or tissue wasting, and the rate of bone formation is determined by both the number of osteoblasts, and the bone matrix activity of the cells. It is becoming increasingly apparent that these properties are due in part to the presence of bioactive polypeptide factors in the extracellular matrix of bone (Canalis, 1985, Hauschka and Hall, 1990, and Mohan and Baylink, 1991). Control of cell proliferation locally, should allow the control of osteogenesis, and the release of growth factors such as GH to the target cells, could stimulate the formation of new osteoid which mineralizes with time, thereby improving the bone material interface.

In this study the polymer system PEM/THFMA was evaluated for the release of GH. This particular growth factor was chosen as there is increasing evidence that GH can have a direct effect on osteoblasts mediated by locally produced Insulin-like Growth Factor-I (Chenu et al., 1990, Nilsson et al., 1990, Kassem, 1993). By releasing the drug locally to the tissues surrounding the implant it is possible to stimulate osteoblast proliferation which may improve osseointegration at the tissue and implant site.

Although a wide range of materials are now available for use as vehicles for drug release, factors such as the complexity of the biological environment in which they have to function and the limitation of the type of drug they can release, have to be considered (Cohen et al., 1991, Cole et al., 1993, Cascone et al., 1994 and Di Silvio et al., 1994). The major disadvantage of current delivery systems is that few systems allow modification of the release once it has commenced. The delivery of drugs to a target site for a sufficient time period can be improved greatly by having a system which primarily releases the drug at the site of action and also allows manipulation to alter the amount of drug released depending on requirement (Kost 1993, Di Silvio et al., 1994).

Many natural and synthetic hydrophilic polymers that are able to absorb and retain large quantities of water have been described, an example is poly-hydroxyethylmethacrylate (HEMA). Studies by Otto Wichterle and co-workers (1960) have shown its successful application for ocular devices, their use for surgical implants however, it is limited and very few clinical trials have been reported. Different polymerization techniques and
different porosities can affect the behaviour of hydrogels, Chirila et al., (1993) showed that increasing the amount of water in the initial monomer mixture affected the cellular invasion and deposition of calcium salts in the implants.

The polymer system used in this work has a further advantage over other hydrophilic polymers of being able to swell in situ and remain rigid, furthermore it does not change structurally (Patel and Braden 1991). By gelling copolymers of poly(EM/THFMA) with hydrophilic monomers such as HEMA and THFMA it is possible to change the hydrophilicity of the system and thus the rate of drug release (the copolymers used in this study were made by Hulda Swai, The London Hospital Medical College).

The extent of the exothermic reaction of any material is of potential clinical importance if it is to be used directly in the aural cavity or elsewhere in the body, as a high exotherm could destroy any added therapeutic agent added to it, apart from the effect of heat per se. For example, the high reaction exotherm of PMMA bone cement limits the choice antibiotics that can be incorporated. The extent of the exothermic reaction is a problem in orthopaedic bone cements; temperatures in excess of 90°C can occur in the femoral cavity resulting in tissue damage. The polymer used in this study has a further advantage over currently available materials of being a room temperature polymerising system, where the temperature increase during polymerization did not exceed 36°C, thus making it suitable for the incorporation of macromolecules such as peptide growth factors.

This polymer system has good intrinsic biological properties and numerous advantages over existing polymers, making it a challenging candidate for validation as a controlled drug-delivery system. This chapter describes the biological evaluation and optimization of the non-degradable polymer PEM/THFMA for use in orthopaedic surgery for controlled local drug delivery. It has potential use as an implant material, capable of site specific delivery which could be used in fracture repair and healing of osteotomies.
Materials and Methods

Different mixing techniques as a means of optimising drug release.

Release of GH and the protein bovine serum albumin (BSA) from differently prepared polymer discs

Recombinant human GH (generous gift from Novo-Nordisk A/S Denmark) was incorporated into the polymer by adding 12IU of GH to 10g of PEM (Bonar Polymers) powder with thorough mixing before the addition of 5ml of THFMA (Ex-Röhm) monomer. Discs containing protein were made by the addition of 1.25g of BSA (Sigma) in 10g polymer with the addition of 5 ml monomer. Several different hand mixing techniques were employed for the mixing stage of both the GH and BSA-loaded discs. The following methods were employed using a mixing time of 45 seconds: (i) Control- normal mix with 2 beats per second, (ii) Slow mix - 1 beat per second, (iii) Fast - 4 beats per second, (iv) Centrifuged - 2 beats per second, followed by centrifugation at 2,500g for 2 minutes, (v) Pressurized - mixed 2 beats per second followed by thumb pressure for 1 minute, (vi) Syringed - as per control mix followed by pressure applied with syringe plunger. These were then cast in PTFE moulds (8mm diameter, 3mm thickness). Elution studies were set up by incubating the respective discs in 5ml phosphate buffered saline (PBS) at 37°C on a continuous rotating mixer. The elution fluid was removed and replaced at regular intervals and stored for assay of the additive.

Growth Hormone Assay

Immunoreactivity of the GH released from the PEM/THFMA system was measured using the in-house ELISA method described in Chapter 2.

Protein Assay

The Bio-Rad protein assay was used for protein estimations. This is a dye-binding assay and works on the differential change of a dye in response to various concentrations of BSA. Samples were assayed in duplicate.

Scanning Electron Microscopy (SEM)

SEM was used to examine both the surface and internal morphology of the differently mixed polymer discs. The discs were mounted onto scanning electron microscope stubs and the surfaces were coated with gold using a Polaron sputter coater prior to examination using a Joel 35C Scanning Electron Microscope.
Stability and Bioactivity

In the second experiment the GH-incorporated discs were made up as per standard protocol and left in a sealed container at the following temperatures: -20°C, 4°C, 20°C (RT), 37°C and 56°C for a period of one month. Elution studies were then performed as above in PBS at 37°C. The diffusion coefficients were calculated as described over the next page.

The GH- bioactivity was measured using the ESTA- bioassay described in Chapter 2

Effect of varying methods of polymer preparation

The effect of changing polymer : monomer ratio was investigated. Copolymers were made with the following EM:THFMA monomer ratios; 30:70, 40:60, 50:50, 60:40, 80:20 and 90:10. The effect of gelling these copolymers with the monomers, THFMA and HEMA was also investigated for their ability to release GH and BSA. In all cases both the immunoreactivity and the bioactivity of the GH were measured. The diffusion coefficients were calculated for the different ratios.

GH Dose-Dependent Release

Two groups of PEM/THFMA discs were prepared as described above, in one group the copolymer ratio 60:40 was used and in the other a 50:50 ratio was used, both were gelled with HEMA. Each group was further subdivided in to three, and different amounts of GH was added; 12, 8 and 4IU per 10g of PEM powder. Elutions were performed as described previously.

Biocompatibility

The biocompatibility of the polymer was tested using HOBS seeded directly onto discs of plain and GH-loaded polymer. These were made as described previously, but under sterile conditions, and were left for 48 hours in a sterile hood to allow evaporation of any residual monomer. Prior to use they were washed extensively in sterile complete DMEM and seeded with 10^5 HOB cells in a 100 µl concentrate (cell preparation described in chapter 2). These were left for one hour to allow the cells to adhere, prior to flooding with 5ml DMEM, they were incubated for a further 24 hours at 37°C, 5% CO₂ in a humidified atmosphere. Following incubation the media was removed and replaced with 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and allowed to fix overnight. The polymer discs could not undergo dehydration in graded alcohols as this affected the polymer, instead, they were air dried and then sputter-coated with gold. All the specimens were examined using a Joel 35C scanning electron microscope.
Results

The effects of mixing techniques on drug release.

GH release from the system was highly dependent on the method of sample preparation, both in terms of the amount released and the diffusion coefficient. Figure 5.1 shows the release profiles for BSA; in all cases an initial rapid release was observed, and was followed by a slower continuous release. A similar initial release pattern was also observed for the release of GH; Figure 5.2 shows the results for the first 72 hours and how by increasing the speed of mixing it dramatically improved the rate of GH release from the polymer. The percentage GH released from the different methods can be seen in Table 5.1. The fast mix polymer released a significantly greater percentage: 240% more GH over a period of 8 days, compared to the control. (Paired Student's t-test, P=0.001). The slow mix and those where pressure was exerted released significantly less GH (P=0.001) compared to the control.

A plot of $M_t / M_\infty$ versus the square root of time was obtained for the different samples. $M_t =$ Total amount of drug released at time $t$, $M_\infty =$ Total amount of drug released at infinite time, $S =$ slope at time $t^{1/2}$, $a =$ radius, $2l =$ length. The slope of the resulting linear graph was found for the early stages of diffusion and used to calculate the diffusion coefficient ($D$) for the differently mixed samples using the following equation (derived from Crank, J., 1993):

$$S = 4 \left( \frac{D}{\Pi} \right)^{1/2} \left[ \frac{2}{2a} + \frac{1}{2l} \right]$$

$$D = \frac{\text{Slope} \times s^2 - x \Pi}{16 \left[ \frac{2}{2a} + \frac{1}{2l} \right]^2}$$

Representative plots of $M_t / M_\infty$ versus the square root of time for the control and fast preparation methods can be seen in Figure 5.3a and 5.3b, respectively.
Table 5.1 Total amount of GH released (%) from the differently prepared polymers.

<table>
<thead>
<tr>
<th>System</th>
<th>( M_\infty ) GH</th>
<th>% Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control mix</td>
<td>213.6</td>
<td>31.5</td>
</tr>
<tr>
<td>Fast mix</td>
<td>512.3</td>
<td>75.4</td>
</tr>
<tr>
<td>Slow mix</td>
<td>17.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Centrifuged</td>
<td>14.6</td>
<td>2.2</td>
</tr>
<tr>
<td>Pressurized</td>
<td>33.8</td>
<td>5.0</td>
</tr>
<tr>
<td>Syringed</td>
<td>23.6</td>
<td>3.5</td>
</tr>
</tbody>
</table>

The slopes were estimated for the early stages of diffusion, and results for the different systems can be seen in Table 5.2. The slope of the graph was used to calculate the diffusion coefficients (D) e.g. Control mix:

\[
D = \frac{\text{Slope} \times \Pi}{16 \left[ \frac{2}{2a + 1/2l} \right]^2}
\]

\[2a = 1.07, \ 2l = 0.59, \ \text{slope} \ (s) \text{ at } t^{1/2} \text{ 0.35 days} = 1.714 \ (\text{units} / \text{day})^{1/2}\]

or \[5.831 \times 10^{-3} \ (\text{units} / \text{sec})^{1/2}\]

\[
D = \frac{\left(5.831 \times 10^{-3}\right)^2 \times \Pi}{16 \left[ \frac{2}{1.07 + 1/0.59} \right]^2}
\]

The Diffusion Coefficient (D) for the control mix = 5.256 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}. The results obtained for D, for the other mixing methods are given in Table 5.2.

The density of the differently mixed polymers was calculated by weighing the discs in air and then in water and the results can be seen in Table 5.2:

\[
\text{Density} = \frac{\text{Weight of disc in air} - \text{Weight of disc in water}}{\text{Weight of disc in air}}
\]
Table 5.2 Diffusion Coefficient and density for the different systems tested.

<table>
<thead>
<tr>
<th>System</th>
<th>Slope (units/sec)$^{1/2}$ x10$^{-3}$</th>
<th>D (cm$^2$.sec$^{-1}$) x10$^{-7}$</th>
<th>Density (g/cm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.87</td>
<td>5.256</td>
<td>1.2073</td>
</tr>
<tr>
<td>Fast</td>
<td>5.35</td>
<td>7.570</td>
<td>1.2620</td>
</tr>
<tr>
<td>Slow</td>
<td>6.80</td>
<td>5.788</td>
<td>1.2498</td>
</tr>
<tr>
<td>Centrifuged</td>
<td>4.37</td>
<td>2.576</td>
<td>1.2221</td>
</tr>
<tr>
<td>Pressurized</td>
<td>4.76</td>
<td>2.109</td>
<td>1.2428</td>
</tr>
<tr>
<td>Syringed</td>
<td>4.37</td>
<td>3.091</td>
<td>1.2310</td>
</tr>
</tbody>
</table>

If a material has many air spaces within it, one would expect the density to be low, however, this was not the case. Interestingly, the fast mix polymer which released the greatest amount of GH also had the highest density, indicating that the density was virtually independent of the method of preparation. The increase in the amount of GH released from the fast mix polymer could be due to the introduction of 'air spaces' or 'pores' within the system, in order to explain the density data, it would appear that these had a very low overall volume. This has led to the conclusion that these are probably flat laminar or disc shaped cracks. Similar types of cracks were observed in epoxy resins following water uptake and have been described by Bucknall et al (1994). Presumably, the plane of the discs is randomly distributed throughout the polymer and can explain the numerous large "pores" seen in the SEMs of the fast mix PEM/THFMA compared to the pressurized mixing methods which had a much smoother appearance with very few "pores" visible. Such disc shaped cracks may create connecting channels or pathways for drug elution.

The diffusion coefficient, a measure of the rate of permeation was calculated for all the preparation methods. This was found to be higher in the fast mix polymer and lower in those polymers where air had been expelled. The amount of GH released from the differently prepared polymers was not however, related to the diffusion coefficient and the small differences observed in the densities were within experimental error and therefore not significant. Additives increase water uptake, depending on solubility and molecular weight; the increase varying inversely with molecular weight. It may be that the diffusion coefficient for GH coming out of the polymer may reflect the diffusion of water entering it. The $D$ values obtained are higher than expected from the individual PEM and P(THFM) (approximately 10$^{-10}$ cm$^2$.sec$^{-1}$- this may possibly be due to some shrinkage occurring when the PEM is dissolved in the THF monomer.
Figure 5.1 The *in-vitro* release of BSA from the PEM/THFMA polymer, prepared using the different methods described. The results plotted are the mean ± SD.

Figure 5.2 The *in-vitro* release of immunoreactive GH for the different mixing methods for PEM/THFMA. The results plotted are the mean value ± SD for the first 72 hours. Paired student's t-test indicates a significant difference in the amount of GH released from the fast mix polymer compared to the control (p = 0.0001). Significantly less GH was released from the pressurized mix polymer compared to the control (p=0.0001).
Figure 5.3a A plot of $M_t/M_\infty$ versus the square root of time, showing an initial rapid release of GH from the control mix PEM/THFMA polymer; 31.5% of the incorporated GH was released.

Figure 5.3b A plot of $M_t/M_\infty$ versus the square root of time, for the release of GH from the fast mix polymer PEM/THFMA polymer, with 75.4% of the incorporated GH being released.
SEM examination, revealed marked differences in the internal morphology of the polymers depending on the mixing technique employed. The control mix polymer showed uniform polymerisation as seen in Figure 5.4a. In contrast the fast mix, Figure 5.4b contained many disc-like cracks which appear as holes or "spaces" possibly as a consequence of the air that had been trapped during the polymerisation step. The method which excluded air did not show cracks to the same extent: centrifuged, Figure 5.4c, pressure mix polymer, Figure 5.4d and syringed polymer Figure 5.4e were less porous and had a smoother appearance with very few pores visible. In the slow mix polymer defects were fewer and the polymer surface appeared more coarse. Obviously, the extent of the pores is limited in the light of the density values, which are very similar for all the different systems studied.

The stability of GH in these polymer systems

The GH release profiles were similar for all but one of the polymers stored at different temperatures. Figure 5.5 shows that in all cases the greatest release of GH was seen during the first 24 hours followed by a slower but sustained release up to 21 days. An interesting finding was that significantly less (30%) immunoreactive GH was detected from the polymer stored at 4°C when compared to the other temperatures (paired Student's t-test p=0.002). This does not however appear to be a kinetic effect as there is no significant increase in the amount of GH released with a rise in temperature; that water is at its most dense at 4°C may have some relevance, but this is thought to be unlikely. No significant difference was observed in the bioactivity of the GH (Figure 5.6).

The fact that GH was unaffected by temperature could be due to the fact that once the drug is dispersed within the PEM particles it becomes 'encapsulated', this may protect the GH and render it stable even after exposure to extremes of temperature. Release profiles for BSA showed no significant differences for the different storage temperatures, indicating that temperature had not affected the stability of the BSA.

A plot of $M_t / M_\infty$ versus the square root of time was obtained for the different temperature samples, and the slope of the graph was found for the early stages of diffusion and used to calculate the diffusion coefficients (D) (Figure 5.7). The results for D were similar for all temperatures indicating that no structural changes had occurred to the polymer on storage. The Glass Transition temperature for PEM is 70°C and the fact that the polymer is unaffected even at temperatures of 200°C may have some relevance. The results for D can be seen in Table 5.3.
Table 5.3 Diffusion coefficients for the polymer system at different temperatures

<table>
<thead>
<tr>
<th>SYSTEM TEMP</th>
<th>SLOPE (units/sec)$^{1/2}$ x10^{-3}</th>
<th>D (cm$^2$.sec$^{-1}$) x10^{-8}</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20°C</td>
<td>2.230</td>
<td>7.688</td>
</tr>
<tr>
<td>4 °C</td>
<td>2.126</td>
<td>6.988</td>
</tr>
<tr>
<td>20°C</td>
<td>2.230</td>
<td>6.313</td>
</tr>
<tr>
<td>37°C</td>
<td>2.021</td>
<td>7.688</td>
</tr>
<tr>
<td>56°C</td>
<td>2.341</td>
<td>8.476</td>
</tr>
</tbody>
</table>

The effect of polymer composition on drug release

Figure 5.8 indicates that all the different copolymer ratios follow a similar release pattern with the exception of the 60:40 ratio. A rapid initial release of GH was seen during the first 24 hrs, followed by a slower but continuous release up to 9 days, indicating that the copolymer (EM/THFMA) gelled with HEMA was a good delivery system for GH. Similar release profiles were obtained for BSA, all the copolymer ratios show an initial rapid release of BSA, but this occurs more rapidly in the copolymers gelled with THFMA. Figure 5.9a, compared with HEMA, Figure 5.9b. Since BSA is water soluble, its release is dependent on the rate of water transport into the system. There was a wide variation in the amount of BSA released from the copolymer ratios for both those gelled with HEMA and THFMA, with no apparent relationship between the THFMA content in the copolymer. The amount of BSA released from the copolymer 60:40 gelled with HEMA was significantly greater than when gelled with THFMA (P<0.05).
Figure 5.4

Figure 5.4a Scanning electron micrograph of PEM/THFMA polymer for the control mixing method. Note the uniform polymerized internal structure, with few pore or defects visible (arrows)

Figure 5.4b Scanning electron micrograph of PEM/THFMA polymer for the fast mixing method. Note the rough appearance of the surface and numerous disc-shaped laminar fissures (arrows)
Figure 5.4

Figure 5.4c Scanning electron micrograph of PEM/THFMA polymer following centrifugation after mixing. Note the absence of pores and the coarse appearance of the surface.

Figure 5.4d Scanning electron micrograph of PEM/THFMA polymer for the pressure mix. The internal structure had a smoother appearance and no defects were visible.
Chapter 5
Figure 5.4

**Figure 5.4e** Scanning electron micrograph of PEM/THFMA for the syringed polymer. Note the very smooth appearance and absence of pores and defects.

**Figure 5.4f** Scanning electron micrograph of PEM/THFMA polymer using the slow mix method. Fewer defects (arrows) were seen, but the surface had a coarser appearance.
Figure 5.5 Total immunoreactive GH released from GH-loaded PEM/THFMA discs following storage at different temperatures. The results plotted are the mean (n=2) ± SD. Paired student’s t-test indicated that significantly less GH was released from the polymer discs stored at 4°C when compared to the other temperatures (p=0.002).

Figure 5.6 Total bioactive GH released from GH-loaded PEM/THFMA discs following storage at different temperatures. The results plotted are the mean (n=2) ± SD. No significant differences were observed in the bioactivity of GH for the different temperatures.
Figure 5.7 A plot of $M_t/M_\infty$ versus the square root of time. The slope of the graph was similar for all the temperatures studied; the diffusion coefficient and the total amount of GH released was also similar, indicating that the GH had remained stable.

Figure 5.8 Accumulative release of GH from the various copolymer systems of EM/THFMA gelled with HEMA. A rapid initial release was seen during the first 24 hours in all cases, and similar release profiles were seen at later stages for all the ratios except 60:40. The release pattern for the 60:40 ratio continued to rise. The results are the mean $(n=4) \pm SD$.
Figure 5.9a  Accumulative release of BSA from the various copolymer systems of EM/THFMA gelled with THFMA. The results are the mean of two experiments (n=4) ± SD.

Figure 5.9b  Accumulative release of BSA from the various copolymer systems of EM/THFMA gelled with HEMA. Although an initial rapid release of GH was seen, it was not as rapid as the copolymers gelled with THFMA. Paired student's t-test showed that the amount of GH released from the 60:40 gelled with HEMA was significantly greater than with the THFMA (p<0.05). The results are the mean of two experiments (n=4) ± SD.
Scanning electron micrographs of the surfaces of the polymers indicated differences in the topography and surface roughness for the different copolymer ratios. Figure 5.10a shows the surface of EM/THFMA in the ratio 30:70 gelled with HEMA, the surface is fairly rough, with an "open" appearance and numerous distinct pores. Figures 5.10b shows the same polymer system in the ratio 60:40; a slightly smoother appearance was seen, with fewer but larger defects. The copolymer ratio 90:10 had a much smoother surface, with only a few very small pores observed (Figure 5.10c). The surfaces of the other copolymer ratios can be seen in Figures 5.10d and 5.10e.
Figure 5.10

Figure 5.10a SEM copolymer EM/THFMA ratio 30:70 gelled with HEMA. Note the fairly rough surface, which has an "open" appearance and numerous large pores (arrows).

Figure 5.10b SEM copolymer EM/THFMA ratio 60:40 gelled with HEMA. The appearance is slightly smoother, and with fewer but larger defects (arrows).
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5.10 a

5.10 b
Figure 5.10

Figure 5.10c SEM copolymer EM/THFMA ratio 90:10 gelled with HEMA. Note the smoother appearance, with only a few small pores visible (arrow).

Figure 5.10d SEM copolymer EM/THFMA ratio 40:60 gelled with HEMA.
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Figure 5.10

Figure 5.10e SEM copolymer EM/THFMA ratio 50:50 gelled with HEMA.
Chapter 5

5.10 e

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>Mean CH</th>
<th>Surface</th>
<th>Release</th>
<th>%RELEASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>30/10</td>
<td>134.3</td>
<td>21.2</td>
<td>32.2</td>
<td>43.5</td>
</tr>
<tr>
<td>30/40</td>
<td>121.2</td>
<td>20.3</td>
<td>18.4</td>
<td>28.6</td>
</tr>
<tr>
<td>60/40</td>
<td>107.6</td>
<td>13.6</td>
<td>15.6</td>
<td>27.3</td>
</tr>
<tr>
<td>90/10</td>
<td>321.9</td>
<td>32.5</td>
<td>27.3</td>
<td>37.4</td>
</tr>
</tbody>
</table>

In conclusion, as shown in Table 5.10, the release of THPMA component increases with the increase of CH. This indicates that the CH plays a significant role in controlling the release profile of THPMA.
The slope of the graph was determined for the early stages of diffusion for all ratios gelled with HEMA, and used to calculate the Diffusion Coefficients (D), the results can be seen in Table 5.4.

Table 5.4 Diffusion Coefficients for Copolymers of (EM/THFMA)

<table>
<thead>
<tr>
<th>SYSTEM</th>
<th>SLOPE (units/sec)(^{1/2}) x10^{-3}</th>
<th>D (cm(^2)-sec^{-1}) x10^{-7}</th>
</tr>
</thead>
<tbody>
<tr>
<td>30:70</td>
<td>4.423</td>
<td>3.024</td>
</tr>
<tr>
<td>40:60</td>
<td>3.402</td>
<td>1.789</td>
</tr>
<tr>
<td>50:50</td>
<td>2.713</td>
<td>1.145</td>
</tr>
<tr>
<td>60:40</td>
<td>1.599</td>
<td>0.241</td>
</tr>
<tr>
<td>90:10</td>
<td>1.249</td>
<td>0.395</td>
</tr>
</tbody>
</table>

Figure 5.11 indicates that the plot \(M_t/M_\infty\) for the copolymer ratio 60:40 went through the origin, indicating that there was no surface release. In comparison, all the other ratios when plotted had positive intercepts, indicating that there was some surface release of the GH; the 90:10 ratio released as much as 36% of the GH from the surface. The composition of the EM/THFMA copolymers does appear to affect the amount of GH released, but not significantly. Overall the 60:40 composition showed the greatest release of GH, but the actual release profile was very different compared to the others. Table 5.5 shows the percentage GH released from the surface of the discs and the total GH released.

Table 5.5 Percentage total and surface release of GH from the different copolymers of (EM/THFMA)

<table>
<thead>
<tr>
<th>SYSTEM</th>
<th>M_\infty GH</th>
<th>% SURFACE RELEASE</th>
<th>% RELEASE DISC</th>
<th>% TOTAL RELEASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>30:70</td>
<td>153.2</td>
<td>26</td>
<td>22.6</td>
<td>48.6</td>
</tr>
<tr>
<td>40:60</td>
<td>125.2</td>
<td>10</td>
<td>18.4</td>
<td>28.4</td>
</tr>
<tr>
<td>50:50</td>
<td>107.6</td>
<td>12</td>
<td>15.8</td>
<td>27.8</td>
</tr>
<tr>
<td>60:40</td>
<td>221.0</td>
<td>0</td>
<td>32.5</td>
<td>32.5</td>
</tr>
<tr>
<td>90:10</td>
<td>83.2</td>
<td>36</td>
<td>12.3</td>
<td>48.3</td>
</tr>
</tbody>
</table>

On examination of D, (Table 5.4) it seems that as the ratio of THFMA component is increased, D also increases, this may be due to the GH being more soluble in THFMA or
that the system is becoming more hydrophilic resulting in more GH being released. HEMA is known to possess a high diffusion coefficient with respect to water, therefore by gelling the copolymer with this monomer, it may increase further the hydrophilicity of the system. THFMA also increases the hydrophilicity of the polymer but not to the same extent as the HEMA.

Figure 5.11 A plot of $\frac{M_t}{M_\infty}$ for the coplymer EM/THFMA, ratios 60:40 and 90:10. The 60:40 ratio goes through the origin indicating that no GH is released from the surface. In comparison the 90:10 has a positive intercept indicating surface release of GH; this was found to be 36%.

**GH Dose-dependent release**

GH can be released from the PEM/THFMA in a dose-dependent manner, with a rapid release during the early phase, followed by a continuous, sustained release for nine days, Figure 5.12. A plot of $\frac{M_t}{M_\infty}$ versus $t^{1/2}$ for the 60:40 ratio gelled with HEMA, goes through the origin for all the GH doses incorporated, indicating that the drug is not released from the surface, but from the inner polymer matrix, once it is placed in the elution fluid (Figure 13a). The 50:50 ratio in contrast has positive intercepts for all doses indicating some release from the surface (Figure 5.13b). In both cases $D$ is seen to decrease with increasing drug loading, i.e. the more GH is incorporated, the lower $D$, indicating that $D$ is a function of concentration of GH. The total amount of GH released from both the 50:50 and 60:40 copolymer ratios is dose-dependent and results for the different doses can be seen in Table 5.6.
Table 5.6 The diffusion coefficients for copolymers loaded with different GH doses

<table>
<thead>
<tr>
<th>SYSTEM</th>
<th>SLOPE (units/day)$^{1/2}$</th>
<th>SLOPE (units/sec)$^{1/2}$ $\times 10^{-3}$</th>
<th>D (cm$^2$-sec$^{-1}$) $\times 10^{-7}$</th>
<th>M$\infty$ GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>12IU 60:40</td>
<td>0.44</td>
<td>1.497</td>
<td>0.346</td>
<td>221</td>
</tr>
<tr>
<td>8IU 60:40</td>
<td>0.90</td>
<td>3.062</td>
<td>1.449</td>
<td>88.6</td>
</tr>
<tr>
<td>4IU 60:40</td>
<td>1.14</td>
<td>3.878</td>
<td>2.325</td>
<td>37.2</td>
</tr>
<tr>
<td>12IU 50:50</td>
<td>0.92</td>
<td>3.130</td>
<td>1.514</td>
<td>107.4</td>
</tr>
<tr>
<td>8IU 50:50</td>
<td>1.12</td>
<td>3.810</td>
<td>2.245</td>
<td>57.5</td>
</tr>
<tr>
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<td>1.18</td>
<td>4.014</td>
<td>2.490</td>
<td>43.6</td>
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</tbody>
</table>

Figure 5.12 The release of GH from PEM/THFMA following different drug loading. A rapid release was seen during the first 24 hours, followed by a sustained release of up to 9 days. The release was dose-dependent. The results plotted are the mean (n=2) ± SD.
Figure 5.13a A plot of $M_t/M_\infty$ for the copolymer EM/THFMA ratios 60:40 goes through the origin for all the doses incorporated, indicating that there was no surface release of GH. The GH was released in a dose-dependent manner.

Figure 5.13b A plot of $M_t/M_\infty$ for the copolymer EM/THFMA ratios 50:50 has a positive intercept and does not go through the origin indicating that there was some surface release of GH. The GH was released in a dose-dependent manner.
Biocompatibility

The PEM/THFMA polymer system has shown good biological and biocompatible responses. SEM of an osteoblast on control Thermanox can be seen in Figure 5.14a. The cells grew well on the plain PEM/THFMA and had a more rounded appearance (Figure 5.14b). The rounded appearance of the cells on the plain PEM/THFMA can be seen at a higher magnification in Figure 5.14c (i). A striking contrast is seen in the morphology of the osteoblast on the GH-loaded PEM/THFMA (Figure 5.14c (ii). The cell had a more flattened appearance with numerous vesicle-like structures, and is surrounded by matrix material. A higher magnification of the osteoblast cell on the plain PEM/THFMA shows rounded morphology Figure 5.14c (iii), the difference in cell morphology or the GH-loaded PEM/THFMA can be seen in Figure 5.14c (iv). Further indication of the polymer biocompatibility is indicated by the fact that the osteoblast cells were able to grow on the monomer THFMA (Figure 5.14 (v). Histochemical staining for ALP activity for the cell growing on the polymer, indicated ALP activity. More ALP positive cells were detected on the GH-loaded polymer (Figure 5.14d) compared to the plain (Figure 5.14e).
Chapter 5

Figure 5.14

Figure 5.14a SEM of HOB cell (O) on control Thermanox.(T) Note the typical osteoblast appearance with numerous processes for attachment.

Figure 5.14b SEM of HOB cell (O) on plain PEM/THFMA.(P) The cell retained its morphological structure and appeared rounded, with cellular processes attaching to the polymer.
Chapter 5

Figure 5.14

Figure 5.14c (i) SEM of a HOB cell (O) on plain PEM/THFMA. The polymer disc was covered with cells. This cell was characteristic of those observed on the polymer, note the rounded appearance of the cell.

Figure 5.14c (ii) SEM of a HOB cell (O) on GH-loaded PEM/THFMA. The polymer disc was covered with cells. This cell was characteristic of those observed on the GH-loaded polymer. Note the more flattened appearance of the cell with numerous vesicle-like structures (arrow), surrounded by matrix (M)
Figure 5.14

Figure 5.14c (iii) SEM of a HOB (O) cell at a higher magnification on plain PEM/THFMA. Note the rounded appearance of the cell.

Figure 5.14c (iv) SEM of a HOB cell (O) at a higher magnification on GH-loaded PEM/THFMA. Note the flattened appearance of the cell.
Chapter 5

5.14 c (iii)

5.14 c (iv)
Figure 5.14

Figure 5.14c (v) SEM of a HOB cell (O) on the THFMA monomer (MN). Note the change in cell shape.
Figure 5.14

Figure 5.14d Light micrograph of osteoblast-like cells (C) positively stained cells for ALP activity on GH-loaded PEM/THFMA. (P) Note the higher number of cells on this polymer compared to the plain.

Figure 5.14e Light micrograph of osteoblast-like cells (C) positively stained cells for ALP on plain PEM/THFMA (P).
Discussion

A non-degradable polymer system has been investigated for potential use as a drug delivery system. Various parameters that may affect the release pattern of GH and BSA have been investigated for the PEM/THFMA and copolymer (EM/THFMA) system. It has proved possible to control both the amount and the pattern of release by varying the preparation method and the copolymer ratios and using different hydrophilic monomers to control hydrophilicity.

Growth hormone can be released in a controlled manner from this polymer. Different methods of mixing had a significant effect on the amount of GH released, fast mixing increased the formation of randomly distributed laminar cracks throughout the polymer. It seems probable that this resulted in the formation of interconnecting channels which resulted in an increase in GH release. This would explain the density values obtained for the differently prepared polymers. A change in the internal structure of the fast mix polymer was confirmed by SEM, which showed surface defects as flat disc shaped cracks, with presumably low volumes. Higher levels of GH were released from those polymers where structural changes had been introduced by different mixing techniques. A possible explanation for this is that drug becomes 'encapsulated' by the polymer particles and the hydrophilic nature of this polymer enables water to enter and form "water clusters" (Barrie 1968), this in turn results in an increase in the drug released out of the polymer. This suggests that the uptake process is governed by the difference between the osmolarities of the external solution and the water clusters within the polymer. Similar systems to this have been described where the active agent is contained within the polymer matrix (Brook and van Noort, 1985). When such systems are placed in an aqueous environment water is imbibed and the agent is dissolved and pumped through the 'holes' or 'crazes' that occur around the drug particles. This type of release, where the agent is released via the filtered water filled channels, and is controlled by the amount of water taken in by the system, has also been described by Marks et al., (1976), Gale et al., (1980) and Wright et al., (1981).

In contrast, the lowest release was seen from the PEM/THFMA polymer system where the formation of "pores" or cracks was reduced by centrifugation or application of pressure. These findings are substantiated by the work of Brook and van Noort (1985), who investigated the release of hydrocortisone from acrylic polymers and were able to show that increasing the porosity by bench curing the acrylic, an increase in both the rate and the total amount of drug eluted was seen. The release of macromolecules via pores and channels formed within the polymer matrix by the dissolution of drug particles has been proposed by Langer et al., (1981), and has also been described by Rhine et al., (1980).
The correlation between the mixing method, diffusion coefficients and density for the different methods of preparation of the PEM/THFMA was very poor. The formation of "pores" or the introduction of air into the polymer system does not fully explain the findings. Although changes in the internal structure of the polymer were evident and "pores" or "air-spaces" were observed by SEM, it does not confirm that this was the only cause of the significant difference observed in the amount of GH released from the fast mix polymer.

Downes et al. (1992) have shown that surface topography of the polymer varies depending on the composition. When gelled with a more hydrophilic monomer HEMA, the polymer had a smoother appearance and this changed during water uptake. Whilst the exact mechanism of drug release remains unclear diffusion of drugs from the polymer matrix appears to play a part (Langer et al., 1981, Lee 1986, Lee and Peppas 1987). The results of this study indicate that the release of GH is influenced in part by internal defects indicated by the fast mixed polymer.

Drug loading, dispersion, and particle size are all factors that affect the release process (Cranks, 1975) and mathematical models where the drug is released via diffusion, through the water filled channels have been described (Gurney et al., 1982). The dispersion of the drug within the polymer matrix may be responsible for the different release patterns observed for the differently prepared polymers. In the fast mix, the drug was well dispersed, and particles are more likely to be in contact with each other. Brook and van Noort (1985), proposed the "crack theory"; that osmotically active drugs dispersed within a polymer system, imbibe water when placed in it and swell; this results in cracking or disruption of the matrix between surrounding drug particles, resulting in the drug being released. The extent to which this occurs is dependent on the defects of the polymer and the amount and the distribution of the incorporated drug. The results of this study indicate that the release of GH is influenced in part by internal defects; thin layered flaws which on SEM appear as holes or pores.

The polymer system used exhibits high water uptake, but the use of EM/THFMA copolymers in the bead phase allowed better control of the hydrophilicity of the system (Patel and Braden 1991, Patel and Braden, 1994). This form of control, was further influenced by gelling the copolymers to more hydrophilic monomers, which were found to be good delivery systems for both GH and BSA. As the monomer component was increased, more GH was released, this effect was probably the result of increased water uptake by the polymer. The different release pattern and behaviour of the 60:40 ratio compared to the other ratios indicates that there is probably an optimal polymer : monomer ratio where the crosslinking properties of THFMA predominate and have more influence than the hydrophilic properties on the amount of drug released.
The release of additives such as GH and BSA from the polymer matrices cannot be explained by diffusion alone and indicates that some other mechanism may be involved. The difference may be attributable to a combination of the effects of crosslinking and water uptake by the polymer. No significant changes were observed by storing the GH at different temperatures, this was confirmed by the fact that the bioactivity had not been destroyed. This finding indicates firstly, that the low exotherm of the polymerisation of the system makes it suitable for the delivery of proteins and peptide hormones, and secondly, for the stability of the GH molecule. The human GH conformation is known to be more stable than the growth hormone from other species (Brems et al., 1990). GH is a protein and proteins need a minimal amount of moisture to shield polar groups; overdrying will lead to exposure of these groups, furthermore, an optimum residual moisture content is required to balance physical and biological stability (Hsu et al., 1992, Pikal et al, 1992). This substantiates the finding that GH stored at the different temperature was unaffected and retained its bioactivity, probably as a result of having some residual moisture once incorporated within the polymer. Pikal et al. (1992), were able to show that the qualitative effect of residual moisture on the stability of human growth hormone was dependent on the temperature of the study.

The PEM/THFMA polymer showed excellent biological response allowing the adherence and growth of HOB cells on the plain and the GH-loaded. SEM revealed that the cells on the plain discs had retained their rounded morphology. In contrast, on the discs containing the GH, there were not only more cells but they had a more flattened morphology with numerous "vesicle-like" structures around them, probably an indication of increased cellular activity. Histochemical staining for alkaline phosphatase, a marker of osteoblast phenotype, indicated that the cells were able to maintain their phenotype in the presence of the polymer. More alkaline phosphatase positive cells were observed on the GH-loaded polymer discs compared to the plain, thus indicating increased cell number and increased cellular activity in the presence of the GH. Cell counts performed on the control and GH disc confirmed a larger number of cells on the GH-loaded discs.

The polymer system investigated in this study has demonstrated many properties which makes it suitable as a potential controlled drug release system. It is hydrophilic, and this property can be exploited as a method of controlling the amount of drug released and furthermore allows the release of water soluble drugs. In addition, it is able to swell within a defect but still be able to retain its rigidity. It has a low exotherm, and is therefore well suited for the incorporation of growth factors and proteins. Further development of this polymer as a drug delivery system may have important clinical applications.
CHAPTER 6

Growth Hormone Regulation of Gene Expression
Current and Future
Chapter 6

Introduction

The data presented in chapter 3 confirms that GH has direct actions on osteoblasts; it is able to stimulate proliferation and increase ALP activity, osteocalcin secretion and the release of IGF-I and IGFBP-3. This type of response is highly suggestive that the cellular effects of GH, are initiated by its binding to specific receptors on the plasma membrane of target cells, in this case, the osteoblast. The direct action of GH on HOB cells is corroborated by the ligand-binding studies used to investigate the regulatory effects of GH at the receptor level. Specific binding of radiolabelled $^{125}$I-GH to cultured HOB cells in the presence of increasing concentrations of unlabelled GH in this present study, suggests the presence of functional receptors. Boivin et al., (1984), and Morel et al., (1993), have demonstrated the localization of GH-immunoreactivity, and the absence of GH mRNA in rat and mouse calvaria, which substantiates the suggestion of an endocrine effect of GH on osteoblasts and the presence of receptors. In order to assess the relative contributions of the systemic and direct actions of GH on osteoblasts, it is necessary to identify the GH-receptor mRNA, assess its distribution, and to examine the factors that regulate its expression in the osteoblasts and determine whether the same receptor exerts different actions in different cell types. The regulation of gene expression could be exerted at different levels, for example, by activation of the gene transcription or by stabilization of mRNA.

The purification of human, rabbit (Leung et al., 1987) and rat (Mathews et al., 1989) GH-receptor cDNA clones has provided important information regarding their structure, expression and regulation (Carlsson et al., 1991, Gluckman et al., 1990). The discovery of high affinity binding sites in tissues other than the liver, as well as the demonstration of direct actions of GH on cartilage in-vivo and in-vitro (Isaksson et al., 1987, Nilsson et al., 1986, Lindahl et al., 1986) suggest that the direct action of GH on peripheral tissues may also be important (Lobie et al., 1990, Hocquette et al., 1990).

Based on the rabbit liver cloned GH receptor cDNAs, the receptor is predicted to be a single membrane-bound receptor. The mature human GH-liver receptor consists of 620 amino-acids, a hydrophobic transmembrane region, and an intracellular domain containing 24 and 350 amino-acids respectively (Leung et al., 1987, Carlsson et al., 1991). Northern blot analysis has shown that GH-receptor probes detect two homologous transcripts in some tissues, for example in the mouse these are 3.9kb and 1.2kb (Smith et al., 1989), while in the rat chondrocytes only the 4.2 transcript has been detected (Nilsson et al., 1990), this longer transcript encodes the full length GH-receptor protein (Leung et al., 1987).

The binding of GH and its specific membrane-bound receptor is believed to initiate numerous events including, metabolic, for example, diabetogenic and lipolytic activities,
and stimulation of gene expression, cell proliferation and differentiation. Although there is no evidence of any additional GH-specific receptors unrelated to the cloned receptor, there is some data to suggest the presence of sub-populations of modified GH receptors (Hocquette et al., 1991, Barnard et al., 1985). Variations in receptor subtypes could explain the differences in dose-response curves, time courses and the different actions of GH (Barnard et al., 1985, Retegui et al., 1982). These responses are in turn dependent on the number of GH receptors, and the amount of GH and secretory patterns and on the presence of GHBP, which will have some effect on the bioavailability of GH.

Binding studies need to be interpreted with some caution however, as GH can potentially bind to proteins found in the cytoplasm and on the cell surface, and three classes of cell surface receptors for hormones of the GH, prolactin and placental lactogen family have been described (Posner et al., 1974). A panel of monoclonal antibodies to rabbit hepatic GH-receptor have been used to demonstrate the presence of immunologically related, but distinct classes of the GH-receptors (Barnard et al., 1985). GH receptors have been demonstrated in rabbit tibia of different ages (Barnard et al., 1988), and also in avian epiphyseal growth plate chondrocytes (Monsonego et al., 1993). Lobie et al., (1990) described a heterogeneous distribution of GH receptors in epithelial components of the gastrointestinal tract, and also a widespread distribution in the reproductive system of the rat. GH receptors in mouse muscle cells have also been described (Adamafio et al., 1991).

There has, however, to date been no report demonstrating GH-receptors on primary human osteoblasts. GH-receptors have been demonstrated in osteoblasts of rat osteosarcoma cell lines (Barnard et al., 1991), but, the receptor density in these cells was lower (K_a = 1.2 x 10^9 M^-1) compared with that in the liver (9-10 x 10^9 M^-1). Furthermore, only one class of receptors was detected and these had a somatogenic specificity. In another study, the interaction of GH with human lymphocytes revealed a single order of binding sites with a similar affinity constant of 1.3 x 10^9 M^-1. Rabbit liver has been identified as having both lactogenic and somatogenic receptors (Posner et al., 1974, Barnard et al., 1985, ). Human GH binds with high affinity to both types of receptors, whereas bovine GH binds only to the somatogenic receptor, while ovine prolactin preferentially binds the lactogenic receptor. The lactogenic receptor binds ^3^I-GH tightly, and is not readily displaced by a large excess of unlabelled GH. In contrast, the somatogenic receptor is readily displaceable and has a lower affinity constant. Binding to rat and rabbit liver microsomes showed two classes of binding sites, with affinity constants of 14 (±4.0) x 10^10 M^-1 and 4.5 (±2.0) x 10^9 M^-1 in the rabbit and 9 (±3.0) x 10^9 M^-1 and 2.5 (±1.0) x 10^8 M^-1 in the rat (Barnard et al., 1985). Werther et al. (1993) were able to show the expression of GH receptors on human foetal mesenchymal tissues; with specific mRNA for the GH receptor being detected in both chondrocytes and fibroblasts.
Chapter 6

The aim of the work described in this chapter was to investigate (i) the presence of GH-receptors in primary human osteoblasts, and (ii) the effect of GH on their expression and also on gene expression of other proteins associated with osteogenesis. By understanding the factors that regulate the GH-receptor, and determining the correct physiological dose required to stimulate osteoblasts, it will be possible to release this locally using the drug delivery systems described in Chapters 4 and 5.

Depending on the cell type, it has been shown that GH is able to stimulate changes in the expression of a variety of genes (Norstedt et al., 1990, for review), and that it is involved in regulating the behaviour of both mature cell types and the induction of the differentiation of progenitor cells (Green et al., 1985). It has been shown biochemically that GH is able to increase the activity of proteins associated with bone remodelling and growth (Chapter 3), it is highly probable that GH is able to do this by directly influencing the expression of their specific genes. The availability of molecular clones for these genes (ALP, osteocalcin and collagen type I) has made it possible to investigate the relationship between GH and gene expression of these proteins.

Materials and Methods

Culture of HOB cells

In order to investigate GH dose-response effect on GH receptor expression; HOB cells were seeded (10^6 cells/in a 75cm^2 flask) and grown to confluency in DMEM (10% FCS) as described in Chapter 3, all media was changed to SFM for 24 hours, followed by the addition of the appropriate GH concentration (50 and 100 ng/ml) and a further 24 hour incubation prior to extraction of total RNA. For the time-course experiment HOB cells were treated as above, but exposed to GH treatment (50ng/ml) for variable time periods (0, 0.75, 1, 2, 4, 6, 24 hours).

HOB RNA Extraction

To avoid destruction of RNA by RNases, all disposable equipment used was sterile, and all glassware and solutions were treated with 0.1% diethyl pyrocarbonate (DEPC) to destroy RNase activity. RNA extraction was performed using the RNAzol B, single step extraction kit, which uses the Guanidinium Thiocyanate-Phenol-Chloroform method (Chomczynski and Sacchi, 1987). The medium was aspirated from the flasks, followed by the addition of 1ml RNAzol (Biogenesis, Bournemouth, UK) for 2-3 minutes maximum, in order to lift the cell layer, which was scraped from the flask and placed in sterile eppendorf tube and 100μl of Chloroform was added. The mixture was cooled on ice for 15 minutes, and centrifuged at maximum speed (13,000rpm) for 15 minutes at 4°C. The supernatant was

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transferred to a clean eppendorf and an equal volume of ice-cold 70% isopropanol (BDH, England) added and cooled for 20 minutes on ice, and centrifuged as before. The RNA pellet was washed three times with 600μl ice-cold 70% ethanol, after the final wash the pellet was resuspended in 30μl of 1mM EDTA (Sigma, Poole, UK). The purity of the RNA was monitored by the ratio of absorbances of the samples at 260nm and 280nm, and integrity of the quantification was confirmed by gel electrophoresis in 1% (w/v) agarose in 0.2M -MOPS (3-[N-Morpholino]propane-sulfonic acid) and 37% formaldehyde minigels stained with ethidium bromide (all reagents from Sigma, Poole, UK).

Preparation of the cDNA Probe for the human growth hormone receptor

The human GH receptor probe cDNA clone pghr.501.1 (kindly provided by Dr W.I. Wood, Genetech, San Francisco, CA, USA), contained a 847 base pair (bp) SacI fragment from the coding region of the hGH-receptor cDNA, in the vector pUC119 (Leung et al., 1987). Amplification of the plasmid was carried out in E.Coli competent cells as per standard transformation protocol (JM109 LlOOl, Promega, UK) (Hanahan 1985). Restriction enzyme analysis was performed using SacI enyzme. A 1kb ladder (Lambda -Hind III, Life Technologies Ltd, Paisley, UK) was used for sizing the linear double-stranded DNA fragments. For Northern blotting [32P] labelled cytidine triphosphate (CTP) (10mCi/ml; Amersham, Life Science, Buckinghamshire, England) labelling of the cDNA GH receptor probe was performed using a Random Primed DNA Labelling Kit and cleaned using sephadex G-25 Quick Spin Columns to remove any unincorporated precursors from the DNA (all reagents from Boehringer Mannheim Biochemica, Lewes, England).

The cDNA probes for human ALP cloned in pUC18 (0.7kb, BamHI+ EcoRI), osteocalcin cloned in pBluescript SK (0.7kb, EcoRI) and collagen type I, alpha 1 cloned in pBR322 (1.8kb, EcoRI) were amplified as above (initial source of probes; The American Type Culture Collection, Philadelphia, USA).

Northern Analysis of RNA

The total RNA (15μg per track) was separated on 1.2 % agarose MOPS-formaldehyde denaturing gel and transferred to Hybond C nitrocellulose filter (Amersham, Life Science, Buckinghamshire, England). Prehybridization was performed for 2 hours at 42°C in the following 'SSC' solution; 5 x SSC, 50% formamide, 5 x Denhart's solution, 0.5% SDS and 500μg/ml of denatured salmon sperm. Hybridization of the labelled probe (20μl) occurred overnight 42°C for 24 hours in a fresh 25 ml of 'SSC' solution. After hybridization, the blots were washed twice with 2 x SSC and 0.1% SDS (0.15 M Na Cl and 0.015M sodium citrate) and once with 1xSSC and 0.1% SDS at 42°C for 30 minutes, and finally with 0.5 x SSC and 0.1% SDS at 60°C. The filter was wrapped in clingfilm.
and then put down against an X-ray film with an image intensifier for 48 hours in the first instance and 1-2 weeks where necessary. Autoradiograms were exposed at -70°C to prevent blurring.

Results

The purity of the RNA extracted can be seen in Figure 6.1a for control HOB cells and those treated with 50 and 100ng/ml of GH. Two distinct bands were visible for all samples indicating intact 28S and 18S subunit bands of ribosomal RNA in both the control, and GH-treated HOBS. The total quantity obtained for the different treatments can be seen in Table 6.1; the cells treated with 50ng/ml of GH had the largest amount of RNA present. Figure 6.1b shows the RNA extracted from HOB cells treated with 50ng/ml for the different time periods. Two distinct subunit bands, 28S and 18S were visible for all time points studied. A very faint band of genomic DNA was detected in all samples, seen as a small band nearest the loading tracks. The total amount of RNA extracted from the HOB cells for the different GH dose-response can be seen in Table 6.1 and for the different time points in Table 6.2 Successful transfer of RNA from the gel to the nitrocellulose filter for both a dose-response (Figures 6.2a) and a time course study (Figure 6.2b). The bands are not as distinct on the nitrocellulose filter, but two bands are visible representing the 28S and 18S subunits. Very little RNA was left on the gel when viewed under ultraviolet light following overnight blotting thus indicating a high transfer efficiency.

Following amplification and purification of the cDNA fragment of the human GH receptor, restriction analysis of the correct fragment size was confirmed by gel electrophoresis in 0.8% (w/v) agarose-TBE gels (0.5M Tris borate, 0.001M EDTA) stained with ethidium bromide. A distinct band was seen between the marker (Lambda-Hind III) 700-1264 bp (track 1) corresponding to the 847bp SacI fragment of the coding region of the human GH receptor cDNA (Figure 6.3a). Restriction analysis of the ALP, osteocalcin, collagen type I, alpha 1 revealed bands corresponding to 0.7kb for ALP and osteocalcin and 1.8kb for the collagen type I, alpha 1(Figure 6.3b).

Table 6.1 Total amount of RNA (in 30µl volume) for control and GH treated HOB cells

<table>
<thead>
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</tr>
<tr>
<td>Control Sample 2</td>
<td>158.6 µg</td>
</tr>
<tr>
<td>GH 50ng/ml</td>
<td>203.5 µg</td>
</tr>
<tr>
<td>GH 100ng/ml</td>
<td>130.1 µg</td>
</tr>
</tbody>
</table>
Table 6.2 Total amount of RNA (in 30μl volume) for HOB cells treated with 50ng/ml GH for different time periods.

<table>
<thead>
<tr>
<th>Track</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
<th>g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (hours)</td>
<td>0</td>
<td>0.75</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA (μg)</td>
<td>74</td>
<td>76</td>
<td>95.3</td>
<td>94.1</td>
<td>108.8</td>
<td>79.8</td>
<td>47.4</td>
</tr>
</tbody>
</table>

GH receptor mRNA

No mRNA was detected for the GH receptor by Northern blot analysis even though the RNA had transferred successfully onto the nitrocellulose filter. There are several possible causes for the unsuccessful hybridization: (i) hybridization conditions may have been too stringent with incorrect salt concentrations, (ii) inefficient labelling of the probe giving rise to a low $^{32}$P-CTP incorporation, (iii) insufficient exposure time of the autoradiogram for detection of a low message, (iv) the expression of GH receptor may be transient and time or media dependent, (v) the method is not sensitive enough to detect the message for GH receptor, or (vi) the GH-receptors are not present on human osteoblasts.
Figure 6.1 a Dose-dependent stimulation of HOB cells; grown to confluency in DMEM (10% FCS) and changed to SFM for 24 hours, prior to the addition of the appropriate GH concentration. At the end of the incubation, total RNA was extracted using the single-step acid guanidium thiocyanate-phenol-chloroform method. The integrity and quantification of the RNA was confirmed by gel electrophoresis of aliquots of each extract in 1% (w/v) agarose-MOPS-formaldehyde minigels stained with ethidium bromide. Each lane was loaded with 15 µg of RNA, purity was determined by the presence of intact 28S (larger arrow) and 18S subunits (small arrow). The RNA concentration and purity was measured by absorbance at 280 and 260nm. Lane 1 - HOB cells stimulated with 50ng/ml of GH, lane 2 and 3 - control cells grown in DMEM (10%FCS), lane 4 - HOB cells stimulated with 100ng/ml of GH. Note that both lanes 1 and 4 had larger 28S bands, indicating that more RNA was present. The cells treated with 50ng/ml of GH had the highest amount of total RNA.

Figure 6.1 b Time-dependent stimulation of HOBS; cells were incubated with 50ng/ml of GH for the time indicated. At the end of the incubation, total RNA was extracted. Each lane was loaded with 15 µg of RNA, the integrity and the amount of RNA was monitored by staining with ethidium bromide, two distinct bands RNA bands 28S (large arrow) and 18S (small arrow) were seen. Some genomic DNA was detected as a small band nearest the loading track. The lanes correspond to the following times: a = 0 hours, b = 0.75 hours, c = 1 hour, d = 2 hours, e = 4 hours, f = 6 hours, g = 24 hours.

Figure 6.2a Northern blot, indicating the transfer of total RNA (15µg) extracted from HOBS following stimulation with GH, from the 1.2% agarose MOPS-formaldehyde denaturing gel to the Hybond nitrocellulose filter, ready for hybridization with the labelled DNA probe for the human GH-receptor. Two bands are visible for the RNA, 28S (large arrow) and 18S (small arrow).
Figure 6.2b Northern blot, indicating the transfer of total RNA (15\(\mu\)g) extracted from HOBS from the time course study from the 1.2% agarose MOPS-formaldehyde denaturing gel to the Hybond nitrocellulose filter, ready for hybridization with the labelled DNA probe for the human GH-receptor. Two bands are visible for the RNA, 28S (large arrow) and 18S (small arrow). The lanes correspond to the following times: \(a = 0\) hours, \(b = 0.75\) hours, \(c = 1\) hour, \(d = 2\) hours, \(e = 4\) hours, \(f = 6\) hours, \(g = 24\) hours.

Figure 6.3a The human GH receptor probe was prepared from the plasmid in the vector pUC119 containing a 847 bp SAC1 fragment of the coding region by amplification of the plasmid in E.Coli competent cells. A mini-preparation of the plasmid was carried out and inserts of cDNA for the GH-receptor were released from the plasmid by restriction enzyme (Sac I) digestion. The correct fragment size was confirmed by gel electrophoresis in 0.8 % (w/v) agarose-TBE gel, stained with ethidium bromide. A distinct band was seen corresponding to approximately the 800bp SacI fragment of the coding region of the human GH-receptor cDNA. The marker (M) used was 1Kb DNA ladder for sizing DNA fragments from 500bp to 12Kb, the larger band is the plasmid DNA (large arrow), the smaller band is the pure cDNA coding for the GH receptor (small arrow) corresponding to approximately 800bp.

Figure 6.3b The cDNA probes for human ALP cloned in pUC18 (restriction enzymes: Bam HI+ EcoRI), osteocalcin cloned in pBluescript SK (restriction enzyme: EcoRI) and collagen type I, alpha 1 cloned in pBR322 (restriction enzyme: EcoRI) were amplified by transformation in E.Coli competent cells. Following digestion with the appropriate restriction enzyme the correct fragment size was confirmed by gel electrophoresis in 0.8 % (w/v) agarose-TBE gel, stained with ethidium bromide. Distinct bands were seen corresponding approximately to 0.7kb for the ALP and osteocalcin and 1.8kb for the collagen type I, alpha 1 corresponding to the correct coding regions. The marker (M) used for sizing the DNA fragments was BstE III, which sizes from a range of approximately 0.5Kb to 8.5 Kb. The large arrow corresponds to 0.7Kb and the smaller arrow to 1.8Kb. Lane 1 = osteocalcin (0.7Kb), lane 2 = alkaline phosphatase (0.7Kb), and lane 3 = collagen type I, alpha 1(1.8 kb).
Discussion

Despite successful transfer of RNA to the nitrocellulose filter, detection of the expression of mRNA for the GH receptor in the HOB cells was unsuccessful using the conditions and methods described above. The hybridization conditions were investigated, and the percentage incorporation of the labelled probe was assessed and found to be satisfactory, indicating that this was not the problem. Increasing the exposure time of the autoradiogram did not have any effect. The absence of the GH-receptor in the HOBS is extremely unlikely, firstly, in view of the direct actions of GH on osteoblast activity demonstrated in this and other studies (Morel et al., 1993, Scheven et al., 1991, Kassem et al., 1993) and secondly, GH receptors have been previously described by Barnard et al. (1991) in clonal rat osteoblast-like UMR 106.06 cells. In their study they also reported that removal of cells from serum-free media to a nutrient-free buffer resulted in a rapid reduction in the binding of labelled $^{125}$I-GH. Other workers have also reported specific buffer effects on expression of receptors. Breier et al. (1988) have shown that the expression of hepatic GH receptor expression in-vivo is dependent on nutritional status; an increase in food intake results in changes in insulin levels, this will in turn influence the GH receptor level.

The expression of the GH receptor is transient, and its half-life is approximately 45 minutes (Norstedt et al., 1990), which might account for some of the difficulty in detecting its expression. However, the time-course for GH receptor expression in this study covered the possible expression time range and the message was still undetectable, indicating a finely balanced situation in this transient expression. Exogenous GH levels in a system will also effect the expression of GH receptors; large doses of GH may cause a down-regulation of receptors. Mullis et al. (1991ii) found that GH in physiological concentrations resulted in an increase in GH-receptor in a human hepatoma cell line. Supraphysiological concentrations however, led to a down-regulation of GH receptor mRNA during the first 3 hours, followed by an increase in receptor mRNA. Their study indicated that this was a result in changes in the rate of transcription of the GH-receptor gene. It would appear therefore, that the GH receptor is a dynamic system which is sensitively balanced to respond to environmental and other influences.

The regulation of GH-receptor expression is more complex than originally thought; the pulsatile nature of GH secretion and the presence of GH-binding protein (GHBp) play an important role in its actions on target tissues and the availability of GH (Carlsson et al., 1991). GH-binding protein is encoded by an mRNA derived by alternative splicing of the GH-receptor mRNA, and is antigenically identical to the GH-receptor. The possible role of GHBp as a modulator of GH action is unknown, but it may have an effect on the interaction between GH and the GH-receptor by changing the half-life of GH or by modulating the free GH concentration to which target cell are exposed during episodes of...
GH secretion. The functions of these proteins at the level of the target cell remain to be resolved.

**Future Work**

The unsuccessful attempt at detecting the mRNA for the GH-receptor in the HOB cells may be due to the fact that the GH-receptor copy number may be low and, if this is the case, then the method used in this study is not sensitive enough to detect the message. More sensitive methods such as in-situ hybridization which allows the investigation of gene expression at the single-cell level or RNase protection assay which allows quantification of the protected RNA bands using RNA riboprobes (synthesized by transcription with T3 RNA polymerase and giving rise to protected fragments) will have to be investigated in the HOB cells.

The sequence of events related to specific stages of osteoblast development and differentiation have been monitored at the protein level biochemically, and, correlation with the expression of the relevant mRNA would confirm this. Whilst the pattern of ALP, osteopontin and osteocalcin expression has been shown in developing rat bone (Weinreb *et al.*, 1990), the effect of GH on the regulation of these markers of differentiation has not to date been shown in human osteoblast and remains to be investigated. Amplification of the ALP, osteocalcin, and collagen type I, alpha 1 cDNA probes has already been performed and so it will be possible to synthesize riboprobes in plasmid vectors. For example, mRNAs translatable into protein products are important in the formation of extracellular matrix, such as ALP, osteocalcin, collagen type I and are expressed during the proliferative phase. By using methods such as in-situ hybridization it will be possible to confirm if the actions of GH on HOB cell proliferation and differentiation are reflected in mRNA changes. Investigation of the localization and the time of appearance of bone-related proteins could enhance our knowledge of osteoblastic differentiation during bone development and help elucidate their role in bone formation. Further investigation will be carried out to confirm this.
General Discussion
The object of this present study was to address two distinct issues: (i) to understand the role of GH in human osteoblasts, the site to be targeted for its local delivery and (ii) to develop and test a degradable and a non-degradable drug delivery system for the release of GH for applications in orthopaedics. Bone metabolism, growth and remodelling is regulated by a complex interaction of numerous factors which direct the behaviour of bone cells. The effects may be mediated by systemic or local hormones and cell-matrix interactions. The release of GH from biomaterials could play a major contribution to the healing process and improve osseointegration at the tissue implant site. In view of this, there is a need to develop a biocompatible controlled drug-delivery system capable of delivering bioactive GH at the local site. This type of approach has a dual effect of improving the biocompatibility of the material and enhancing the proliferation of cells at the interface between the bone and the implant resulting in an earlier induction of bone formation around the implant thereby improving the stability of the prosthesis.

Biomaterials are widely used in orthopaedics for the replacement of joints and the fixation of prostheses. One of the major problems facing orthopaedic surgeons is the poor biocompatibility of the fixing materials presently available. Ideally, in situations such as bone-implant incorporation and bone repair, where rapid osteogenesis is required, it would be advantageous to have a large number of osteogenic precursor cells present. The biomaterials investigated in this study have been considered both for their orthopaedic function and their use as local drug-delivery systems, so that the control of local proliferation, should allow control of new bone formation.

Cultures of osteoblast-like cells have been widely used to study the effects of osteotropic hormones and other growth factors, but the majority described are transformed rodent or human cells. These exhibit differences in hormonal responsiveness, species and maturity of cells. In view of this, one of the objectives of this study was to develop an in-vitro culture model that closely mimicked the in-vivo situation. Cellular events at the implant or defect site play a critical role in determining the fate of the implant, and the time taken for the tissue and wound to repair at the defect site. Primary, human non-transformed bone cells were used in this study and modifications in the culture method ensured a defined and well-characterized cell population in order to obtain a better understanding of the cellular changes that occurred in the presence of GH.

The work presented in this thesis has shown that GH and IGF-I stimulate osteoblastic proliferation and differentiation, indicative of a major role in the local regulation of bone remodelling. An increase in cellular proliferation was confirmed by $^3$H-thymidine and bromodeoxyuridine incorporation. In addition, a significant increase in ALP activity was observed in the presence of GH, followed by an increase in the production and release of osteocalcin, an indicator of a mature differentiated bone cell population and also
procollagen type I. Whilst others have shown that GH has mitogenic effects (Stracke et al.,
1984, Morel et al., 1993), it has not been confirmed whether this is due to just an increase
in proliferation or an increase in the markers of differentiation. The results from this present
study have shown that GH is able to directly stimulate cellular proliferation and in addition
stimulate the expression of protein markers of osteoblast activity. Furthermore, the
presence of these markers is seen much earlier in GH treated cells, indicating that GH has
differentiating-promoting properties. Intermediate actions of GH via IGF-I have also been
demonstrated; de-novo synthesis of IGF-I and an increase in IGFBP-3 release was
observed following treatment with GH. Both IGF-I and IGFBP-3 independently had a
significant effect on osteoblast proliferation, a positive correlation was observed between
these indicating a physiological significance of IGF-I regulated IGFBP-3 for bone
formation. These effects observed at the cellular level are corroborated by studies such as
Brixen et al. (1993), who have shown that GH can affect bone remodelling in-vivo, and
increases were observed in the biochemical markers of bone formation in adults with
osteoporosis.

The effects of GH on the HOBS has indicated a direct action of a paracrine and autocrine
nature, and that the actions of GH are via its binding with specific receptors on the
osteoblasts. Barnard et al., (1991) described GH receptors in a rat osteoblast cell line and
the presence of high affinity GH receptors is consistent with the data obtained in this study
and are suggestive of the presence of receptors. Scatchard analysis has indicated specific
binding of GH to the HOB cells and that there are receptors present, this has not been
previously documented in primary non-transformed human osteoblasts. Whilst all
indications are, that there are GH receptors on the HOBS, the detection of mRNA for the
GH-receptor was unsuccessful in this instance. This appears to be more a methodological
problem , rather than their absence. Future work will involve the use of more sensitive
methods such as in-situ hybridization, which can look at the message for the GH-receptor
on a single cell basis, other methods to be investigated include mRNase protection assay's
or polymerase chain reaction (PCR) for quantification of the signal.

A recognized limitation of currently available drug-delivery systems in the field of
orthopaedics is their inability to release high molecular weight compounds, and few allow
the amount of drug released to be altered once it has commenced. The aim of this study
was to develop two drug delivery systems to release biologically active GH, a
macromolecule, to the appropriate cells in a localized therapeutic dose, thereby, avoiding
any systemic effects. By selectively targeting the GH using the HOB model a local effect
has been demonstrated. Stimulation of bone formation at the trauma site could compensate
for the damage caused during orthopaedic surgery and also accelerate the repair process
during the critical post-operative period.
The degradable system comprised GH dispersed within a gelatin matrix; was tested both in the form of microspheres and as a coating. Bioactive GH was readily released from the gelatin in both forms, the mode of release from the microspheres was partially due to diffusion; followed by a second phase controlled by the rate of degradation of the gelatin, determined by the extent of crosslinking. The method used for its preparation does not involve the addition of any toxic agent, and this was reflected in the excellent biocompatibility of the gelatin in-vitro. Water plays an important role in the biodegradation rate of polymer-based drug-delivery systems, the solubility of gelatin in water makes it ideal for the absorption of tissue fluids and proteins from the surrounding bone matrix and marrow. The amount of GH released was significantly increased following exposure to ultrasound. Few systems allow this type of non-invasive modulation of drug release depending on clinical requirements; a major contribution in orthopaedics and particularly useful for the release and efficacy of hormones and peptides such as GH, by allowing a more subtle and physiological delivery pattern.

The biodegradable gelatin drug-delivery system could have numerous clinical applications both in the form of microspheres and as a coating on prostheses. A degradable system is particularly relevant in paediatric orthopaedics, where bone formation and tissue repair is rapid, and all that may be required is a short-term polymer able to release a local dose of GH to stimulate the cells sufficiently to speed up the repair process. The microspheres could be used directly at the bone-implant site, for example, for filling bone defects, or they could be incorporated into allograft material to stimulate tissue regeneration. The major advantage is that once the incorporated agent has been exhausted, it does not have to remain in-situ.

The non-degradable biomaterial is novel, and has properties that make it a suitable candidate as an orthopaedic material for drug-delivery. It polymerizes at room temperature and is therefore suitable for the incorporation of peptides and proteins; GH retained its bioactivity, indicating the stability of the polymer-drug complex. Its high water uptake in-vitro allows water soluble drugs to be readily released from it, and by manipulation of the copolymer ratios, a method of control was attained. The hydrophilic nature of the polymer would be particularly advantageous in-vivo, as it will be able to absorb tissue fluids and growth factors from the surrounding bone matrix, marrow and synovial fluid, creating an ideal environment for bone remodelling and cartilage repair. Furthermore, this polymer has shown good biocompatibility and the ability to swell but still remain rigid, therefore by swelling in-situ it will provide a good bond and stable interface with bone.

This study has confirmed that it is possible to influence specific cells locally to proliferate and to differentiate. The use of these systems in orthopaedics, either as a fixative for
prosthetic implants or simply as a delivery system, will be to target the release of GH directly to the osteoblasts.

This type of control would be an obvious advantage compared to systemic treatment, and would allow the control of bone repair directly at the target site where factors such as GH could be used to stimulate bone growth and remodelling. This is important in devising new therapeutic regimens to control bone formation based on the novel regulatory mechanisms discussed in this thesis. Depending on requirements, that is, long or short term, and whether mechanical stability is required or not, the drug delivery systems discussed could be used in a variety of clinical applications. Examples include; the treatment of non-union of fractures, bone grafting, allografts, or in children with growth problems as a result of epiphyseal arrest. Naturally, duration, timing and dose are all factors that have to be considered depending on the intended use.

The information gained in this study will help to advance the concept that GH and other GFs or drugs such as antibiotics can be incorporated into drug delivery systems to be delivered at target sites singly or in combination, so that the appropriate cells can be stimulated for a sufficient period using therapeutic doses in order to accelerate and improve repair. This type of approach may have future applications not only in the treatment of bone and cartilage disease, but also in other situations where tissue regeneration is required.
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Appendix I

Poly(ethylmethacrylate) - PEM

\[ \text{C}_2\text{H}_5 - \text{O} - \text{C} - \text{C} = \text{CH}_2 \]

Tetrahydrofuryl methacrylate - THEMA

2-Hydroxyethyl methacrylate - HEMA

\[ \text{OHC}_2\text{H}_4 - \text{O} - \text{C} - \text{C} = \text{CH}_2 \]


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Biodegradable microspheres: a new delivery system for growth hormone

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A drug delivery system for biologically active agents targeted to specific cells could be used to improve tissue repair in orthopaedics. The system should be controllable and capable of drug release over an extended period of time. Biodegradable, membrane-moderated, monolithic microspheres for the controlled release of growth hormone (GH) were developed and the release of GH was monitored in vitro. Cross-linked gelatin microspheres were used as the vehicle, with the drug dispersed within the gelatin. The amount of GH released from the microspheres was increased following ultrasonication. The release of growth hormone was monitored in phosphate buffered saline and horse serum. Interestingly, a higher level of GH was detected in the phosphate buffered saline than in serum. In addition, both pH and enzyme-induced degradation had an effect on the swelling kinetics of the gelatin microspheres. The release of GH from the microspheres was diffusion controlled, during the time period studied. Biomaterials (1994) 15, (11) 931-936

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Controlled drug delivery has caused considerable interest in recent years, particularly in the development of biodegradable controlled-release systems. A problem common to most systems is that they display either a constant release rate or the release is dependent upon degradation of the system with time. Methods of drug delivery described in the literature include encapsulation, membrane-enclosed 'reservoir' devices, and monolithic systems. These have, however, been few reports on methods for modulating the release rate with time. The approach used was to develop a monolithic microspherical drug delivery system in which growth hormone (GH) is dispersed within a biodegradable, biocompatible matrix. The delivery of GH from biomaterials can be an important therapeutic factor in the healing process of many tissues. It is known that GH can have both direct and indirect effects on osteoblast proliferation and differentiation, and it also plays a role in the regulation of bone remodelling. It has been shown previously that GH can be delivered from bone cement, ceramics and polymer systems. These biomaterials were selected primarily for their function in orthopaedics, and were modified further to allow drug delivery as a secondary function. In this work, we have specifically designed a release system for GH and other growth factors. Gelatin, a naturally occurring non-toxic polymer, was used to deliver the GH in a controllable manner, with the further advantage of being able to alter the amount of drug released on demand.

MATERIALS AND METHODS

Preparation of the spheres

A 20% gelatin solution was prepared using 300 Bloom gelatin (Swine skin type 1, Sigma, Poole, Dorset, UK) dissolved in sterile water. This was mixed, and the gelatin left to dissolve at 40°C. The solution was then divided into two aliquots, one of which was left plain as a control. Growth hormone solution (Novo Nordisk, Gentofte, Denmark) was added to the second aliquot at a concentration of 8 lU in 10 ml of sterile water. The microspheres were formed by forcing the gelatin mixture through a pre-heated syringe with a 21G needle into chilled paraffin oil in a 30 cm cooled column. The microspheres solidified as they passed through the column and were collected at the bottom. The paraffin oil was removed, and the microspheres were washed several times in 200 ml of phosphate buffered saline (PBS), and dried in a stream of cool air overnight. Although several other methods of cross-linking were tested, this was found to be the optimal method.

Optimization of microsphere stabilization

Various methods for cross-linking the microspheres were investigated prior to choosing the method.
described above. The gelatin was prepared as described, and each of the following cross-linking agents were tested: glycylid acrylate 4% (Aldrich, Gillingham, Dorset, UK), acyl azide (Sigma) and carbodiimide (Sigma) using concentrations of 0.5 and 1 mg/ml, respectively. In each case the respective cross-linking agent was added to the gelatin and mixed thoroughly at room temperature for 2 h with gentle heating until the gelatin was dissolved. The different mixtures were then divided into two aliquots, and GH was added to one half of each gelatin batch containing the different cross-linker. The microspheres were then made by the method described above.

**Elution of growth hormone from the spheres**

The release of GH was monitored in PBS (Oxoid, Unipath, Basingstoke, UK) and horse serum (Gibco, Paisley, UK), by adding 2.5 ml of either solution to each 0.1 g of microspheres. The release of GH was carried out at 37°C on a continuous rotating mixer. The PBS and horse serum was removed and replaced with 2.5 ml of fresh PBS or horse serum after 1 h, and then daily. The elution fluid was frozen at −20°C for GH assay.

**Effect of ultrasonication**

The microspheres were divided into two groups, one in PBS and one in horse serum. These groups were then subdivided into a control group and an ultrasonication group. Those microspheres that were ultrasonicated were exposed for 2 min at an ultrasonic frequency of 40 kHz (using a DAWE 6441 ultrasonication bath) prior to sampling of the PBS or horse serum. The temperature of the microspheres was monitored and not allowed to exceed 37°C, the PBS and horse serum were replaced as above at each time point. The elution media was retained and frozen for GH assay.

**Effect of pH**

The effect of changing the pH of the dissolution medium was tested for both plain and GH-loaded microspheres. This was done by examining the swelling kinetics of the microspheres in PBS at pH 2.4, pH 7.2 and pH 10.5. The microspheres were weighed dry, and 0.1 g of gelatin microspheres (mean = 28 microspheres) were placed in 2.5 ml of PBS at the different pHs. At different time points, the microspheres were weighed and the change in weight recorded for their respective media. The swelling ratios were calculated by comparing the weights of the swollen microspheres to the dry weight of the microspheres with time.

**Enzymatic degradation**

The effect of pepsin was investigated on both plain and GH-loaded microspheres. In order to establish the optimal concentration of pepsin, a range from 20-660 IU/ml was tested. A final concentration of 660 IU/ ml of pepsin was used to test for degradation. Prior to the start of the experiment, the microspheres were weighed and a dry weight of 0.1 g of beads was used. PBS (2.5 ml) was added to each set of microspheres containing varying concentrations of pepsin. These were incubated at 37°C and, at hourly intervals, the change in weight for each test sample was recorded.

**Growth hormone assay**

The assay used for GH was an in-house, enzyme-linked, immunosorbent assay (ELISA). This assay uses two polyclonal antibodies, the coating antibody is a guinea-pig immunoglobulin G fraction and the conjugate, a peroxidase-labelled Fab' fragment of guinea-pig anti-human GH with 3,3'-5,5'-tetramethylbenzidine (TMB) as the substrate for the enzymatic reaction. The optical density was measured at 490 nm with a reference wavelength of 650 nm. The standard used was 22K-recombinant human GH (antibodies and standard GH were supplied by Novo Nordisk A/S, Gentofte, Denmark).

**Assay validation**

Accurate dilutions of the GH standard were performed in both PBS and horse serum (Gibco) to ensure that adequate recovery was observed in parallel dilution, and so confirm the absence of cross-reactivity within the GH assay. Further GH recovery studies were performed in 'binding protein-free' horse serum (briefly, removal of binding proteins was achieved by using an acid/ethanol extraction procedure), to determine whether the presence of binding proteins affected the amount of measurable GH.

**Biocompatibility**

The gelatin microspheres were washed several times in complete Dulbecco’s Minimum Essential Medium (10% fetal calf serum, supplemented with 1% L-glutamine, 1% non-essential amino acids, 2% HEPES and streptomycin, from Gibco, UK). In order to observe their biocompatibility primary human osteoblasts were then seeded onto the gelatin microspheres and left in culture in DMEM at 37°C, in a humidified atmosphere of 5% CO₂. The gelatin microspheres were examined using SEM (Joel JSM 35) at 5 d post-seeding.

**RESULTS**

**Gelatin spheres**

The method described produced homogeneous and transparent microspheres with a diameter between 0.3 and 0.5 mm. The spheres possessed a uniform, spherical shape with a smooth surface. Although various methods were tested in order to achieve suitably cross-linked microspheres, the addition of cross-linkers such as glycidyl acrylate, acyl azide and carbodiimide proved unsuccessful with complete degradation of the microspheres at 37°C within a few hours of commencing the elution study. The optimal cross-linking for this system was achieved in the presence of 25% glutaraldehyde vapour. Complete cross-linking of the microspheres occurred within 48 h, with a colour change from pale yellow to deep yellow. Drying of the microspheres in a stream of cool air in a fume cupboard, allowed evaporation of any residual chloro-
form or glutaraldehyde. This method provided microspheres that lasted for over 2 months in vitro in the absence of cells and over 2 wks in the presence of cells in culture.

**Growth hormone release**

The microspheres released high concentrations of GH into both PBS and horse serum. The results indicated that the release of the GH from the microspheres was diffusion controlled. A plot of the rate of release versus the square root of time was linear during the early stages of GH release for the period studied, in both PBS and horse serum (Figures 1 and 2). It was noted that the plot line did not go through the origin, which suggests that release of GH also occurs from the surface. Ultrasonication of the microspheres in PBS resulted in a release of approximately 23% of the total GH incorporated, compared with 10% in those microspheres which were not exposed to ultrasonication. However, only 15% of the total GH incorporated was released from the ultrasonicated microspheres in horse serum compared with 8% from the control group. Although a difference was observed between the release of GH in PBS and the horse serum in the control samples, it was not significant. There was, however, significantly more GH detected in the PBS than in the horse serum ($P = 0.017$, Student's t-test) indicating that ultrasonication of the microspheres had increased the amount of GH released.

Parallelism was observed in both the PBS and the horse serum standard curve indicating that there was no cross-reactivity. Studies using 'neat' horse serum and 'GH-binding-protein free' horse serum spiked with GH gave recoveries of 73% and 88%, respectively. These figures were not significant, thus indicating that the difference in the amount of GH detected was not due to the presence of GH-binding proteins.

**Effect of pH**

These results indicate that the swelling of the microspheres is pH sensitive for both plain and GH-loaded microspheres (Figures 3 and 4). The pH of the media (2.4, 7.2, 10.5) did not affect the swelling ratio.
during the first 3 h of the experiment, and a rapid increase was seen in all cases. This was followed, however, by a marked fall in the swelling of the microspheres in pH 2.4 and 10.5. At pH 7.2, a very slow fall was observed in the swelling kinetics with a much lower diffusivity of GH. These findings were the same for both GH-loaded and plain microspheres.

Enzymatic degradation

Pepsin had a marked effect on the swelling kinetics of both plain and GH loaded microspheres. The plain microspheres in PBS showed a swelling ratio of approximately 7.9 after 7 h compared with 6.6 in the presence of pepsin (Figure 5). In contrast, a swelling ratio of 5.7 was observed in the absence of pepsin compared with 3.9 for the GH-loaded microspheres (Figure 6). This experiment indicates that the extent of cross-linking is important, and that the degradation of the microspheres by a proteolytic enzyme is dependent on this.

Biocompatibility

The microspheres showed no evidence of cytotoxicity. Scanning electron microscopy showed that viable cells were able to grow on the gelatin microspheres, indicating that they were biocompatible and not toxic to the osteoblasts (Figure 7). Good cell adhesion was observed in vitro, with penetration and invasion of the cells within the gelatin microspheres (Figure 8). Although some degree of degradation was visible, the microspheres were still not fully degraded even after 2 wks in culture in the presence of cells (Figure 9).

DISCUSSION

The concept of sustained release of therapeutic agents has been in existence for many years and many systems have been described, including encapsulated beads or pellets, coatings and porous materials containing the dispersed drug. Many of the swellable systems are dependent on the polymer swelling and releasing its contents into the surrounding fluid, this can, however, result in problems with solute stability.

Figure 7 Scanning electron micrograph of a gelatin microsphere showing the adherence of numerous human osteoblasts 5 d post-seeding.

Figure 8 Scanning electron micrograph of a human osteoblast cell on a gelatin microsphere, penetration of the cells within the gelatin matrix was visible 5 d post-seeding.
Biodegradable microspheres: L. Di Silvio et al.

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The release of agents from monolithic systems usually has zero-order kinetics, with release rates diminishing with time. Mathematical models for sustained release of water soluble drugs have been described, which indicate that the release is dependent upon the amount of drug loaded and upon dissolution and diffusion. In part these can be ascribed to Fick’s Law where the diffusion coefficient is constant with time, and the dimensions of the system remain the same.

Figure 9 Scanning electron micrograph of gelatin microspheres following 5 d in culture with human osteoblasts. Cells are visible on the microspheres and some degree of degradation was noticeable. The microsphere examined in Figures 7 and 8 is indicated by

The release of agents from monolithic systems usually has zero-order kinetics, with release rates diminishing with time. Mathematical models for sustained release of water soluble drugs have been described, which indicate that the release is dependent upon the amount of drug loaded and upon dissolution and diffusion. In part these can be ascribed to Fick’s Law where the diffusion coefficient is constant with time, and the dimensions of the system remain the same.

In this study, we have shown that microspheres can be used as a delivery system for GH. The drug was dispersed uniformly within the gelatin matrix. The release of GH from the microspheres is partially due to diffusion. If the release process is diffusion controlled, then the theory for diffusion from a sphere should apply:

\[ \frac{M_t}{M_{\infty}} = C_o \left( \frac{Dt}{a^2} \right)^{1/2} \]

\[ \left\{ \frac{1}{\Pi^{1/2}} + 2 \sum_{n=1}^{\infty} \text{ierfc} \left( \frac{n}{\sqrt{Dt}} \right) \right\} - 3Dt/a^2 \]  

(1)

In which \( M_t = \) total amount of drug released at time \( t \); \( M_{\infty} = \) total amount released at infinite time; \( C_o = \) initial drug loading; \( t = \) time; \( a = \) radius of microsphere; \( D = \) diffusion coefficient; and \( \text{ierfc} = \) the integrated error function.

However, as the data presented represent a loss of approximately 22% of the total drug incorporated (the remainder retained probably as a result of cross-linking), and in view of the fact that the plots in Figures 1 and 2 are linear, it is reasonable to assume that

\[ \text{ierfc} \left( \frac{n}{\sqrt{Dt}} \right) < \frac{1}{\Pi^{1/2}} \]

Equation (1) then reduces to:

\[ \frac{M_t}{M_{\infty}} = 6 \left( \frac{Dt}{\Pi a^2} \right)^{1/2} - 3Dt/a^2 \]  

(2)

This equation is a general quadratic, but because of the linearity of the \( t^{1/2} \) plot, it is further assumed that the second term is much less than the first. This transpires to be the case. For the highest diffusion coefficient taken and for a time of 4 d:

\[ 3Dt/a^2 = 0.6, \quad \text{and} \quad 6 \left( \frac{Dt}{\Pi a^2} \right)^{1/2} = 1.51 \]

Also noting that:

\[ M_{\infty} = \frac{4}{3} \Pi a^3 C_o \]

in which \( C_o = \) the concentration of the drug incorporated.

Equation (2) then reduces to:

\[ M_t = 8 C_o a^2 (\Pi Dt)^{1/2} \]

(3)

However, a number of microspheres are used experimentally, hence if there are \( n_i \) microspheres of radius \( a_i \), then Equation (3) becomes:

\[ M_t = 8 C_o (\Pi Dt)^{1/2} \sum_{i=1}^{n_i} n_i a_i^2 \]  

(4)

Because the range of microsphere sizes is 0.3–0.5 mm, Equation (4) can be rewritten as:

\[ M_t = 8 C_o (\Pi Dt)^{1/2} n a^2 \]

(5)

in which \( n = \) total number of microspheres; and \( a = \) mean radius (mm).

The slope (\( s \)) of the \( t^{1/2} \) plot is:

\[ s = 8 C_o (\Pi Dt)^{1/2} n a^2 \]

\[ D = \frac{s^2}{64 C_o^2 \Pi n^2 a^4} \]  

(6)

The outward diffusion of the drug is dependent upon inward diffusion of water, so the process is more complex than the above. The diffusion of the water into the gelatin microsphere will be concentration dependent and possibly time dependent as well. In view of the linear plots for the release of the GH, this was not time dependent over the period studied, but may have been concentration dependent.

More GH was detected in PBS than in serum indicating that the media into which the microspheres were placed had an effect on both the rate and the amount of drug released. There are several possible explanations for this observation. Proteins in the horse serum could have been adsorbed onto the gelatin microspheres resulting in a protective proteinaceous coating around them. It has been shown that coatings on drug delivery vehicles can affect the release rates and dissolution of the incorporated drugs. This seems unlikely because different diffusion coefficients would be expected, depending upon the medium, and this was not the case. Another possibility could be that GH was released in the same quantities in both cases, but in the horse serum the GH may have formed a complex with the GH-binding proteins, thereby preventing all the immunoreactive GH from being detected. Recovery studies, however, showed this not to be the case. The most probable explanation for the difference observed was due to the presence of .

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enzymes in the horse serum causing a more rapid degradation of the GH released.

Kost et al.\(^\text{26,27}\) have shown that it is possible to increase significantly the degradation rate of polymers and the release rate of incorporated agents using ultrasound. Ultrasonication of the microspheres was used in our system to increase the amount of GH released. The structure of the gelatin microspheres was probably broken down by the ultrasonication. This in turn causes a change in the kinetics resulting in further degradation, with an increase in the amount of water penetrating into the matrix, causing an increase in the diffusion coefficient and in the release of incorporated GH. Modulation of drug delivery by an external means may improve the release pattern and the efficacy of peptides such as GH which is usually released in a pulsatile manner. Such release systems are potentially useful in a variety of clinical applications.

CONCLUSIONS

The monolithic gelatin microspheres in our system provide a simple and effective drug delivery system. The advantages of the system are that the matrix used is a natural polymer, which is both biodegradable and biocompatible, and its preparation does not involve the addition of any toxic component. It can respond to an external stimulus, and the amount of drug released can be increased when required.

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Validation and Optimization of a Polymer System for Potential Use as a Controlled Drug-Delivery System

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Abstract: A room temperature polymerizing system consisting of polyethylmethacrylate (PEM) and tetrahydrofurfuryl methacrylate (THFMA) monomer was validated for use as a drug-delivery system. The effect of gelling PEM/THFMA copolymers with a more hydrophilic monomer, hydroxyethyl methacrylate (HEMA), was also investigated. The release of growth hormone (GH) and bovine serum albumin (BSA) protein from this polymer has been studied. The polymer has the advantage of high water absorption and low shrinkage properties. Changes in release profiles have been studied by introducing structural differences in the polymer by changing the mixing technique. The stability and bioactivity of the GH incorporated have been examined at various temperatures. To optimize release profiles further, the possibility of attaining a more sustained and controllable release by varying the ratios of polymer used has also been investigated.

INTRODUCTION

The clinical success of orthopaedic biomaterials requires bony ingrowth for integration and fixation. Whilst bioactive materials have been described which enhance bony ingrowth, another approach is the controlled release of incorporated growth factors or osteogenic promoters to improve bone bonding at the interface. The design of controlled drug-delivery systems, whereby a drug is delivered in the correct dose to the target cells for the appropriate length of time, is particularly advantageous for growth hormone and other growth factors. Growth hormone (GH) was used because of the increasing evidence that it can have a direct effect on osteoblasts mediated by locally produced insulin-like growth factor-1 (IGF-1) which is subsequently released to the surrounding tissues, stimulating osteoblast proliferation and thereby improving osseointegration at the tissue and implant site. We have previously shown that GH-loaded biomaterials are able to stimulate osteoid formation.

Although a wide range of materials are now available for use as vehicles for drug release, the complexity of the biological environment in which they have to function makes their design a difficult task. The delivery of drugs to a target site for a sufficient time period can be greatly improved by having a system whereby the drug incorporated can be manipulated to release primarily at the site of action and not indiscriminately to the whole body. The disadvantage of current delivery systems is that the majority of them are dependent on constant release rates or decay with time.

The polymer system that we have studied was originally developed for aural and dental applications. It has low polymerization shrinkage, a consequence of the high molar volume of the THFMA; this is in contrast with other conventional systems currently available such as polymethylmethacrylate (PMMA) which has a high polymerization shrinkage. An advantage of our system is its hydrophilicity, and as water plays an important part in the permeability and biodegradation rate of polymer-based drug-delivery systems, it is ideal for the absorption of tissue fluids and proteins from the surrounding bone matrix and
marrow. The water absorption of polymers in clinical applications is important; the PEM/THFMA system studied exhibits a high water uptake of 34% in 2 years, making it suitable as a drug-release system and for the incorporation of water-soluble agents such as growth hormone, which diffuse out of the polymer system into the surrounding tissue and so encourages repair.\textsuperscript{16,17}

There are many natural and synthetic hydrophilic polymers that are able to absorb and retain large quantities of water.\textsuperscript{18} Among the synthetic hydrogels, poly(HEMA) has proved a valuable biomaterial. Work by Otto Wichterle\textsuperscript{19} has shown its successful application for ocular devices; its use for surgical implants, however, is limited and very few clinical trials have been reported.

Different polymerization techniques and different porosities can affect the behaviour of hydrogels. Chirila et al.\textsuperscript{20} showed that increasing the amount of water in the initial monomer mixture affected the cellular invasion and deposition of calcium salts in the implants. The system that we have studied has a further advantage over other hydrophilic polymers of being able to swell in situ and still remain rigid, and furthermore it does not change structurally. We have also studied a system comprising copolymers of PEM/THFMA containing N,N-dimethyl paratoluidine 2.5% (v/v) gelled to HEMA.

The extent of the exothermic reaction of any material is of potential clinical importance if it is to be used directly in the aural cavity or elsewhere in the body, as a high exotherm could destroy any added therapeutic agent. For example, the high reaction exotherm of PMMA limits the possible antibiotics that can be incorporated. The extent of the exothermic reaction is a problem in orthopaedic bone cements; for example, temperatures in excess of 90 °C can occur in the femoral cavity resulting in tissue damage.\textsuperscript{21} The polymer we have studied has the major advantage over currently available materials of being a room temperature polymerizing system, thus making it suitable for the incorporation of peptide growth factors. Studies by Patel and Braden,\textsuperscript{22} where exotherms of different polymer systems were investigated, indicated that the temperature during polymerization did not exceed 36 °C. Growth hormone and BSA were incorporated and their release profiles under various conditions were investigated. This paper describes the validation and optimization of a polymer system incorporating GH, with a view to using it for site-specific delivery in fracture repair and healing of osteotomies.

**MATERIALS AND METHODS**

**Different mixing techniques as a means of optimizing drug release**

Human GH was incorporated into the polymer by adding 12 IU of GH to 10 g of PEM powder with thorough mixing before the addition of 5 ml of THFMA monomer. Discs containing BSA were made by the addition of 1.25 g of albumin in 10 g polymer with the addition of 5 ml monomer. Several different hand mixing techniques were employed for the mixing stage of both the GH- and BSA-loaded discs. The following methods were used and the mixing time was 45 s: (i) control, normal mix with 2 beats per second; (ii) slow mix, 1 beat per second; (iii) fast, 4 beats per second; (iv) centrifuged, 2 beats per second, followed by centrifugation at 2500 × g for 2 min; (v) pressurized, mixed 2 beats per second followed by thumb pressure for 1 min. These were then cast in PTFE moulds (8 mm diameter, 3 mm thickness). Elution studies were set up by incubating the respective discs in 5 ml phosphate-buffered saline (PBS) at 37 °C on a continuous rotating mixer. The elution fluid was removed at regular intervals and stored for assay of the additive. This was replaced with a fresh aliquot of PBS.

**Growth hormone assay**

Immunoreactivity of the GH released was measured using an in-house sandwich enzyme-linked immunosorbent assay (ELISA). This assay uses a polyclonal guinea-pig anti-human GH antiserum as the coating antibody. The second antibody conjugate is a peroxidase-labelled guinea-pig Fab' fragment. The substrate for the enzymatic reaction is 3,3',5,5'-tetramethylbenzidine (TMB). The standard was recombinant biosynthetic 22K human growth hormone (Novo-Nordisk A/S, Denmark). Optical density was measured at 490 nm with a reference wavelength of 650 nm.

**Protein assay**

Bio-Rad protein assay was used for protein estimations. This is a dye-binding assay and works on the differential change of a dye in response to various concentrations of protein.
Scanning electron microscopy (SEM)

SEM was used to examine both the surface and internal morphology of the differently mixed polymer discs. The surfaces were sputter-coated with gold prior to examination using a Jeol 35C scanning electron microscope.

Stability and bioactivity

In the second experiment the GH-incorporated discs were made up as per standard protocol and left in a sealed container at the various temperatures being investigated for one month. Elution studies were then performed as above in PBS at 37 °C. The GH bioactivity was measured using an ESTA (Eluted Stain Assay). The cytochemical assay uses NB2 rat lymphoma cells and utilizes the reduction of a tetrazolium salt to a formazan by intracellular dehydrogenase.

Effect of varying methods of polymer preparation

The effect of changing polymer:monomer ratio was investigated. The ratios tested were 30:70, 90:10, 40:60, 80:20, 50:50 and 60:40. The effect of gelling copolymers of PEM/THFMA with a hydrophilic monomer HEMA was also investigated. In all instances, both the immunoreactivity and the bioactivity of the GH were measured.

RESULTS

The effects of mixing techniques on drug release

The methods of mixing the polymers significantly affected the rate of GH released. All the release profiles showed an initial rapid release followed by a slower continuous release (Fig. 1). Increasing the speed of mixing dramatically improved the rate of GH release from the polymer. The fast mix polymer released a significantly greater percentage, 240% more GH over a period of 7 days, compared to the control (Student's t-test, \( P = 0.001 \)). The slow mix and those where pressure was exerted released significantly less GH (\( P = 0.001 \)). Release profiles for BSA followed a similar pattern, with a rapid initial phase followed by a slower continuous release (Fig. 2). Although approximately 25% more GH was released from the pressurized and syringe-mixed polymer, there was no significant difference in the amount of GH released when compared to other methods of mixing. SEM examination revealed marked differences in the internal morphology of the polymers depending on the mixing technique that was used. The normal mix polymer showed uniform polymerization (Fig. 3). In contrast, the fast mix contained many 'air-spaces' or pores, indicating that air had been trapped during the polymerization step (Fig. 4). The centrifuged and pressure mix polymer was less porous and had a smoother appearance as most of the air had been expelled during the mixing process (Figs 5 and 6).

The stability of GH in these polymer systems

The GH release profiles were similar for all but one of the polymers stored at different temperatures. In all cases the greatest release of GH was seen during the first 24 h, followed by a slower but sustained release up to 21 days. An interesting finding was that significantly less (30%) immunoreactive GH

Fig. 1. The \textit{in vitro} release of immunoreactive growth hormone from differently mixed polymers (\( n = 2, \) test specimens).
was released from the polymer stored at 4 °C compared to the other temperatures (Fig. 7) (Student's \( t \)-test, \( P = 0.002 \)). This does not, however, appear to be a kinetic effect as there is no significant increase in the amount of GH released with a rise in temperature; the fact that water is at its most dense at 4 °C may have some relevance, but this is thought to be unlikely. No specific explanation can be offered for the difference observed at this temperature; the fact that no significant difference was observed in the bioactivity indicates that this was probably an assay artifact for the polymer stored at 4 °C. Release profiles for BSA showed no significant differences for the different storage temperatures.

The effect of polymer composition on drug release

A rapid initial release of GH during the first 24 h, followed by a slower but continuous release up to 9 days, was seen for all the different ratios of polymer mix, indicating that the copolymer PEM/THFMA gelled with HEMA was a good delivery system for GH and BSA. The greatest release was seen at the copolymer ratios 60:40 and 30:70 respectively. For the most part it appears that as the ratio of THFMA component was increased in the copolymer gelled with HEMA monomer, an increase in the amount of GH released was seen, with the exception of the 60:40 ratio. Overall, this ratio composition showed the greatest release of GH, but the actual release profile was different compared to the other ratios (Fig. 8).
Fig. 5. Scanning electron micrograph of the polymer following pressurization through a syringe. Note the smooth internal appearance with the absence of any pores.

The GH incorporated in the polymer system remained bioactive even when subjected to storage at different temperatures, indicating that storage had not had an adverse effect on its stability (Fig. 9).

**DISCUSSION**

The effect of different parameters on the release profiles of the polymer has been studied. We have shown that GH hormone and protein can be released in a controlled manner from these polymers. Different methods of mixing had a significant effect on the amount of GH released; fast mixing increased the porosity of the polymer and subsequent increase in GH release was probably a direct effect of this. Change in the internal structure of the fast mix polymer was confirmed by SEM. This showed surface defects, termed 'pores', that were observed within the polymer system whilst in the liquid state. Downes et al. have shown that the surface topography of the polymer varies depending on the composition. When gelled to the more hydrophilic monomer HEMA the polymer had a smoother appearance and this changed during water uptake. Whilst the exact mechanism of drug release remains unclear, diffusion of drugs from the polymer matrix appears to play a part.

The release of the drug via pores or channels has also been described in the literature. Our findings substantiate that the release of GH is influenced in part by internal porosity as indicated

![Graph](image-url)  

**Fig. 7.** Total growth hormone released following storage of the loaded discs at different temperatures ($n = 2$, test specimens).
by the fast mix polymer. The lowest release was seen from those polymers where porosity was reduced by centrifugation or application of pressure. Formation of copolymers of PEM/THFMA gelled with HEMA resulted in a more hydrophilic polymer which was found to be a good delivery system for GH and BSA. As the monomer component was increased, more GH was released. This effect was probably the result of increased water uptake by the polymer. Our results indicate that the release of additives from the polymer matrices cannot be explained by diffusion alone and that some other mechanism may be involved.

Higher levels of GH were released from those polymers where structural changes had been introduced by different mixing techniques. A possible explanation is that the drug becomes 'encapsulated' by the polymer particles and the hydrophilic nature of this polymer enables water to enter within them and form 'water clusters'. This in turn results in an increase in the drug released out of the polymer. Systems similar to ours have been described where the active agent is contained within the polymer matrix.\textsuperscript{31,32} When such systems are placed in an aqueous environment and water is imbibed, the agent is dissolved and pumped through 'holes' or 'pores', the amount released being controlled by the amount of water taken in by the system. This type of release has been described by Marks \textit{et al.}\textsuperscript{33} where the agent is released via the filtered water-filled channels.

No significant changes were observed by storing the GH at different temperatures, and this was confirmed by the fact that it had lost no bioactivity. The human GH conformation is known to be more stable than the growth hormone from other species.\textsuperscript{34-37} This finding indicates, firstly, that the low exotherm of the polymerization system makes it suitable for the delivery of proteins and peptide hormones. Secondly, it indicates the
stability of GH, with no changes seen when subjected to various storage temperatures.

CONCLUSION

The polymer system investigated in this study demonstrated many properties which make it suitable as a potential controlled drug-release system. Successful development of this polymer as a drug-delivery system may have important clinical applications.

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The use of gelatin as a vehicle for drug and peptide delivery

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Gelatin, a naturally occurring polymer, has been investigated as a vehicle for drug delivery in two different delivery systems: microspheres and as a coating on titanium implants. The gelatin was loaded with recombinant human growth hormone (hGH) which was dispersed within the polymer matrix prior to crosslinking; it was then made into microspheres or coated onto the implants. The release of hGH was monitored in vitro using an "in-house" ELISA system. The effects of pH on the swelling kinetics and the physical properties of the loaded gelatin in the microsphere system were studied. In addition, the effect of ultrasound on the microspheres was investigated as a possible method for controlling the rate of release of hGH, it was demonstrated that exposure to ultrasound significantly increased hGH release. Biocompatibility of the gelatin was determined using both primary human (HOB) and rabbit (ROB) osteoblast-like cells in culture.

1. Introduction

The delivery of growth factors such as hGH can be an important contributing factor in the healing process of bone and the repair of cartilage. It has been shown that hGH can have both direct and indirect effects on osteoblast differentiation and proliferation [1–5] and we have previously demonstrated that hGH can be delivered from bone cement [6, 7], ceramics [8] and other polymer systems [9, 10]. One of the key factors in the design of controlled drug release systems is the choice of an appropriate carrier or vehicle, as this influences the release rate of the incorporated drug. Erodable matrices are controlled by both chemical reactions and diffusion [11, 12], whereas monolithic devices (where the drug is dispersed within the polymer matrix) are osmotically controlled with zero-order drug delivery kinetics [13, 14]. The properties of drug delivery systems can be selected to provide an optimal release rate for the additive, and assure physical and chemical stability of the system. There are numerous polymeric materials available, but only a few have successfully been used for drug delivery [15]. In general, previously studied degradable and non-degradable delivery systems have some limitations; for example, most are able to release only low molecular weight compounds, they display release rates that are either constant or decay with time and their release cannot be modulated once it has commenced [16–19]. Biodegradable polymers are becoming increasingly important in the design of controlled release systems, as they have the major advantage that once the drug has been exhausted they are readily degraded [20, 21]. A variety of biodegradable drug delivery systems have been introduced for controlled drug release and examples include liposomes, gel beads, microcapsules, microspheres and hydrogels [22–26]. In the present study, we have examined the biocompatibility and release kinetics of the natural polymer, gelatin, in microsphere form and as a coating on titanium screws, as a vehicle for the release of hGH. In addition, we have investigated the biocompatibility of the gelatin and examined the effect of ultrasound as a non-invasive method for modifying the amount of hGH released from the microsphere system.

2. Materials and methods

2.1. Preparation of GH-loaded gelatin microspheres

A 20% gelatin solution (300 Bloom, Swine skin type 1) was prepared in sterile water at 37 °C. The solution was divided in two: hGH (8IU) was added to provide a loaded solution to one and the other was a control without hGH. Each solution was then used to prepare microspheres; they were placed in a pre-heated syringe and forced through a 23G needle directly on to ice-cold paraffin oil in a long column, where the microspheres solidified as they collected at the bottom. They were then washed three times in chloroform, crosslinked in 25% glutaraldehyde vapour, under vacuum, for 48 h and dried in a stream of cool air overnight. Crosslinking of the microspheres was confirmed by a deep yellow colouration; and the mean diameter was 0.4 mm.

2.2. The release of GH from the microspheres

The microspheres were placed in 5 ml phosphate-buffered saline (PBS) and mixed at 37 °C on a continuous rotating mixer. The PBS was changed after 1 h
and then daily; the eluant was frozen at \(-20{\degree}C\) until assayed for immuno- and bioactivity of the released hGH.

2.3. The effect of ultrasound
The microspheres were prepared as described and divided into control and test groups, each comprising 100 mg of microspheres in 5 ml PBS. Those in the test group were exposed to an ultrasonic frequency of 40 kHz (using a DAWE 6441 ultrasonic bath) for 2 min; the PBS was then removed and retained for hGH measurement and replaced. The temperature of the microspheres was monitored and did not exceed 37{\degree}C.

2.4. Preparation of gelatin-coated implants
Commercially pure titanium screw implants, 2 mm \(\times\) 4 mm, were supplied by Nobelpharma Ltd. and were coated by dipping the screws into a 20% gelatin solution (300 Bloom, Swine skin type 1, Sigma, Poole, UK) containing GH (1 U/ml) and then immediately plunging them into cold water. The gelatin coating was crosslinked in 25% glutaraldehyde vapour, under vacuum, for 6 h and the screws were then placed under a UV lamp overnight. This resulted in an even dry coating of gelatin between the threads of the screws (Fig. 2c). The release of hGH into PBS from the screws was monitored for 14 days to determine the pattern, and total amount of hGH released.

2.5. Effect of pH
The effect of pH on hGH release and degradation was tested for both plain and growth-hormone-loaded microspheres. The swelling kinetics of the microspheres were examined in PBS at pH 2.4, 7.2 and 10.5. 100 mg of dry gelatin microspheres, 28 in number, were placed in 2.5 ml PBS at the appropriate pH. The microspheres were weighed at different time points and the change in weight recorded. The swelling ratios were calculated by comparing the weights of the swollen microspheres to the dry weight of the microspheres with time.

2.6. Assays for growth hormone
An "in-house" ELISA, previously validated to confirm the absence of cross-reactivity and optimized for use with the different elution media [27], was used to measure immunoreactive hGH (antibodies were generously supplied by Novo Nordisk A/S, Gentofte, Denmark).

The bioactivity of the hGH released was measured using an ESTA (eluted stain assay). This cytochemical assay uses NB\(_2\) rat lymphoma cells and relies on the reduction of a tetrazolium salt to a formazan by intracellular dehydrogenase [28].

2.7. Tissue culture
2.7.1. Preparation of gelatin coated tissue culture dishes
Gelatin was prepared as described above, without the addition of hGH, and poured into twelve 35 \(\times\) 10 mm tissue culture dishes to form a uniform 2 mm layer. The dishes were crosslinked in 25% glutaraldehyde vapour for 48 h.

2.7.2. Biocompatibility
The biocompatibility of the system was investigated using both HOB and ROB osteoblast-like cells. One group of each cell type was seeded (50 000 cells/dish) in Dulbecco's Modified Eagles Medium (Gibco, 10% foetal calf serum) directly onto the gelatin coated dishes. The gelatin-coated titanium implants were placed in a tissue culture dish and seeded with ROB cells (50 000 cells/dish). All materials were maintained in culture at 37{\degree}C, in a humidified atmosphere of 5% CO\(_2\), for a period of 2 weeks. The microspheres were seeded with HOB cells as above and examined using scanning electron microscopy (SEM, Joel JSM 35) 5 days and 2 weeks post-seeding. During this period, all dishes were examined frequently under a light microscope to observe any changes in cellular morphology.

3. Results
3.1. Release of GH from the microspheres and the effect of ultrasound
Both the control and test group readily released high levels of hGH into PBS, with a marked enhancement and significantly more hGH detected in the test group following exposure to ultrasound prior to sampling (students t-test, \(p = 0.017\)) (Fig. 1a). The hGH released from the microspheres remained bioactive, indicating that it had not been adversely effected (Fig. 1b).

3.2. hGH release from screw fixtures
The hGH-loaded gelatin coated screws, were able to release hGH \textit{in vitro}, with the bulk elution occurring during the first 4 h. This was followed by a much slower continuous release for up to 5 days. The rate at which the hGH is released is dependent on the thickness of the coating and the extent of crosslinking (Fig. 1c).

3.3. Effect of pH
The swelling kinetics of both the control and hGH-loaded microspheres were affected by pH. During the first 3 h, the microspheres swelled in all cases, resulting in an increase in weight and swelling ratio. This initial increase was followed by a marked fall in the swelling ratio of those microspheres at pH 2.4 and pH 10.5, at approximately 9 days, with only a small drop observed for the microspheres at pH 7.2. These findings were consistent for both hGH-loaded (Fig. 1d) and the control microspheres.
3.4. Biocompatibility
The gelatin-coated tissue culture plates supported growth of both human and rabbit osteoblast-like cells (Fig. 2a and b). In the 48 h crosslinked dishes, HOB cells grew to confluency and although there was visible evidence of degradation of the gelatin after 21 days in culture, the cells retained their typical morphology and appeared unaffected by any degradation products. The ROB cells grew rapidly on the gelatin coated screws, with a large number around the screw threads. Once again there was no evidence that even after 14 days in culture, degradation products affected cell growth (Fig. 2d).

Scanning electron microscopy (SEM) showed that HOB cells grew well on the microspheres, and after several days in culture the cells appeared to infiltrate the gelatin matrix (Fig. 3a). Areas of degradation were evident around the cells 2 weeks after seeding; probably a direct effect of enzymatic degradation by the cells (Fig. 3b).

4. Discussion
Gelatin is a natural biocompatible polymer, and we have shown that gelatin can be used as a vehicle for the delivery of hGH in microsphere form and as a coating. The biological environment in which the delivery system is to be used will have an effect on both the rate and the amount of the additive released. Gelatin has a setting temperature of 35 °C and pH limits between 3 and 10, thus making it suitable for the incorporation and delivery of hGH and other growth factors that may be susceptible to damage by excessive changes in temperature and pH [29–32]. Although the solubility of gelatin in water may appear to be a disadvantage with regard to the rate of hydrolysis, careful manipulation of the preparation conditions and crosslinking time results in minimal destruction of the gelatin molecule [33]. Water plays an important role in the biodegradation rate of polymer-based drug-delivery systems, the solubility of gelatin in water makes it ideal for the absorption of tissue fluids and proteins from the surrounding bone matrix and marrow. Gelatin microspheres and coatings could be used to release therapeutic doses of growth factors to a target site, and have the further advantage that once the drug is released they are degraded. The mode of release of hGH from the microspheres has previously been described, and is partially due to diffusion;
followed by a second phase which is controlled by the rate of degradation, and the extent of the initial crosslinking of the gelatin [34]. Our results show that hGH can be released from the microspheres, with a twofold increase in the amount of hGH released following exposure to ultrasound. One possible explanation for the enhancement seen, is that cavitation, induced by the ultrasonic waves results in an increase
in the penetration of water, thereby promoting hydrolytic degradation and the release of more hGH. This type of modulation may prove useful for the release and efficacy of peptides such as hGH, by allowing a more subtle and physiological delivery pattern. Kost et al. have demonstrated that ultrasound affects the release rates of implantable controlled delivery devices [35, 36].

The rate of cell proliferation and bone formation at a trauma site could be improved by the release of growth factors locally. In general, these factors have a short biological half-life, and their actions are mediated through autocrine and/or paracrine pathways from locally stimulated cells [37]. An increase in local release rates of implantable controlled delivery devices could have numerous clinical applications, both in the form of microspheres and as a coating on prostheses.

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Collagen and hyaluronic acid based polymeric blends as drug delivery systems for the release of physiological concentrations of growth hormone

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Two synthetic polymers, poly(vinyl alcohol) (PVA) and poly(acrylic acid) (PAA) were blended, in different ratios, with two biological polymers, collagen (C) and hyaluronic acid (HA). These blends were used to prepare two different materials, sponges and hydrogels, which were loaded with growth hormone (GH). The GH released, was monitored in vitro using a specific enzyme-linked immunoadsorbent (ELISA) assay. The results show that GH is released in a dose-dependent manner, from HA/PAA sponges and from HA/PVA and C/PVA hydrogels. The amount of GH released was proportional to the percentage of the natural polymer (HA and C). The release of GH from HA/PAA sponges was constant with time, whereas in HA/PVA hydrogels it was linear for the first 3 days followed by a slower release. The GH release pattern in C/PVA hydrogels was different, with a slow release for the first 3 days followed by a more rapid release. The concentrations of GH released from the materials were within a physiological range and sufficient to have a local effect on cellular proliferation. The effects of GH were tested in vitro using primary human osteoblast-like cells (HOBS) and measuring cell proliferation and alkaline phosphatase (ALP), a biochemical marker of HOB cell differentiation.

1. Introduction
The success of synthetic polymers as biomaterials is mainly related to their wide range of mechanical properties, processing methods and low production costs, but their interaction with living tissues is a major problem. On the other hand, many biological polymers of interest as biomaterials (namely, collagen, elastin and glycosaminoglycans) possess good biocompatibility but their mechanical properties are often inadequate. An interesting set of new materials have been made by combining synthetic polymers with naturally occurring polymers [1-3]. We have investigated the potential use of such polymer blends as drug delivery systems for the release of GH. Our aim was to measure the GH release and to determine if the rate of GH release from these materials was sufficient to have a local effect on cellular growth. Bone remodelling is a process that occurs throughout life and is dependent on cellular activity; this is controlled by numerous systemic and local factors [4, 5]. Although it has been well documented that GH is a major regulator of skeletal growth, and that it stimulates longitudinal bone growth in a dose-dependent manner [6, 7], there is increasing evidence that GH has a direct and indirect effect via insulin-like growth factors on bone cell proliferation and differentiation [8-10].

2. Materials and methods
2.1. Hyaluronic acid/poly(acrylic acid) sponges
Solutions of 5% hyaluronic acid (supplied as sodium salt by Fidia Advanced Biopolymers SpA-Italy) and 5% PAA with molecular weight 250,000 (Aldrich) were prepared in water, dissolved at 50°C. The two solutions were blended in three different HA/PAA ratios: 20/80, 40/60, 60/40. The samples were then lyophylized. After crosslinking by thermal treatment at 130°C under vacuum for 24 h, GH was added. One sponge was used as a control and GH was added to the other four to produce final concentrations of 25, 50, 75 and 100 mIU GH per ml of HA. The samples were lyophylized again and the release of GH was monitored in vitro.

2.2. Collagen/poly(vinyl alcohol) hydrogels
1.5 g of collagen was dissolved in 100 ml of 0.5 M acetic acid, in an ice bath, to obtain a final collagen
solution of 1.5%. 10 g of PVA was added to 100 ml of distilled water and dissolved in an autoclave for 1 h at 120°C to obtain a final concentration of 10% PVA. The two solutions were blended in three different C/PVA ratios: 30/70, 20/80, 10/90. One well was used as a control, and GH was added to the other four to produce final concentrations of 25, 50, 75, 100 mIU GH per ml of collagen. After GH addition, samples underwent eight cycles of freeze–thawing to obtain hydrogels. Each cycle, with the exception of the first one, consisted of 1 h at \(-20°C\) and 30 min at room temperature. The first cycle differed from the others due to a longer standing time at \(-20°C\) (overnight).

2.3. Hyaluronic acid/poly(vinyl alcohol) hydrogels

A 5% hyaluronic acid solution was made in distilled water at 50°C. 5 g of PVA was added to 100 ml of distilled water and dissolved in an autoclave for 1 h at 120°C to obtain a final concentration of 5% PVA. The two solutions were blended in three different HA/PVA ratios: 30/70, 20/80, 10/90. One well was used as a control, and GH was added to the other four to produce final concentrations of 25, 50, 75, 100 mIU GH per ml of hyaluronic acid. After GH addition, samples underwent eight cycles of freeze–thawing to obtain hydrogels. Each cycle, with the exception of the first one, consisted of 1 h at \(-20°C\) and 30 min at room temperature. The first cycle differed from the others due to a longer standing time at \(-20°C\) (overnight).

2.4. Elution studies

The GH eluate from the samples were monitored in vitro. The hydrogels and sponges were each placed in 3 ml of phosphate-buffered saline (PBS) in individual universals at 37°C. The elution fluids were removed at regular time intervals (every day for 7 days and then every 2 days for a further 7 days), stored at \(-20°C\), and returned back to 37°C.

2.5. GH assay

Elution fluids were assayed for the GH using ELISA, as previously described [8]. The microtitre plates were read using a 96-well fluorescent plate reader (MR. 700 Dynatec Microplate Reader). Optical density was measured at 490 nm with a reference wavelength of 650 nm. The standard used was 22K rhGH (Novo-Nordisk, Gentofte, Denmark).

2.6. Cell culture

The primary HOBS were isolated from femoral heads obtained from patients undergoing surgery for total joint replacement. Trabecular bone fragments were dissected under sterile conditions, washed several times in calcium and magnesium-free PBS and cultured in complete Dulbeccos Modified Eagles Medium (DMEM, supplemented 10% foetal calf serum, 1% non-essential amino acids, 1% ascorbic acid, 1% L-glutamine, 2% hepes and 50 U/ml penicillin, 0.05 mg/ml streptomycin), in a humidified atmosphere with 5% CO₂ at 37°C, for a period of 4–5 days, with one media change during this time, thus allowing the removal of any non-adherent cells. The bone fragments were then digested using a collagenase (100 U/ml) and trypsin (0.02%) digest mixture and were incubated for 20 min at 37°C in this solution, in order to allow the release of bone cells from within the bone fragments. Following digestion the “osteoblast-rich” supernatant was transferred to a sterile universal and spun down to obtain a cell pellet. The cells were washed several times in complete DMEM to remove all traces of the digestion media. The cells were counted and seeded to obtain an appropriate number of cells for the different proliferation assays.

2.7. Cell proliferation

Dose-dependent stimulation of HOBS was assessed using the bromodeoxyuridine (BrdU) assay (Amerham); this assay determines the number of S-phase cells. The cells were seeded (40,000/well) in a 96-well plate in complete medium. After a 24 h incubation the medium was replaced with serum-free medium containing different physiological concentrations of GH (3.125–100 ng/ml). (The conversion factor for GH is 100 ng = 0.3 mIU.) BrdU (200 ml) was added to all the wells and incubated for a further period of 2 h. The medium was drawn off and retained for biochemical analysis, the cells were then fixed and a further incubation with a monoclonal anti-BrdU followed. The incorporation of BrdU by the cells was detected using a peroxidase-labelled anti-mouse IgG₂a substrate. The reaction was stopped and read spectrophotometrically at 410 nm.

2.8. ^3H-thymidine incorporation

HOBS cells were seeded as above and grown in culture for 24 h, after which the medium was replaced with serum-free medium (SFM) to arrest cell growth. The medium was then changed and the cells incubated in the presence of GH at concentrations of 0–50 ng/ml (generously donated by Novo/Nordisk A/S Denmark). ^3H-thymidine (1 mCi/ml, Amersham) was added for a further 24 h incubation. After washing in DMEM containing cold thymidine (5 mg/ml), the cells were papain digested (1 ml/ml) and an aliquot was counted in the scintillation counter.

2.9. Assay for alkaline phosphatase

ALP production by the cells stimulated with GH was measured using the substrate p-nitrophenyl phosphate dissolved in diethanolamine buffer pH 9.8 (Diagnostics Merck) on a COBAS BIO centrifugal analyser.

3. Results

3.1. Hyaluronic acid/poly(acrylic acid) sponges

The rates of GH release from samples prepared using the three different HA/PAA blends were linear. Interestingly, those sponges with higher hyaluronic acid
content but the same GH concentration released more GH, i.e. there was significantly more GH released from samples prepared using the 60/40 (HA/PAA) blend than the samples prepared using the 40/60 (HA/PAA) blend ($p < 0.05$ paired $t$-test) and in turn samples prepared using the 20/80 (HA/PAA) blend ($p < 0.05$ paired $t$-test). In addition, there was more GH released from the sponges with higher initial GH concentrations. Fig. 1 shows the release curves for GH from sponges with the same hyaluronic acid content (60%) but different GH concentrations.

### 3.2. Collagen/poly(vinyl alcohol) hydrogels

GH was released from the C/PVA hydrogels with a slow lag phase during the first 3 days followed by a faster burst of GH release. The initial GH concentration did not affect the pattern of release but did, however, affect the total amount released. There was a direct relationship between the amount of GH incorporated in the hydrogels and the total amount of growth hormone released. Fig. 2a shows the release curves for GH from hydrogels with the same collagen content (20%) but different GH concentrations. It was also observed that increasing the collagen content of the hydrogels loaded with the same amount of GH (25 mIU), increased the total amount of GH released (Fig. 2b). Samples from the 30/70 blend released significantly more GH ($p < 0.05$, paired $t$-test) than samples from the 20/80 blend, which in turn, released significantly more GH ($p < 0.05$, paired $t$-test) than samples from the 10/90 blend.

### 3.3. Hyaluronic acid/poly(vinyl alcohol) hydrogels

During the first 3 days GH was released from the HA/PVA hydrogels in a constant linear manner which subsequently reached a plateau (Fig. 3). The 30/70 blend polymer released significantly more GH ($p < 0.05$, paired $t$-test) than the 20/80 blend and these in turn released significantly more GH ($p < 0.05$, paired $t$-test) than the 10/90 blend. The initial GH concentration did not affect the pattern of release but did affect the total amount released.

### 3.4. Effect of GH on HOB cell proliferation and ALP production

GH stimulated proliferation of the HOB cells in a dose-dependent manner. Maximal stimulation occurred between GH concentrations of 70 ng/ml and
and tissue repair in a variety of biomedical applications. Systemic treatment with GH has been shown to cause an increase in bone formation, and direct stimulation of chondrocytes [12–15]. The effects of GH have been shown to go beyond the control of longitudinal bone growth to the level of bone turnover [16–20]. Our results have indicated that HOB cells can be stimulated by GH in a dose-dependent manner, and at physiological dose ranges of the hormone, an increase was seen in the uptake of BrdU, ^3H-thymidine incorporation and the production of ALP in response to GH. The polymer systems we have investigated could be used to release GH and/or other growth factors directly to a target site, for a sufficient time period, in the appropriate concentration. We have shown that HA/PAA sponges proved to be an excellent delivery system for GH. They are able to release GH at physiological concentrations that can stimulate the proliferation of cells. The release of GH was linear with time and the GH released was directly related to the initial amount incorporated in the sponges. Examination of the scanning electron micrographs of both the surface and the internal structure of the sponges has shown porous structures composed of sheets of the polymers with interconnecting channels [21]. This type of structure may facilitate the transport of GH from the polymers into the surrounding environment as a result of an increased surface area. The hydrogels also proved to be useful for GH delivery. In C/PVA hydrogels there was an initial lag phase followed by a burst of GH release, whereas the HA/PVA hydrogels exhibited a rapid release followed by a plateau. Although HA/PVA hydrogels released more GH than C/PVA hydrogels, the amount of GH released from the latter was within a more physiological range. In addition all the blends of polymers studied indicated that by increasing the biological component (collagen or hyaluronic acid) it was possible to increase the amount of GH released, thus indicating that this could be used as a control mechanism. In conclusion, we have successfully combined natural and synthetic polymers to produce new systems for the delivery of GH and other growth factors in physiological quantities.

4. Discussion

The development of controllable, long-term, effective, release systems for the delivery of growth hormone and other growth factors may improve wound healing

100 ng/ml as indicated by both the BrdU assay (Fig. 4a) and ^3H-thymidine incorporation (Fig. 4b). GH had a significant effect on the production of ALP at days 7 and 12 post-seeding (p < 0.05) (Fig. 4c).

References