Expression of the locally produced IGF-I splice variants in damaged-regenerating and stretched and stimulated skeletal muscle

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ABSTRACT

Muscle is a mechanical tissue in which new nuclei are obtained by the fusion of satellite cells with damaged muscle fibres following local damage. Up regulation of the expression of growth factors is essential to nurture the proliferation of these muscle precursor cells. Increased expression of IGF-I was found to be associated with regeneration of muscle fibres that is produced by muscle. Two alternatively spliced isoforms of IGF-I are produced locally in active muscle and appear to be positive regulators of muscle hypertrophy; IGF-IEa with a similar structure to the major endocrine form produced by liver and IGF-IEb or MGF that has an extra 52bp insert in exon 5 in rodents and was found to be up regulated in exercised muscle. Muscle cells are known to produce and secrete IGFBPs that are important in modifying the availability and biological effects of IGFs. In order to study the mechanism of local muscle repair, two systems of inducing local damage were applied; muscle stretch combined with an initial bout of electrical stimulation and injection of a myotoxic agent, bupivacaine.

Using Real-Time PCR with primers that distinguish these spliced forms of IGF-I, their expression and the expression of MyoD, M-cadherin, IGFBP-4 and IGFBP-5 were measured in the damaged tibialis anterior muscle, its contralateral muscle as well as sham and normal muscles from age matched controls. The appearance and localisation of their proteins was investigated by immunohistochemistry.

Both models resulted in extensive damage followed by activation of satellite cells, which decreased after muscle regeneration commenced. The two IGF-I isoforms were differentially regulated at the RNA level. A significant, acute surge of MGF in the damaged muscle was associated with the activation of satellite cells and an increased expression of IGFBP-5. IGF-IEa mRNA and protein expression appeared to be related to the long-term recovery of muscle.
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ABBREVIATIONS

A          adenine
ALS        acid labile subunit
ATP        adenosine 5' triphosphate
ATPase     adenosine triphosphatase
bHLH       basic Helix-loops-Helix
BI         bupivacaine-injected
BMDM       bone marrow-derived macrophages
BMPs       bone morphogenic proteins
bp         base pairs
BSA        bovine serum albumin
C          cytosine
Ca^{2+}    calcium ions
cAMP       cyclic Adenosine 3', 5'-Monophosphate
cdMAF      cadherin-dependent microtubule-associated factor
cDNA       complementary DNA
DEPC       diethyl pyrocarbonate
DIG        digoxigenin
DNA        deoxyribonucleic acid
dNTP       deoxynucleotide triphosphate
dsDNA      double stranded DNA
DTT        dithiothreitol
E          E-domain
EDL        extensor digitorum longus muscle
EDTA       ethylenediaminetetraacetic acid
FGF        fibroblast growth factor
G          guanine
GAPDH      glyceraldehyde-3-phosphate dehydrogenase
GH         growth hormone
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>GHR</td>
<td>growth hormone receptor</td>
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<tr>
<td>GHRH</td>
<td>growth hormone releasing hormone</td>
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<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin counterstain</td>
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<tr>
<td>HEPES</td>
<td>2-hydroxyethyl-piperazine-N-2'-ethanelfonic acid</td>
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<tr>
<td>HGF/SF</td>
<td>hepatocyte growth factor/scatter factor</td>
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<td>IGF</td>
<td>insulin-like growth factor</td>
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<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>IGFBP</td>
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<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MAP-kinase</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>Met</td>
<td>methionine</td>
</tr>
<tr>
<td>MEF2</td>
<td>myocyte-specific enhancer binding factor 2</td>
</tr>
<tr>
<td>ml</td>
<td>millilitres</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-N-(morpholino) propanesulfonic acid</td>
</tr>
<tr>
<td>mpcs</td>
<td>myogenic precursor cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MRF</td>
<td>myogenic regulatory factor</td>
</tr>
<tr>
<td>MSCs</td>
<td>muscle stem cells</td>
</tr>
<tr>
<td>MyHC</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium ions</td>
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<tr>
<td>ng</td>
<td>nanograms</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>N-CAM</td>
<td>neural cell adhesion molecule</td>
</tr>
<tr>
<td>NF-AT</td>
<td>nuclear factor of activated T cell</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NI</td>
<td>non-injected</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localisation signal</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>pg</td>
<td>picograms</td>
</tr>
<tr>
<td>PMNL</td>
<td>polymorphonuclear lymphocytes</td>
</tr>
<tr>
<td>poly A</td>
<td>polyadenylic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SI</td>
<td>saline injected</td>
</tr>
<tr>
<td>Shh</td>
<td>sonic hedgehog</td>
</tr>
<tr>
<td>SOL</td>
<td>soleus muscle</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline triphosphate</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single stranded DNA</td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TA</td>
<td>tibialis anterior muscle</td>
</tr>
<tr>
<td>Taq</td>
<td>thermus aquaticus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TGF-beta</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>TE</td>
<td>tris-HCl, EDTA</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminoethane</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>tris(hydroxymethyl)aminoethane hydrochloride</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5' triphosphate</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
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CHAPTER I

General Introduction
1. Introduction
Skeletal muscle is a remarkably plastic tissue. It is able to retain and adapt its structure, function and metabolism during normal growth and following a variety of mechanical stimuli and traumas to which it is subjected to throughout its lifetime. Regeneration of skeletal muscles after different levels of damage is testimony to their great potential for repair. Following direct mechanical (Carlson, 1986) or chemical trauma (Foster & Carlson, 1980; Couteaux et al., 1988), after severe exercise (Irintchev & Wernig, 1987) or during the course of myopathic or neuromuscular disease (Banker & Engel, 1986), skeletal muscle fibres degenerate partially or completely and regenerate in a stereotyped manner which restores some of their normal structure and function (Carlson and Faulkner, 1983).

1.1. Skeletal muscle regeneration
1.1.1. Types of regeneration
   a) Epimorphic regeneration
This type of regeneration occurs in animals capable of re-growing a lost appendage. Amongst vertebrates only reptiles and amphibians have the capacity for this kind of regeneration.

   b) Tissue regeneration
The repair of the damaged muscle fibres uses the viable remnants of the original fibre and occurs independently of other tissues. Fibre repair occurs from viable segments of partially damaged fibres (continuous tissue regeneration) or from a population of proliferating myoblasts when the original fibres are severely damaged (discontinuous tissue regeneration).

1.1.2. Response of skeletal muscle to injury
1.1.2.1. Degeneration
Muscle degeneration occurs in two distinct phases:
   a) A non-inflammatory necrosis occurs within the injured fibre also known as intrinsic degeneration. In this phase, the myofibrills break up into individual sarcomeres, sarcoplasmic organelles are disrupted and part or all of the sarcolemma is lysed (Carlson & Faulkner, 1983)
b) This is followed by the second phase of degeneration in which the damaged fibre is infiltrated by circulating macrophages, which remove the necrotic debris, and this is also known as extrinsic degeneration.

1.1.2.2. Factors affecting muscle regeneration

Regeneration following injury of adult muscles is a process of generating new muscle tissue and which to a large extent recapitulates embryonic myogenesis. Four significant components at least, of skeletal muscles contribute to the extent to which damaged muscle fibres regenerate. These are the nerve supply, blood supply, the basal membranes and the satellite cells (Marechal, 1986).

- **Vasculature**
  The preservation of a functional muscle microvasculature or the ingrowth of capillaries when the original vascular bed has degenerated is vital if the macrophage-mediated removal of the necrotic debris is to take place and in addition, it helps regeneration process to be completed more rapidly. In contrast, in the absence of vascular reintegration, phagocytosis will not take place, and regeneration will be suspended (Bischoff, 1975) and necrotic tissue will be replaced by connective tissue (Carlson, 1983).

- **Basal Lamina**
  An intact basal lamina is believed to enhance fibre regeneration by its ability to act as a selective barrier, to “safeguard the memory” of the topographic relationship between the original muscle fibres. Furthermore, to assist with cellular orientation and organized regeneration of the myotube (Vracko and Benditt, 1972) and to guide sprouting nerve fibres towards their previous synaptic sites (Marshall et al., 1977).

- **Nerve supply**
  Absence of innervation prevents final muscle differentiation and maturation from occurring (Carlson, 1986).

- **Satellite cells**
  Adult skeletal muscle tissue consists of multinucleated, contractile myofibres covered in connective tissue through which the blood vessels and nerves course. Satellite cells are mononucleated myogenic precursor cells that reside between the basal lamina and muscle fibre sarcolemma. They are involved in the postnatal growth of muscle and are the means of providing the extra nuclei for the formation of the adult muscle mass.
Chapter 1

(Schultz, 1989, 1996). The descendants of activated satellite cells, called myogenic precursor cells (mpcs) undergo multiple rounds of division prior to fusing with existing or new myofibres. Satellite cells appear to form a population of stem cells that are distinct from their daughter mpcs (Grounds and Yablonka-Reuveni, 1993). Satellite cells in adult skeletal muscle are normally mitotically quiescent but are activated (i.e. commence multiple rounds of proliferation) in response to different stimuli like injury, denervation, exercise or stretching (Grounds, 1998).

1.1.3. Experimental models of muscle regeneration

A number of experimental models have been developed to study aspects of skeletal muscle degeneration and regeneration. Some of these were designed to mimic trauma or disease and some to elucidate the mechanisms of degeneration and regeneration. These methods are grouped according to the site of damage:

a) Damage to the sarcolemma (a collagenous envelope closely adherent to the cell membrane which is constituted by the basal lamina and the endomysial collagen / reticulin fibrils together) by mincing with auto-grafting (Studitsky, 1964; Carlson, 1972) and transecting (Gay and Hunt, 1954) a muscle will induce fibre degeneration. This method denervates and devascularizes the muscle and regeneration occurs in a disorderly manner with variable and limited recovery.

b) Damage to the plasmalemma (or known as cell membrane) by thermal injuries induced by freezing (Sartore et al., 1982) or heating (Shafiq, 1970); mechanical injuries by mild compression or crush (Järvinen and Sorvari, 1975); by exposure to myotoxic agents including intramuscular and subcutaneous injections of local anaesthetics (Benoit and Belt, 1970), subcutaneous injections of cardiotoxic snake venoms (d’Albis et al., 1989) and by hot Ringer’s solution (Schmalbruch, 1976). Thermal and mechanical injuries cause local damage to the muscle. Subcutaneous injections of cardiotoxins and hot Ringer’s solution produce widespread damage and intramuscular injections of local anaesthetics cause massive muscle damage. The myotoxic agents don’t seem to involve satellite cells and have little effect on the vascular supply of the muscle, which leads to a rapid regeneration.

c) Ischaemia: free and nerve-intact grafting (Carlson et al., 1981) cause muscle damage by ischaemia. In this model, degeneration and regeneration occur
along a centripetal gradient, parallel with the degeneration and re-growth of capillaries.

1.1.3.1. Morphological studies of bupivacaine

The local anaesthetic bupivacaine is a potent and specific myotoxic agent that produces an immediate and massive myonecrosis followed by phagocytosis of necrotic debris and a rapid and apparently complete regeneration of muscle fibres after 3-4 weeks after injection (Hall-Craggs, 1974). Electron microscopic observations on rat and mouse muscle injected with bupivacaine have given morphological details: Intrinsic degeneration begins within 15 minutes after injection of bupivacaine into fast or slow muscle. There is disruption of plasmalemma, hypercontraction of myofibrills, dilatation of the sarcoplasmic reticulum, and pyknosis of myonuclei (Bradley, 1979). During the next 12 hours there is a loss of the myofilament-banding pattern, lysis of the Z-band, rupturing of the myofibrills, fragmentation of the cytoplasm, and dissolution of the plasmalemma (Hall-Craggs, 1974; Yoshimura and Schotland, 1987). Macrophages engulf the necrotic fibres at 12hr, infiltrate them by 24hr and phagocytose the debris during the next 3 days (Nonaka et al., 1983; Wakayama and Shibuya, 1986). Myoblasts are observed at the end of the first day, and by the second day the population of these mononucleated cells has increased substantially. By the third day the myoblasts fuse to form multinucleate myotubes. After 4 days, myoblasts are no longer present, and the developing myotubes now contain chains of centrally positioned myonuclei. Growth and differentiation of the fibres occur during the next 7 to 14 days during which time the fibres appear polygonal in cross-section. By about 3 weeks, fibre maturation is complete and the size of the fibres is normal (Hall-Craggs, 1974; Sadeh, 1988), as shown in figure 1. The persistence of centrally located myonuclei in many of the fibres appears to be the only residue of the injury. The exposure of muscles to local anaesthetics has been used extensively since it is relatively simple, the damage caused is generally selective for muscle fibres and both degeneration and regeneration are rapid and reproducible. The effects of bupivacaine are quite similar to those of cardiotoxins and hot Ringer’s solution but there are some significant differences, like the damage to the microvasculature by cardiotoxin and the damage of intramuscular nerves by hot Ringer’s solution.
Figure 1. Schematic diagram showing the myogenic differentiation pathway that involves: (1) determination of mesodermal precursors as myoblasts, (2) differentiation of myoblasts into multinucleate myotubes and (3) maturation of myotubes into skeletal myofibres.

1.1.3.2. Mechanisms of local anaesthetics in causing damage

The mechanisms by which tertiary amine local anaesthetics damage muscle fibres is uncertain, however it appears that the disruption in the regulation of sarcoplasmic Ca\(^{2+}\) concentrations by the sarcoplasmic reticulum and sarcolemma partly mediates their mode of action (Bianchi and Bolton, 1967). Tertiary amine local anaesthetics are membrane-permeant giving them access to the interior of muscle fibres (Bianchi and Bolton, 1967). At low concentrations, they decrease Ca\(^{2+}\) diffusion across sarcoplasmic reticulum membranes, whereas at higher concentrations they increase the release of calcium from, and inhibit calcium uptake by, the sarcoplasmic reticulum (Johnson and Inesi, 1969). Moreover, they competitively bind to and displace calcium from membrane binding sites that increase the ionic flux across the sarcolemma (Madieri and Carvalho, 1972).
1.1.4 Snake Venoms

Snake venoms are a complex mixture of active proteins with many cytotoxic effects. Muscle damage is one of the most common symptoms produced by venoms from species in the Viperidae family. When venom components have a specific direct action on skeletal muscle affecting muscle fibres only and leaving other tissue structures such as connective tissue and nerves unharmed, they are called myotoxins. However, some observations have shown that intra-muscular injections of crude venoms or isolated toxins in mice selectively affect some muscle cells while leaving others undamaged (Ownby et al., 1976, 1982). Sensitivity to myotoxic components of snake venoms is relative to the muscle type i.e. fast or slow. Several groups observed initially, that fast muscle like EDL was more sensitive than the slow Soleus muscle to the in vivo administration of snake venoms, based on light microscopic observation of the presence of damaged muscle cells (Harris et al., 1975; Harris and Johnson, 1978; Brook et al., 1987). However, more recent investigations based on the rate of creatine kinase release from the affected muscle, showed that murine EDL muscle was more sensitive to snake venoms or isolated toxins than the Soleus muscle (Melo and Ownby, 1996). A possible explanation to this sensitivity of EDL muscle comes from observations by Nagaoka et al. (1994) that EDL has a higher mobilization of Na\(^+\) to the intracellular medium than Soleus muscle when stimulated at low frequencies or during contractile activity. The increase in intracellular Na\(^+\) results in an interruption of the exchange between extracellular Na\(^+\) and intracellular Ca\(^{2+}\), which can over-activate intracellular proteases or other enzymes that can facilitate muscle cell damage (Jackson, 1993; McArdle and Jackson, 1994).

The effects produced by bupivacaine are quite similar to those of certain cardiotoxins and hot Ringer's solution. However, there are some differences. The majority of cardiotoxin snake venoms cause damage to the microvasculature (Gutierez, Ownby and Odell, 1994), while regeneration following exposure to others (Naja mossambica mossambica) produce neuromuscular junctions with morphological irregularities (Couteaux et al. 1988). Hot Ringer's solution damages the intramuscular nerves. Additionally, the extent of damage cause by those is less than that caused by bupivacaine intramuscular injection.
1.1.5. Satellite Cells

1.1.5.1. Developmental Origin of Satellite cells

Skeletal muscles of vertebrates derive from the dorsa-lateral domain of somites. These are epithelial structures that are formed in a craniocaudal sequence from the paraxial mesoderm and are flanking the axial neural tube on both sides (Christ and Ordahl, 1995). This evidence comes from quail-chick chimera experiments that showed the presence of satellite cell nuclei derived from implanted quail somite associated with host chick myofibres (Armand et al., 1983) and verified by in vitro explant culture in birds and mammals (Cossu et al., 1996). However, more recent analyses suggested that the somitic origin of satellite cells is not exclusive. It was shown that this cell population might at least in part, arise from progenitors in the dorsal aorta of embryonic mice (DeAngelis et al., 1999). Co-expression of endothelial (VE-cadherin, P-selectin, smooth α-actin, αM-integrin, β3 integrin) and myogenic markers (MyoD, Myf5, desmin, c-Met, and M-cadherin) in cells derived from the dorsal aorta support the above hypothesis suggesting that these myogenic cells may be derived from true endothelial cells or from a common precursor. In addition, these cells are able to contribute to regenerating muscle (DeAngelis et al., 1999).

1.1.5.2. Activation of satellite cells during muscle regeneration

The molecular mechanisms that regulate the activation of satellite cells in response to diverse stimuli, leading to their entry into the cell cycle remains under investigation. Amongst several mechanisms, the inflammatory response and the release of growth factors have been implicated. Molecular events that have been implicated in satellite cell activation are as follows:

A. Cell-cell interactions mediated by VCAM-1 (a cell surface integrin molecule) and VLA-4 (integrin α4β1) ligation may initiate genetic responses within satellite cells and immune cells to promote regeneration (Jesse et al., 1998).

B. Another candidate is the hepatocyte growth factor/scatter factor or HGF/SF, a large disulfide-linked heterodimer originally discovered in regenerating liver as a factor that could stimulate hepatocyte proliferation. Its receptor, the c-met proto-oncogene, a membrane bound disulfide-linked heterodimer has an
intracellular tyrosine kinase domain. HGF/SF mRNA was first reported to be present in regenerating muscle (Jennische et al., 1993) therefore, it could be responsible for the activation of satellite cells and/or expansion of the myogenic precursor cells before the new myofibres start to form. Immunolocalization studies with adult rat skeletal muscle (Tatsumi et al., 1998) showed the presence of HGF/SF in the extracellular matrix surrounding the muscle fibres and c-met localized in putative cells. Therefore, the activation of satellite cells by exogenous HGF/SF released from the matrix may stimulate endogenous production of HGF/SF responsible for the stimulation of satellite cell proliferation. In addition, continuous regeneration of mdx mouse muscle, in the same study, showed both HGF/SF and c-met to be present. However, an equal expression of c-met and HGF in growing myoblasts and myotubes has been demonstrated (Gal-Levi et al., 1998) where myoblasts expressed c-met alone and newly formed myotubes HGF alone. The secretion of HGF by intact myotubes could promote mpc proliferation in regenerating muscle. Although a mechanism for secretion of HGF in damaged muscle is not yet confirmed, there is strong evidence to suggest that HGF/c-met signal transduction plays a fundamental role in muscle regeneration.

C. Macrophages (activated monocytes) and polymorphonuclear lymphocytes migrate to the site of injury a few hours after damage. Macrophages are the main immune cells present in the damaged area 48hrs post-injury (Tidball, 1995). They phagocytose necrotic cell debris and secrete a soluble growth factor, not yet characterized, but shown to exert a mitogenic effect on myoblasts (Cantini and Carraro, 1995; Merly et al., 1999). Their essential role in muscle regeneration was drawn from results showing impaired myogenesis in their absence (Lescaudron et al., 1999).

D. Cytokines like interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF) have been shown to stimulate the proliferation of mpcs in culture (Kurek et al., 1996). Results from the same study demonstrated a significant increase of LIF expression 3hrs after muscle injury, indicating that damaged muscle produces LIF prior to infiltration of immune cells. In addition, the source of LIF mRNA expressed in regenerating diaphragm of mdx mice was revealed to be muscle
and surrounding non-muscle cells (Kurek et al., 1996). In contrast, IL-6 was shown to be produced by infiltrating immune cells at 12 to 24hrs after injury (Kurek et al., 1996).

E. A role for IGF-I has also been implicated in the activation of satellite cells. In vitro studies suggested that IGF-I stimulates proliferation, differentiation and fusion of satellite cells into growing myotubes (Florini et al., 1991; Delany et al., 1994; Goldspink et al., 1995). Moreover, when IGF-I was directly introduced into the tibialis anterior muscles of adult rats, total muscle protein and DNA content were increased. This was regarded as indicative of skeletal muscle hypertrophy relating to satellite cell activation (Rosenblatt et al., 1994; Adams and Haddad, 1996; Adams and McCue, 1998). Recent findings from Chakravarthy et al. (2000), reported that IGF-I extended the in vitro replicative life span of skeletal muscle satellite cells by enhancing G1/S cell cycle progression via the activation of phosphatidylinositol 3'-kinase /Akt signalling pathway. With these observations a mode of IGF-I action stimulating satellite cell activation and prolonging their cell division capacity has been revealed.

F. Fibroblast growth factor 2 (FGF2) is also implicated in promoting recruitment of skeletal muscle satellite cells in young and old rats (Yablonka-Reuveni et al., 1999). In agreement to this finding, Floss et al., (1997) showed that FGF6 is critical in vivo for muscle regeneration. This was demonstrated by a muscle regeneration deficit when using FGF 6(-/-) knockout mice. He also proposed that FGF6 could play a role in stimulating or activating satellite cells. Both FGF2 and FGF6 might be involved in the specific recruitment of satellite cells because they are both present in the adult muscle and promote activation / proliferation of satellite cells in isolated fibres in similar fashion (Yablonka-Reuveni et al., 1999). Therefore, it is possible that different FGFs can be involved in the recruitment of satellite cells.
Figure 2. A summary of molecular events implicated in satellite cell activation, during skeletal muscle regeneration. (A) Satellite cell activation may result from the ligation of VLA-4 integrin molecule on infiltrating polymorphonuclear leukocytes (PMNL) and VCAM-1 on resident satellite cells. (B) & (C) HGF is postulated to activate satellite cells through its receptor c-Met, expressed in quiescent satellite cells. (B) HGF may be produced by undamaged myofibres in response to physiological stimuli. (C) Basal lamina damage and extracellular matrix of myofibres may release HGF that is normally sequestered here. Activation of quiescent satellite cells results in expression of MyoD and/or Myf5 and the generation of daughter mpcs. Several growth factors have been implicated in expansion of the mpc compartment, including HGF, PDGF, LIF, IL-6, FGF and IGF-I. In addition, a growth factor (possibly LIF) secreted from the infiltrating macrophages is crucial for mpc proliferation. Following proliferation, mpcs undergo terminal differentiation in the formation of myocytes that requires myogenin and MRF4 expression before fusing with existing or new fibres. IGF-I has also been suggested to induce muscle hypertrophy in fibres, independent of its role in mpc proliferation (adapted from Seale and Rudnicki, 2000).
1.1.5.3. Satellite cell self-renewal

The number of quiescent satellite cells in adult muscles remains relatively constant over multiple cycles of degeneration and regeneration, demonstrating an inherent capacity for self-renewal (Gibson and Schultz, 1983). However, the mechanism by which satellite cells undergo self-renewal remains elusive. Three possible models mentioned underneath could co-exist together to maintain a steady number of satellite cells:

1) Muscle stem cells may undergo asymmetric cell division to generate two daughter cells: a committed myogenic precursor cell and a pluripotent "self".

2) Self-renewal of the satellite cell compartment may be Myf-5 (the earliest marker of myogenic commitment) dependent. RT-PCR analysis reveals that activated satellite cells express Myf-5 first alone or MyoD alone before expressing both at the same time (Cornelison and Wold, 1997). MyoD-/- myogenic cells represent an intermediate stage between a quiescent satellite cell and a myogenic precursor cell (Yablonka-Reuveni et al., 1999) and activation of Myf-5 without MyoD expression defines a capacity for satellite cells to undergo self-renewal.

3) De-differentiation of committed myogenic precursor cells, expressing myoblast markers like MyoD, Myf-5 and desmin could lead to quiescent satellite cells.

1.1.5.4. Satellite cells as pluripotential stem cells

The study of muscle satellite cells as stem cells, has been investigated thoroughly however, several intriguing questions are still raised. Experiments by Gussoni et al., (1999), using muscle stem cells (MSCs), showed that by intravenous injection into mice, MSCs gave rise to satellite cells in recently regenerated host fibres. In addition, MSCs reconstituted the whole of the haematopoietic system when they were injected into lethally irradiated recipients. This was also supported by studies done by Jackson et al. (1999) that revealed the contribution of MSCs to all major blood lineages 3 months after the injection. Whether these MSCs are the satellite cell themselves or the direct progenitors of satellite cells or even an independent stem cell population still remains unresolved and molecular and biochemical analysis will need to answer these questions. Recent experiments by Pye and Watt (2001) demonstrated that dermal fibroblasts could contribute to muscle fibre formation when implanted into the
regenerating muscle of a normal mouse, however not indicating whether they contribute to the satellite cell population.

The ability of tissue-specific cells to activate various genetic programmes in response to environmental cues is very exciting. This finding could raise the possibility of stem cell therapy for a variety of degenerative diseases, including muscular dystrophy by the pluripotent nature of adult stem cells isolated from diverse tissues and being delivered through the circulation.

1.1.6. Cadherins involved in development of striated muscle

Cadherins are calcium-dependent, transmembrane intercellular adhesion proteins with morphoregulatory functions in the development and maintenance of tissues. The most studied cadherins are N- (neural), E- (epithelial) and P- (placental), B- (brain) and M- (muscle). Although each one of the cadherins show a distinct pattern of tissue distribution and developmental regulation, the expression pattern of some of these can show an overlap and can coexist in the same tissue or even in the same cell (Moore and Walsh, 1993). Cadherins are involved in processes like cell recognition and sorting, cell polarization, formation of junctional complexes and tumour metastasis (Takeichi, 1990, 1991). It has been shown that some of the cadherins can perform only if connected to the catenins in their cytoplasmic tails. Another class of cell adhesion molecules, N-CAM (neural cell adhesion molecule) has been found on activated satellite cells (myoblast stage) and later on in myotubes during the regeneration process or on the entire sarcolemma of muscle fibres after denervation (Covault and Sanes, 1985). In normal adult muscle, N-CAM is expressed at the neuromuscular junction (Covault and Sanes, 1986).

1.1.6.1. Expression pattern of M-cadherin during prenatal and postnatal skeletal muscle development

In mouse development, M-cadherin mRNA can be first detected in somites at day 8.5 of gestation with a delay of M-cadherin protein visible at day 10.3 in myotomal cells (Moore and Walsh, 1993). At day 16 of gestation when primary and secondary myotubes are formed, M-cadherin clusters appear on the cell surface of all myotubes (Rose et al., 1994). Therefore, M-cadherin could be important for the adhesion at the different stages in myotube formation. In postnatal skeletal muscle, M-cadherin is expressed in satellite cells of intact muscle (Bornemann and Schmalbruch, 1994;
Irintchev et al., 1994; Reimann et al., 2000) and immunoelectron microscopy demonstrated its location on myofibres underneath the satellite cells (Bornemann and Schmalbruch, 1994) as shown in figure 3.

**Figure 3.** Localization of M-cadherin (M-cad), laminin and N-CAM on muscle fibre. The proportions of the depicted structures do not necessarily correspond to their actual size. N-CAM was observed rarely in intact muscles (adapted from McGeachie and Grounds, 1999).

Therefore, M-cadherin might serve as a molecular link between the satellite cell and the muscle fibres and any disruption of this link could result in the satellite cells being activated and entering into the muscle fibre.

In regenerating skeletal muscle fibres, M-cadherin is rapidly up regulated (Irintchev et al., 1994) with an initial expression in myoblasts exclusively. Down-regulation of M-cadherin expression occurs when myoblasts fuse into myotubes and its expression is barely detectable in mature myotubes surrounded by basal lamina sheaths (Irintchev et al., 1994). These results signify the role of M-cadherin in myogenic morphogenesis both in the embryo and in the adult organism during regeneration. The expression pattern of N-CAM and desmin, both muscle-specific marker proteins, differed significantly from that of M-cadherin and from each other. Desmin was found in both mpcs (satellite cells and myoblasts) and in the differentiated multinucleated cells...
(myotubes and muscle fibres), which appears to agree with previous work by Bomemann and Schmalbruch (1992) therefore, presenting desmin as a marker of post-embryonic striated muscle cells in all stages of regeneration.

Double immunostaining carried out in denervated mouse muscle, for M-cadherin and laminin (a major component of the basal lamina of blood vessels and muscle fibres) showed that M-cadherin positive cells were muscle satellite cells enclosed within the basal lamina of parent muscle fibres (Irintchev et al., 1994). M-cadherin expression was also compared with the expression of N-CAM found to be up regulated in activated rodent satellite cells (Grounds, 1991). It was shown that both of them were more intense at the side of satellite cells facing the muscle fibre suggesting that M-cadherin and N-CAM are localized on both muscle fibre and satellite cell membranes as shown in figure 3.

1.16.2. The M-cadherin-catenin complex with the cytoskeleton during muscle differentiation

In skeletal muscle cells, M-cadherin interacts with β-catenin or plakoglobin and via these proteins with α-catenin. Kaufmann et al., (1999) proposed a model of interaction of M-cadherin with the microtubules implicating that they are both essential to keep myoblasts aligned during fusion. Initially in myoblasts, microtubules spread out from the centrosomal region to the cell membrane and start orientating in parallel to the longitudinal axis of aligned myoblasts when myogenic differentiation is underway. It is speculated that a muscle-specific and cadherin-dependent microtubule-associated factor (cdMAF) mediates the connection of the M-cadherin catenin complex to the microtubular system. However, whether cdMAF directly interacts with the microtubules or not is still unclear. The mechanism by which the cdMAF mediates the interaction of M-cadherin/catenin and the microtubules is still elusive. One possibility could be that cdMAF binds directly to the cytoplasmic domain of M-cadherin. Another likelihood could be that one of the catenins, mentioned above, generates the molecular link between the M-cadherin-catenin complex and the microtubules by interacting with the cdMAF. Based on this model, it was shown that cutting of the microtubules by nocodazole (an alkaloid solution that depolymerises microtubules) did not destroy the M-cadherin/catenin complex (Kaufmann et al., 1999).
1.2. Skeletal myogenesis

Skeletal myogenesis begins in the embryo as a result of signalling molecules from surrounding tissues that denote the myogenic cell fate. The existence of distinct molecular markers such as the myogenic regulatory factors (MRF) allows detailed analyses of myogenic determination, myoblast proliferation and terminal differentiation.

Gene targeting and transgenic mice have revealed the temporal and spatial expression patterns of these muscle regulatory factors during early somitogenesis providing an insight in muscle cell determination, differentiation and gene expression.

1.2.1. The Myogenic Regulatory Factors (MRFs)

The MRFs belong to the basic helix-loop-helix (bHLH) superfamily of transcription factors. The MRFs contain an evolutionary conserved core consisting of a basic region, involved in DNA binding, and the HLH domain that aids their dimerization.

A large number of bHLH proteins bind to the consensus E-box (CANNTG) DNA sequence found in the promoters of many of muscle specific genes (Lassar et al., 1989). Four MRFs have been cloned, MyoD, Myf-5, myogenin and MRF4.

Overexpression of these factors converts a wide range of cultured cells to a muscle phenotype, demonstrating their role in myogenic lineage determination and differentiation. The MRFs functions in vivo have been investigated by the establishment of their pattern of expression and by gene targeting.

1.2.2. Temporal and Spatial Patterns of MRFs' expression during embryogenesis

During development, the order of expression of MRF genes varies according to muscle origin and among species (Yun and Wold, 1996). Experiments using knockout mice progressively clarified the hierarchical relationships between the MRFs. In situ hybridisation shows that MRF expression occurs sequentially during somitogenesis and myogenesis and in slightly different patterns in epaxial versus hypaxial muscle (figure 4). Myf5 mRNA is the first to be detected at embryonic day 8 in the dorsomedial portion of the somite and at day 9.5 in the hypaxial domain of the somite until day 14 (Ott et al., 1991). Early expression of Myf5 in the un-compartmentalized somite suggests a role in the initial commitment of cells to the myogenic lineage. The formation of the myotome at day 8.5-post coitum is parallel with the initial
appearance of myogenin transcripts, which remains detectable in the myotomal cells as they differentiate (Sassoon et al., 1989). MRF4 mRNA is detected transiently in the somite between days 10 and 11 and then re-expressed at day 16 throughout the muscle-forming regions of the embryo to become the predominant amongst the MRFs expressed after birth (Hinterberger et al., 1991). MyoD mRNA is first detected around day 10.5 in the hypaxial somite and is present throughout embryonic development (Sassoon et al., 1989). The expression pattern of the MRFs recurs as each somite differentiates.

In the limb bud, the temporal appearance of the MRFs is different from that in the somite. Although Myf5 mRNA is detected first, co-expression of myogenin and MyoD mRNAs follow very quickly from day E10.5 onward. MRF4 mRNA is not expressed in the limb until late in development, first appearing at day E16 and becomes the principal MRF expressed in the adult (Bober et al., 1991). The migratory myoblasts leaving the ventro-lateral domain of the somite to colonize the limb and body wall do not show MRF being expressed until they reach their final destination (Sassoon et al., 1989). Therefore, this indicates that the signals responsible in activating the MRFs in the medial somite are distinct from those that activate the MRF expression in the migratory myoblasts arising from the lateral somite. In vivo, the temporal expression of the MRFs does not run through in the same manner as in vitro.
Figure 4. Schematic representation of myotome formation by delamination of some of the cells of the dermamyotome and further migration to the developing limbs. Somites form in a rostral to caudal direction. As development proceeds, somites become subdivided into the ventral sclerotome and dorsal dermomyotomal domains (B). The two red arrows in (A) figure represent the migratory muscle precursor cells (mpcs). Dermomyotomal expansion (top red arrow) leads to the extension of cells to a position beneath the dermomyotome. This marks the formation of the epaxial myotome, which can be identified by Myf5 expression. A similar extension occurs forming the hypaxial mesoderm (not shown here) where cells predominantly express MyoD. Nt= neural tube, n= notochord, D=dorsal, V=ventral. (Adapted from Perry and Rudnicki, 2000).

1.2.3. Gene targeting experiments in mice

In order to gain insight into the nature of lineage determination and maintenance and clarify the functions of the MRFs during myogenesis in vivo, homologous recombination was used to disrupt each MRF in mice. The following summary of events depicts the results of the experiments and shown in table 1:

Mice homozygous null for MyoD showed no obvious muscle defect (Rudnicki, et al., 1992). Targeted inactivation of the Myf-5 gene results in morphologically normal muscle and survival to birth, but in contrast to MyoD-null mice, they die perinatally.
due to defective rib development and inability for normal respiration (Braun et al., 1992). In addition, myotome formation is delayed and this causes the abnormal differentiation of the sclerotome from which the axial skeleton and ribs derive from (Braun et al., 1992).

Since Myf-5 is the first MRF to be expressed in the mouse embryo (Ott et al., 1991) and MyoD is not expressed until later (Sassoon et al., 1989), Myf-5 must be required for the normal formation and interactive events of the early myotome. Moreover, since Myf-5 is expressed in the epithelial somite before it compartmentalizes, it may also play a role in the sclerotomal lineage formation. This suggests a possible explanation for the rib-deficient phenotype of Myf-5-null mice. Mice lacking both MyoD and Myf-5 genes show a complete absence of myoblasts and muscle fibres.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype</th>
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<tr>
<td>MyoD (-/-)</td>
<td>Rudnicki et al., 1992</td>
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<td>Myf-5 (-/-)</td>
<td>Braun et al., 1992</td>
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<td>MRF4 (-/-)</td>
<td>Zhang et al., 1995</td>
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<td>Myf-5 (-/-) MRF4 (-/-)</td>
<td>Patapoutian et al., 1995</td>
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<td>Myogenin (-/-)</td>
<td>Braun &amp; Arnold, 1995</td>
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<td>Myogenin (-/-) MRF4 (-/-)</td>
<td>Hasty et al., 1993</td>
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<td>MyoD(-/-)MRF4(-/-)</td>
<td>Nabeshima et al., 1993</td>
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Table 1. Summary of targeted mutations of the MRFs in mice by homologous recombination. X denotes the stage of myogenic differentiation achieved.
When the myogenin gene was disrupted, mice died perinatally like Myf-5-null mice also showing a distinct defective rib cage, but there is also a severe deficiency of differentiated muscle fibres (Rudnicki et al., 1993). Myogenesis is independent of myogenin during embryonic stages, but requires myogenin for the second wave of myogenic differentiation that occurs late in embryogenesis (Venuti et al., 1995).

Three different groups have inactivated the MRF4 gene producing a range of defective rib cage phenotypes (Braun & Arnold, 1995; Patapoutian et al., 1995; Zhang et al., 1995). The difference in severity of the rib phenotype is linked with alterations in Myf-5 expression. The two genes are located around 6kb apart, therefore cis-regulatory elements important for Myf-5 gene regulation may have been altered during the generation of this mutant allele (Yoon et al., 1997). Starting from the most severe rib defects being observed in mice lacking Myf-5 (Braun & Arnold, 1995), moderate severity is seen with defects in myotome formation resulting in inviability but with no alteration in myogenic differentiation (Patapoutian et al., 1995). Mice with normal Myf5 expression are born healthy and fertile with minor rib defects and myogenin mRNA expression is shown to be up regulated four-fold in the adult skeletal muscle relative to the wild-type expression (Zhang et al., 1995). This suggests that myogenin can compensate for MRF4 in adult muscles.

Combinations of null mutations in the MRFs or when coding sequence of one MRF knock-in to the locus of another, have been generated to unmask their functional relationships. These gene-targeting experiments together, propose that MyoD and Myf5 act to determine the myoblast lineage while myogenin and MRF4 are essential for differentiation and maintenance of the terminally differentiated state (Rudnicki and Jaenische, 1995).

1.2.4. Regulation of myogenesis during development

Somite formation and determination of cell lineages are both influenced by a number of factors expressed in axial and lateral regions of the developing embryo. These factors secreted either from the axial structures or the lateral mesoderm and dorsal ectoderm include: sonic hedgehog (Shh), transforming growth factor-beta (TGF-beta)-like molecules, fibroblast growth factors (FGFs) and bone morphogenic proteins (BMPs). Regulation of myogenic determination and differentiation results from the expression of a whole variety of positive and negative factors.
1.3. Skeletal muscle fibre types

Mammalian skeletal muscle fibres differ widely with respect to their biochemical and functional characteristics and due to their plasticity they can undergo extensive remodelling to meet new functional requirements. This high adaptability appears to reside in the muscle fibre’s ability to transcribe different isoforms of its contractile proteins. Myosin, actin, troponin and tropomyosin are responsible for the contractile function of the muscle. Myosin consists of two heavy chains each of which is associated with two light chains. The myosin heavy chains (MyHCs) exist as a number of isoforms encoded by distinct genes. At least six MyHCs are expressed in rat skeletal muscles (Schiaffino and Reggiani, 1996). Two developmental isoforms, embryonic and neonatal and four adult MyHC isoforms designated as slow type I, fast IIA, IIX, and IIB have been identified. The expression pattern of these isoforms is developmentally regulated and can be modulated by hormonal, metabolic and mechanical factors in a tissue-specific manner. The myosin molecule has the ability to catalyse the hydrolysis of ATP. Cross-bridges linking actin and myosin increases the adenosine triphosphatase (ATPase) activity of myosin resulting in production of force and movement coupled to ATP hydrolysis. It is now established (Rome et al., 1990; Larsson and Moss, 1993) that the ATPase activity of the different MyHC isoforms is a major determinant of the differences in the speed of contraction of muscle fibres. This combined with the rate at which the ATP levels are replenished within the muscle fibres (their oxidative capacity), have led to the classification of distinct fibre types in mammalian skeletal muscle. Two types of muscle fibres have been distinguished: the slow-twitch fibre or type I and the fast-twitch fibre or type II. Type I fibres are slow contracting with a low specific myosin ATPase and high oxidative enzymatic activity. Therefore, they are fatigue resistant and suitable for slow, repetitive, prolonged activity. Type II fibres are classified in three subtypes as mentioned above. The IIA fibres have a high oxidative capacity. The IIB type constitutes the main fast type in rodent muscles and the IIX is the predominant fast type in mammalian muscles, including man (Ennion et al., 1995). Both IIB and IIX have a high glycolytic capacity giving them a capacity for short-term phasic movements, as the onset of fatigue is rapid.
1.3.1. Cellular mechanisms involved in muscle growth

Growth of muscle can occur in two ways: a) by an increase in muscle cell numbers or hyperplasia, limited in prenatal muscle fibres and b) increase in muscle cell size or hypertrophy, in postnatal muscle. The latter can occur in two ways: 1) by increase in length where fibres increasing in length is a consequence of additional sarcomeres (Williams & Goldspink, 1971), 2) by increase in girth which occurs in a discontinuous way (Alnaqeeb & Goldspink, 1987). This can be described as the increase in number and size of myofibrils. Examination of muscle fibres at different stages of growth, have demonstrated that the proliferation of myofibrils is the consequence of longitudinal splitting of the existing myofibrils, as soon as they reach a certain size.

1.3.2. Factors causing changes in muscle mass and phenotype of skeletal muscle

A. Nerve activity

The nerve is known to play a fundamental role in the diversification of muscle fibre phenotypes. Reducing neuronal activity in a slow muscle i.e. soleus by denervation, switches on genes associated with a fast muscle phenotype (Loughna, *et al.*, 1990; Loughna and Morgan, 1999). On the other hand, denervating a fast muscle such as EDL causes the fast fibres to convert to a slower contractile phenotype, with a higher proportion of type IIa and slow type I fibres appearing (Pette and Vrbova, 1985; Hoh, 1991; Windisch *et al.*, 1998). In addition, denervation also induces the re-expression of neonatal fast myosin (Butler-Brown *et al.*, 1982). It has been suggested that the MRFs especially MyoD and myogenin, confer the effects of the nerve on muscle phenotype in postnatal skeletal muscle. Short-term denervation (48hr) in correlation to the MRFs, was highly phenotype dependent, with the least dramatic effects in the slow soleus and significant changes seen in the fast phasic EDL, plantaris and tibialis anterior muscles (Walters *et al.*, 2000). A longer period of denervation reduces the ability of the muscle to become functionally re-innervated and the muscle continues to degenerate (Sunderland, 1978).

Cross-innervation experiments gave further evidence of muscle phenotype modulation by nervous activity (Close, 1965; Mommaerts *et al.*, 1977) by showing that cutting and implanting a nerve normally innervating a slow muscle to a fast one, the re-innervated muscle acquires properties of the slow muscle.

Several different applications of stimulation of nerves have also been used in a number of studies where stimulation of a fast muscle with a slow pattern causes a
transformation to slow muscle (Pette and Vrbova, 1992). Chronic low frequency stimulation has been applied in several studies where it effectively transforms fast fatigue-able muscle into slow fatigue-resistant ones but this induces muscle atrophy over a long-term protocol. MyHC analyses on single fibres from the vastus lateralis muscle of spinal-cord injured patients exposed to electrical stimulation for up to a year, displayed pronounced increases in MyHCIIa but never in MyHCI (Andersen et al., 1996). However, hormonal or genetic factors could also interfere with the muscle response to stimulation and cause the phenotype change.

B. Stretch

Stretch or increased tension on a muscle is a major component contributing to muscle mass increase. For example, the fast TA of a rabbit shows a 30% increase in mass when stretched and immobilised with the use of a plaster cast (Goldspink et al., 1992). In addition, fast to slow phenotype change occurs and muscle adapts to an economic phenotype for a more postural type (Goldspink et al., 1992). The effects of chronic muscle disuse in different models that disrupt (i.e. spinal cord transection and neural application of toxins) or leave intact (hind limb suspension and limb immobilisation) communication between the muscle and central nervous system have been studied. The effect of immobilization could cause partial fibre type conversion (Booth and Kelso, 1973; Gardiner and Lapointe, 1982), and muscle fibre atrophy. Immobilization by pinning the knee and ankle joints, which resembles the clinical condition of restricted mobility due to plaster casting and arthritic /surgical pain, showed changes in muscle mass and fibre cross-sectional area (Ibebunjo and Martyn, 1999) with fibre atrophy explaining the muscle weakness.

C. Exercise

Many histochemical studies on endurance training in humans have demonstrated that mATPase increases in type IIa fibres and decreases in type IIb fibres (Andersen and Henriksson, 1977; Harridge, 1996). Equally, endurance training in rats has been shown to cause a decrease in MyHCIIb together with an increase in MyHCIIa (Sugiura et al., 1992). Sprint training in rats and humans results in transformation from both myosins IIb and I to myosin IIa (Allemeier et al., 1994; Andersen et al., 1994).
D. Loading

A well-studied factor is the load supported by muscle. Overloading is responsible for muscle hypertrophy and slower phenotype, whereas unloading results in muscle atrophy and a slow to fast phenotype change.

1. Unloading

Using the tail suspension method, Awede et al., (1999a) showed that there was a 30% decrease in the soleus muscle mass and phenotype change from slow-to-fast, by unloading the hind limb of 2-month-old mice for two weeks.

2. Overload

There are several methods generating muscle overload:

- Denervation of synergistic muscles where re-innervation can occur after a few weeks suppressing the increased load (Binkhorst and van’t Hof, 1973).
- Tenotomy: when the tendon of a synergistic muscle is detached causing a compensatory hypertrophy where the muscle markedly increases (Mackova and Hnik, 1971; James, 1976). The tendon however, tends to reattach or adhere to the remaining musculature after 10-14 days, so that the reattached muscle becomes functional again and abolishes the overload process (Timson, 1990).
- Ablation of synergistic muscle creates overload of skeletal muscle in rodents where the intervention acts locally on, or close to, the muscle of interest and often provokes an inflammatory process.

By inserting blocks of lead with weight equivalent to the value of body mass, underneath the skin of the lower part of the mice back, over a period of 4 weeks Awede et al., (1999a) showed a 17% increase in soleus mass. In addition, this method caused changes in mechanical properties of soleus muscles and increased MyHC type I fibres with a reduced number of MyHCIIa fibres.

E. Hormones

Thyroid hormones induce changes in muscle fibre phenotype; Injection of T3 triggering of hyperthyroidism, results in a shift from slow-to-fast fibre whereas, hypothyroidism has the opposite effect (d’Albis et al., 1993; Xiaopeng and Larsson, 1997).

Androgens are anabolic agents, known to increase muscle mass (Ferrando et al., 1998) and also affecting muscle phenotype (Holmäng et al., 1990). Androgens have been used in studies with immobilised and non-immobilised muscles causing an increase in muscle protein synthesis and therefore an increase in muscle mass (Taylor
et al., 1999). When administered to animals diet anabolic agents such as β2-adrenoceptor agonists induce skeletal muscle hypertrophy after a few days. When clenbuterol, a β2-adrenoceptor agonist was given to female rats over 4 weeks, soleus showed an increase of 16% with slow-to-fast phenotype trend (Awede et al., 2002).

1.4. Insulin-like growth factor system

The importance of peptide hormones or growth factors in growth and development is well established. IGF-I and -II are multifunctional growth factors that have shown to elicit a broad range of functions in the embryo, foetus and adult.

1.4.1. Source of production

Insulin is synthesized in the beta cells of the pancreas as pro-insulin, which is cleaved to form insulin and C peptide. The IGFs are primarily produced by the liver, retain the C peptide and have an extended carboxy-terminus (Daughaday and Rotwein, 1989). They share approximately 50% homology with insulin. Insulin circulates in very low concentrations (picomoles) and has a half-life of minutes, whereas the IGFs circulate at higher concentrations (nanomoles) and are bound to one of the six IGF-binding proteins that modulate IGF activity. These binding proteins are synthesized primarily in the liver. Most tissues also locally produce both IGFs and their binding proteins, where they act in an autocrine or paracrine manner.

1.4.2. IGF-I gene and mRNA structure

IGF-I is a 70-residue, single chain-peptide that is structurally related to insulin and IGF-II (Humbel, 1990). Human and rat IGF-I consist of 6 exons spanning more than 80 kilobases (kb) of genomic DNA and are separated by 5 introns ranging in size from 1.9 to 50kb (Daughaday and Rotwein, 1989; Humbel, 1990; Hepler and Lund, 1990). Exons 1 and 2 have multiple transcription sites that define two distinct leader exons (Adamo et al., 1991; Kim et al., 1991) that encode various 5'-untranslated regions (UTRs) and distinct amino-terminal putative signal peptides (see figure 5). Exon 3 and 4 encode the common carboxy-terminal amino acids (aa) of the signal peptides, the mature IGF-I peptide and the amino-terminal 16aa of the E peptide, which is presumably cleaved during processing of the pro-hormone. All IGF-I protein molecules contain four domain structures termed B, C, A and D based on the
homology to B, C, and A domains of insulin. Exon 3 encodes the first part of the B domain. Exon 4 encodes the remainder of the B, C, A and D domains (D domain does not have an homologue in the insulin molecule) and the initial part of the E peptide as mentioned above. In the rat and mouse IGF-I gene exon 5 is an alternative spliced “cassette” of 52 nucleotides, whose inclusion in the IGF-I mRNA changes the reading frame, and the remainder of the E peptide sequence from that encoded by IGF-I mRNAs in which exon 4 is spliced directly to exon 6. Exon 6 encodes the carboxy-terminus of the E peptide and the presence of multiple polyadenylation sites produces 3'-UTRs (>6kb) of variable lengths (Lund et al., 1989).

1.4.3. Regulation of IGF-I gene transcription

Two promoters control the transcription of IGF-I gene, one adjacent to exon 1 and the other 5' to exon 2. Both promoters regulate transcription at multiple initiation sites, and both lack a “TATAA” box, a “CCAAT” box and other typical proximal control elements, yet neither of them resemble a GC-rich “housekeeping” promoter (Adamo et al., 1991; Jansen et al., 1991; Hall et al., 1992).

1.4.4. IGF-I mRNA heterogeneity

The IGF-I mRNA heterogeneity has been best characterized in the rat (Lund et al., 1991) and exists in two levels (also in man and mouse):

a) Size heterogeneity: multiple IGF-I mRNAs arise primarily as a result of variation in the length of the 3'-untranslated sequence; i.e. in the rat IGF-I, exon 6 has at least three polyadenylation signals.

b) Coding sequence heterogeneity: while all the IGF-I mRNAs encode the same mature IGF-I, they may differ in coding sequences for amino-terminal or carboxy-terminal precursor peptides that flank the mature IGF-I sequence (Daughaday and Rotwein, 1989; Hepler and Lund, 1990).
**1.4.5. IGF-I mRNAs encoding alternate E peptides**

Alternate splicing involving exons 5 and 6 results in IGF-I mRNAs encoding different E peptides. These E peptides are cleaved from nascent pre-pro-IGF-I during its processing, since they are not found in the mature circulating form of IGF-I. 3’ alternative splicing of the primary IGF-I gene results in six different IGF-I mRNAs in humans. These are:

- Class 1 IGF-IEa (exons 1, 3, 4 and 6) (Sussenbach *et al.*, 1992; Gilmour, 1994)
- Class 1 IGF-IEb (exons 1, 3, 4, and 5) (Rotwein *et al.*, 1986; Gilmour, 1994)
- Class 1 IGF-IEc (exons 1, 3, 4, part of 5 and 6) (Chew *et al.*, 1995)
- Class 2 IGF-IEa (exons 2, 3, 4, and 6)
- Class 2 IGF-IEb (exons 2, 3, 4, and 5)
- Class 2 IGF-IEc (exons 2, 3, 4, part of 5 and 6)
In rat IGF-I gene, 3’ alternative splicing by inclusion of exon 5 (i.e. splicing of exons 4-5-6) or exclusion of exon 5 (splicing of exons 4-6) results in IGF-I Ea and IGF-I Eb mRNAs, respectively. Class 1 and 2 sequences are each associated with Ea or Eb 3’ coding sequences, therefore rat IGF-I gene encodes class 1 Ea, class 1 Eb, class 2 Ea and class 2 Eb mRNAs (or otherwise classified as IGF-IA or IGF-IB mRNAs).

IGF-I Ea encodes a 35 amino acid E domain in contrast to the IGF-I Eb that codes for a different 41 amino acid E domain due to the inclusion of the cassette exon (see figure 7). This occurs because inclusion of the mini-exon results in a shift in translation reading frame and stop codon (Daughaday and Rotwein, 1989; Hepler and Lund, 1990).

In the rat, the splicing pattern of exon 5 is different from that in the human; exon 4-6 or exon 4-5-6 splicing occurs (Roberts et al., 1987; Shimatsu and Rotwein, 1987) and exon 5 is a cassette exon of 52bp, rather than a terminal exon. The original classification of the different IGF-I isoforms were based on those expressed by the liver. The nomenclature is however often confusing. When it is applied to IGF-I splice variants derived from non-hepatic cells and also between species, IGF-I Eb in the rat is equivalent to IGF-I Ec in the human. Comparison between the human and rat cDNA and deduced peptide sequences suggests that the exon 4-5-6 splices in both species are equivalent, shown in figure 6 below (Roberts et al., 1987; Shimatsu and Rotwein, 1987). The deduced E-peptide sequence of the rat and human 4-5-6 mRNAs show 73% homology. A number of experiments have postulated that the rat hepatic IGF-I Eb mRNA may produce circulating and GH-responsive IGF-I (Lowe et al., 1988; Lund, 1994). Rat exon 5 contains four-dispersed purine-rich repeats (GGAAG) within 300bp down-stream of the donor site which have been shown to enhance splicing in the bovine GH gene by interacting with protein splicing factors (Sun et al., 1993). Human exon 5 has a polyadenylation site; this is absent in the rat exon 5.
Figure 6. Human exon 4-5 (Eb) and exon 4-5-6 (Ec) cDNA sequences aligned with rat exon 4-5-6 (Eb). Homology to human EC is shown with asterisks, exon boundaries are marked by slashes and in-frame stop codons are underlined in human Ec and rat Eb. The human Eb sequence is the one cloned by Rotwein (1986). Taken from Chew et al., (1995).

1.4.6. Role of E peptides

Until recently, the biological roles of the E peptides have been overlooked. It was generally believed that they were biologically inert. The Ea type E domain contains two potential N linked glycosylation sites and is glycosylated in vitro (Bach et al., 1990). The Eb E domain is not glycosylated in vitro (Bach et al., 1990) and is highly basic. These structural characteristics suggest different biological roles for each. The Ea type E domain is highly conserved among vertebrates (Shamblott et al., 1993) whereas the Eb types are conserved in the first 15 amino acids and differ thereafter among humans, rats and mice. The conservation of the E-peptide sequence implies that a selection pressure exists against random mutations in these and puts forward a possibility for functionality in E peptides. A synthetic amide (IBE1) of 22-amino
residues encoded within the variable region of the human IGF-IEb, was shown to act as a mitogen in normal and malignant bronchial epithelial cells (Siegfried et al., 1992). In addition, Tian et al. (1999) demonstrated that E-peptides of rainbow trout pro-IGF-I possess mitogenic activity in heterologous systems. To date, to our knowledge, there has not been any report on the presence of free pro-IGF-I E peptides in the circulation of any vertebrate. The co-translated and possibly co-secreted E-peptides in accordance with the mature IGF-I could exert their mitogenic activities in a similar endocrine, paracrine / autocrine fashion and together with IGF-I may also play important roles in regulating growth and differentiation.

1.4.7. Different 5’ sequences in IGF-I cDNAs and mRNAs
Characterization of IGF-I cDNAs from mouse, human, rat and sheep liver with different 5'-UTR/pre-peptide sequences (Bell et al., 1986; Roberts, et al., 1987; Wong et al., 1989; Tobin et al., 1990) gave the initial evidence for the alternate splicing in the 5'-UTR region. At least three distinct sequences are found 5’ to the identical region in IGF-I cDNAs and mRNAs; one 5’ sequence termed type I or Class C (Roberts et al., 1987), a variant type 1/Class C cDNA –type 1 del- with an external deletion of 186 bases (Shimatsu and Rotwein, 1987) and a type 2 or class B (Roberts et al., 1987). The 5’ leader designated Class C is specified by exon 1 whereas the 5’ leader of class B by exon 2 in the rat IGF-I gene and corresponding IGF-I mRNAs are termed respectively Class 1 and Class 2 mRNAs (Holthuizen et al., 1991).

1.4.8. Different IGF-I mRNA 5’ sequences may specify different leader peptides
Class 1 mRNAs have in-frame AUGs at position –22 and –48 relative to the IGF-I sequence (Roberts et al., 1987; Shimatsu and Rotwein, 1987). The –48 AUG is conserved across a number of species including human, rat and chicken Class 1 IGF-I mRNAs (Kajimoto and Rotwein, 1990; Tobin et al., 1990; Jansen et al., 1992). A reported Class 2 rat IGF-I cDNA with larger 5’ sequence than class 2 mRNAs contain in-frame AUG codons at –71, -61 and AUGs at –32 and -22(Roberts et al, 1987). In addition, there is an in-frame CUG codon, at position –44 (Roberts et al, 1987) shown to serve as one of the translation initiation codons in mRNAs encoding basic fibroblast growth factor (Prats et al., 1989). As in rat, human, chicken and xenopus class 1 IGF-I mRNAs initiate translation preferentially at a –48 AUG in cell-free systems (Rotwein et al., 1987; Kajimoto and Rotwein, 1989; Kajimoto and Rotwein,
1990) rather than at downstream AUG codons. The resulting 5’ sequences may encode different IGF-I pre-peptides. The most common pre-peptides arising from class 1 and 2 mRNAs contain 48 and 32 amino acid signal peptides, respectively. Pre-peptides arising from use of the other in-frame AUG sequences are possible, depending on the site of transcription initiation, as shown in figure 7 below with minor arrows above exons 1, 2 and 3.

**Figure 7.** Schematic representations of alternative transcription units and the predicted protein precursors of IGF-I mRNA. Boxed regions represent exons and blue areas represent open reading frames. Transcription initiates from a disperse series of sites within exon 1 and less within exon 2. The resulting 5’ sequences may encode different IGF-I pre-peptides. The most common pre-peptides arising from class 1 and 2 mRNAs contain 48 and 32aa signal peptides, respectively. Pre-peptides arising from the use of other in-frame AUG sequences are possible, depending on the site of transcription initiation and the minor arrows above exons 1, 2, and 3 in the transcription units indicate these. The conserved regions of the mature IGF-I peptide are designated B, C, A and D (taken from Arkins et al., 1994).
1.4.9. Translatability of different IGF-I mRNAs is affected by the different 5'-UTRs

It has been shown that the 5'-UTR can affect mRNA translation due to its length, secondary structure, and/or presence of AUG codons upstream of the reading frame (Kozak, 1991a; Kozak, 1991b). Yang et al. (1995) showed, using synthetic IGF-I RNAs including the different 5'UTR sequences with mutations at specific bases, that the translational efficiency of IGF-I RNAs was inversely proportional to the length of the 5'UTRs. Nonetheless, IGF-I mRNAs potentially encode multiple forms of pre-pro-IGF and that specific differences in their 5'-UTRs provide a molecular basis for translational control of IGF-I biosynthesis (H. Yang et al., 1995).

1.4.10. IGF-I mRNAs with alternate leader sequences are differentially expressed in a tissue-specific manner

IGF-I mRNAs with class 1 5' sequences are expressed in rat liver and a wide range of non-hepatic tissues both prenatally and postnatally (Hoyt et al., 1988; Adamo et al., 1989). Postnatally, rat liver expresses much higher levels of class 1 IGF-1 mRNAs than fetal liver and as with most non-hepatic tissues of rats in fetal or postnatal periods (Hoyt et al., 1988; Adamo et al., 1989).

IGF-I mRNAs with class 2 5' sequences are undetectable or barely detectable in non-hepatic tissues (Hoyt et al., 1988; Adamo et al., 1991). This suggests that the basal promoter associated with exon 1 is active in a wide range of tissues.

1.4.11. Control of IGF-I mRNA stability by the various lengths of the 3'UTR region

Apart from the variability at 5'ends, IGF-I mRNAs can differ in the length and sequence of the 3'-UTR due to the presence of alternative splicing of exons 5 and 6 and the existence of multiple polyadenylation sites in exon 6. The existence of these alternative polyadenylation sites in exon 6 gives rise to IGF-I mRNA species ranging in size from <1Kb to around 7.5Kb in length. RNase H mapping experiments have established that differential polyadenylation site usage is mainly responsible for size variation in IGF-I mRNAs (Lund et al., 1989). The exon 6 sequence has been determined for the human (Steenbergh et al., 1991) and rat (Hoyt et al., 1992) genes. The existence of A-U-rich sites is likely to destabilize sequences in the more distal region of this exon. The high molecular weight IGF-I mRNAs produced, would be
expected to contain such sequences whereas the lower molecular weight ones
generated by use of the more proximal polyadenylation sites, would be relatively
deficient in these motifs. Use of hypophysectomized rats in order to examine the in
vivo half-life of all the rat liver IGF-I mRNAs showed that by injecting growth
hormone, the half-life (the time taken for the mRNAs to decay from maximum
abundance induced by GH) of the 7.5-7Kb IGF-I mRNAs was much shorter than that
of the smaller ones (Hepler et al., 1990). Therefore, the larger molecular weight IGF-I
mRNAs are less stable, suggesting that the size heterogeneity of IGF-I mRNAs
provides a mechanism for post-transcriptional down-regulation of IGF-I synthesis at
the level of mRNA stability.

1.4.12. Growth hormone and IGF-I axis; related and independent roles of GH
Investigations of how somatic growth is regulated by the pituitary gland led to the
discovery of growth hormone (GH). The most abundant cell type of the pituitary
gland is acidophil GH secreting cells, otherwise known as somatotrophs. Two
peptides from the hypothalamus, growth hormone-releasing hormone (GHRH) and
somatostatin (somatotropin release-inhibiting factor), act as the regulators of GH
production and secretion by these cells (McCormick et al., 1990). Somatostatin
inhibits GH release from the anterior pituitary gland, whereas GHRH stimulates its
release.

Stress, sleep and exercise can promote synthesis of GHRH, therefore increasing GH
serum levels released into the circulation. There, it can bind to cell surface receptors
(GHRs) on target tissues like liver, muscle, adipose and bone. This results in an
increase in the serum concentration of IGF-I.

1.4.13. The Somatomedin hypothesis 2000
The term somatomedin 50 years ago, came to reflect the existence of a growth –
promoting substance, later on characterized as IGF-I. Both IGFs were termed insulin-
like because they can stimulate glucose uptake into fat cells and muscle. The original
concept of the somatomedin hypothesis back in 1950s was that GH stimulates the
release and synthesis of IGF-I solely from the liver and that IGF-1 reaches the target
tissues via the circulation in an endocrine manner. This hypothesis was revised in
1980s when it was found that both IGF-I and IGF-II were produced in most tissues
therefore, including an autocrine/paracrine function. However, it was still believed
that non-hepatic production of IGF-1 was entirely GH-dependent and all GH effects were mediated by IGF-I whether IGF-I was liver or other tissue derived. The Cre-loxP model of gene deletion was used to compare the role of local autocrine-paracrine IGF-I with those of endocrine (circulating) IGF-I. Exon 4 of the IGF-I gene was flanked by two loxP sequences in a targeting vector and was transfected into embryonic stem cells of one mouse line. Using homologous recombination, the endogenous region of the gene was replaced with the loxP-contained allele. When this mouse line was mated with transgenic mice expressing liver-specific Cre recombinase with an albumin enhancer and promoter (Sjogren et al., 1999; Yakar et al., 1999), Cre recognized the loxP sequences, which caused recombination and exon 4 was excised. The result was that recombination was only detected in liver and IGF-I was expressed in other tissues but not in liver. Circulating IGF-I was reduced by 75% with no compensatory increase in IGF-I expression by other tissues and levels of GH raised four-fold. However, the pups were born with normal body weights, were fertile and grew at normal rate. Therefore, this suggested that liver-derived IGF-I is not necessary for postnatal growth and development (Sjogren et al., 1999; Yakar et al., 1999). A schematic representation of the somatomedin hypothesis is shown in figure 8 below.
Figure 8. The Somatomedin hypothesis. (A) The original hypothesis suggested that the effects of GH on postnatal growth were mediated by IGF-I (originally termed as somatomedin) derived from the liver and secreted into the circulation. (B) Later on, the hypothesis was corrected suggesting that most tissues produced IGF-I and that GH affected circulating levels of liver-derived IGF-I and the stability of circulating IGF-I via the IGFBP-3-ALS complex. GH was also capable of influencing local tissue production of IGF-I, thereby maintaining its overall control on postnatal growth. (C) Finally, following studies in mice the hypothesis showed that liver-derived IGF-I is not essential for postnatal growth and development. Abbreviations: ALS, GH, IGF-I, IGFBP-3 (adapted from LeRoith et al., 2001).
1.4.14. IGF receptors

1.4.14.1. Characterization of IGF receptors in skeletal muscle

The IGFs act on cells by binding with high affinity to a membrane receptor. Two cell surface receptors the IGF-I (or type I receptor) and the IGF-II (or type II) receptor are known. Type II receptor is identical to the cation-dependent mannose-6 phosphate receptor and it functions in the trafficking of lysosomal enzymes, but has no known IGF signalling function in contrast to the type I receptor. The IGF-I receptor is similar in structure and sequence to the insulin receptor therefore receptor hybrids can form, which can be found in several tissues and cell types including skeletal muscle (Moxham et al., 1989).

Both IGF receptors have been found in skeletal muscle cells from humans (Shimizu et al., 1986) and rodents (De Vroede et al., 1984), although in avian muscle only IGF-I receptor binds to IGF-I (Bassas et al., 1988; McFarland et al., 1992). The IGF-I receptor is a hetero-tetrameric transmembrane protein comprising of two identical extracellular α-subunits containing a cysteine-rich IGF-binding site and two β-subunits that contain an ATP-binding site (Siddle, 1992). In addition, the existence of three tyrosines at close positions; these are rapidly phosphorylated upon IGF binding and are essential for activation of tyrosine kinase activity. Extra tyrosine residues in the C-terminal domains of the β-subunit are also essential in signal transduction by the IGF-I receptor (Kato et al., 1993). In skeletal muscle there appears to be developmentally regulated changes in the IGF receptors; at birth IGF-II receptor in the rat declined rapidly while IGF-I receptor remained steady for about 1 month before declining in the adult (Alexandrides et al., 1989). Furthermore, while the IGF-I receptor in adult human muscle contains a 95kDa β-subunit, fetal muscle contains a novel 105kDa β-subunit and the 95kDa β-subunit (Alexandrides and Smith, 1989). These novel receptors exhibit increased tyrosine phosphorylation when compared with adult muscle receptors and may play an important role in early muscle development.
1.4.14.2. Cytoplasmic signal transduction pathways

The IGF receptor becomes activated upon ligand binding which results in a conformational change leading to auto-phosphorylation of tyrosine residues. These serve as recruiting sites for specific cytoplasmic proteins containing SH2 (src homology 2) domains. Insulin receptor substrate (IRS-1) interacts directly with insulin and IGF-I receptors (Sun et al., 1992; Myers and White, 1993; Myers et al., 1993) and serves as a multi-component docking platform where binding with multiple SH2 domain-containing proteins takes place. Amongst these proteins are Grb2, Nck (an adaptor protein), c-Crk (a Grb2 and Nck homolog), Syp a protein tyrosine phosphatase (SH-PTP2) and the p85 subunit of PI3-kinase. Recruitment of Grb-2 via IRS-1 leads to the activation of the Ras -mitogen-activated protein (MAP) kinase pathway. Although the phosphotyrosine phosphatase (PTPase) Syp, has not been shown to be directly phosphorylated by the insulin or IGF-I receptor, phosphorylation of IRS-1 results in the recruitment and binding of Syp with IRS-1 via its SH2 domain. Syp is a positive regulator of growth since expression of dominant negative Syp blocked the stimulation of MAP kinase by insulin (Milarski and Saltiel, 1994). Other phosphatases may also be involved in IGF-I or insulin signalling, playing an important role in modulating phosphorylation states, thus regulating growth factor-induced signalling. Activation of PI3-kinase leads to activation of the enzyme p70 S6 kinase, which may be involved in mitogenesis (Cheatham, and Kahn, 1995). Transgenic mice lacking IRS-1 exhibit growth retardation and are mildly insulin and IGF-I resistant (Patti et al., 1995). Although IGF-I acts via the same receptor, myoblast proliferation and differentiation are stimulated via two distinct signal transduction pathways. MAPK pathway induces myoblast proliferation whereas PI3-kinase pathway stimulates myoblast differentiation to myotubes (Coolican et al., 1996). Recent studies have reported nuclear localization of IGF receptors (Chen and Roy, 1996) and transport of exogenous IGF-I into the nucleus (Li et al., 1997). This suggests that IGF-I could exert its biological effects via a nuclear transduction pathway.

1.4.15. Insulin-like growth factor binding proteins (IGFBPs)

The IGFBP superfamily consist of six proteins (1 to 6) that bind to IGFs with high affinity. They are ubiquitously produced in virtually all tissues. IGFBP proteases modify IGFBPs by reducing the affinity with which they bind IGFs, therefore
increasing IGF bioavailability. In serum, most of the IGF-I and IGF-II are found in a 150kDa ternary complex formed by an IGF, IGFBP3 and a glycoprotein, the ALS. IGFBP-5 also forms a ternary complex with ALS but to a lesser degree (Twigg and Baxter, 1998). A small proportion of IGFs is carried by other IGFBPs and less than 1% circulates in the free form.

Structurally, the IGFBPs have three distinct regions of approximately equal size. The highly conserved N-terminal region contains 12 (in IGFBP-1 to IGFBP-5) or 10 (IGFBP-6) conserved cysteine residues and the highly conserved C-terminal region contains 6 conserved cysteine residues. Intra-domain disulphide bonds form between the cysteine residues within the C-or N-terminal regions (Forbes et al., 1998). In contrast, the linking central domain of the IGFBPs (55-95aa) is highly variable (Zapf, 1995). Each of the IGFBPs is subjected to post-translational modification such as N- or O-glycosylation or phosphorylation (table 2). The physiological significance of these is not completely understood. However, phosphorylation of IGFBP-1 has been shown to enhance its affinity for IGF-I and may augment the inhibitory effect of this protein in various in vitro situations (Jones and Clemmons, 1995).

1.4.15.1. Biological actions of the IGFBPs

IGFBPs modulate the actions of IGFs by competing with the IGF receptors for ligand binding. IGFs bind to the IGFBPs via their amino terminal region that does not interact with the IGF receptor. Cell culture experiments indicated that IGFBP-2, -4, -6 are predominantly inhibitory, whereas IGFBP-5 potentiates IGF-I action (Mohan et al., 1995). IGFBP-5 binds with high affinity to the extracellular matrix, which decreases its affinity for IGF-I and protects it from proteolysis. IGFBP-1 and -3 can either augment or hinder the actions of IGF-I depending on the cells and culture conditions used (Elgin et al., 1987).

The abundance of IGFBPs in different tissues and body fluids varies as shown in table 2 below. In terms of serum concentrations, IGFBPs remain fairly stable with minimal change in response to physiological perturbations, except for IGFBP-1.

It has been shown that the IGFBPs, particularly IGFBP-3, clearly have a transport role for the IGFs, which can explain how IGF-I can be involved in the endocrine and local growth responses following injury.
<table>
<thead>
<tr>
<th>Properties</th>
<th>IGFBP-1</th>
<th>IGFBP-2</th>
<th>IGFBP-3</th>
<th>IGFBP-4</th>
<th>IGFBP-5</th>
<th>IGFBP-6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IGF affinity</strong></td>
<td>IGF-I&gt;IGF-II</td>
<td>IGF-II&gt;IGF-I</td>
<td>IGF-I&gt;IGF-II</td>
<td>IGF-I&gt;IGF-II</td>
<td>IGF-I&gt;IGF-II</td>
<td>IGF-I&gt;IGF-II</td>
</tr>
<tr>
<td><strong>Molecular weight</strong></td>
<td>25 kDa</td>
<td>31 kDa</td>
<td>43-45 kDa</td>
<td>24 kDa</td>
<td>29 kDa</td>
<td>28-30 kDa</td>
</tr>
<tr>
<td><strong>Serum concentration</strong></td>
<td>2-15 nM</td>
<td>2-15 nM</td>
<td>100 nM</td>
<td>2-15 nM</td>
<td>2-15 nM</td>
<td>2-15 nM</td>
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<tr>
<td><strong>Bind ALS</strong></td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><strong>Nuclear target sequence</strong></td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><strong>Heparin binding residues</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>ECM binding</strong></td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><strong>N-glycosylation</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>O-glycosylation</strong></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><strong>Serine phosphorylation sites</strong></td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2. Properties and affinities of each IGF binding protein with IGF-I and IGF-II.

1.4.15.2. IGFBP related proteins

IGFBP related proteins are cysteine-rich proteins that share structural similarities with the IGFBPs. Their difference in structure lies in the middle region and C-terminus (Hwa et al., 1999). They also bind IGFs but with lower affinity and specifically IGFBP-rP1 has been shown to modulate IGF effects on myoblast proliferation and differentiation (Haugk et al., 2000).
1.4.16. Effect of IGF-I in skeletal muscle development

Mitogenesis (cell proliferation) and myogenesis (differentiation) during muscle development are complex processes. IGF-I has the ability to stimulate both processes in cell culture (Florini, 1987; Florini et al., 1991; Florini et al., 1996). In a cell culture model where C2C12 cells were stably transfected with IGF-I, skeletal muscle hypertrophy was established, using quantitative morphometric analysis, showing 67% increase in myotube area compared to non-transfected cells (Semsarian et al., 1999a). In the transgenic mouse, Coleman et al., (1995) found that over-expression of IGF-I (47-fold increase) in muscle leads to hypertrophy of muscles but not other tissues. On the other hand, newborn mice homozygous for a targeted disruption of IGF-I exhibit growth deficiency and marked hypoplasia (Liu et al., 1993).

1.4.17. Autocrine / Paracrine IGF-I function in muscle regeneration

During regeneration of skeletal muscle in young rats, usually after ischaemia, elevated IGF-I expression has been reported (Jennische and Hansson, 1987; Edwall et al., 1989). Bupivacaine injection to the tibialis anterior muscle of young, adult and old rats, showed increased IGF-I mRNA levels after 5 days which was diminished by the 15th day of recovery (Marsh et al., 1997). However, sustained levels of IGF-I mRNA in adult and old rat muscle until the 15th day associated with the inability to regenerate completely were present.

Skeletal α-actin is a highly conserved protein required to interact with myosin molecules for the production of muscle contractile force. During the initial 5-10 days of regeneration after bupivacaine injection, the skeletal α-actin promoter is activated and IGF-I and myogenin mRNA levels are elevated. By 16 days post-bupivacaine injection, the activity of the promoter in regenerating muscle is similar to controls and levels of IGF-I and myogenin mRNAs are also diminished, however muscle mass continues to increase until day 24 (Marsh et al., 1998). The actions of IGF-I and IGF-II during myogenic differentiation are mediated through the IGF-I receptor in an autocrine manner (Florini et al., 1996). During muscle maturation, reports have shown post-receptor modifications that result in signal transduction breakdown in the IGF-I pathway (Dardevet et al., 1994). Musaro et al. (2001) generated a model of persistent, functional myocyte hypertrophy using a tissue-restricted transgene encoding a locally acting isoform of IGF-I expressed in skeletal muscle (mIGF-1). Transgene embryos
developed normally and postnatal increases in muscle mass and strength were not accompanied by the additional pathological changes seen in other IGF-I transgenic models. In addition, preservation of muscle architecture and age-independent regenerative capacity through the localised mIGF-I transgene expression suggests clinical approaches for the treatment of age or disease-related muscle frailty (Musarò et al., 2001).

Expression of IGF-I and IGF-II was reported only in regenerating fibres after mechanically induced fibre damage without additional delivery of IGF to the tissue by inflammatory cells (Keller et al., 1999). This was consistent with the appearance of both IGF-I and IGF-II mRNA in muscles undergoing compensatory hypertrophy (DeVol et al., 1990) and recovering from myotoxin-induced injury (Marsh et al., 1997).

1.4.18. Muscle IGF-I and Hypertrophy

In vitro models demonstrate that IGF-I stimulates anabolic, mitogenic and myogenic processes in muscle cell lines and in primary cell cultures (Florini et al., 1996). Vandenburgh et al. (1991) found that myoblasts isolated from avian skeletal muscle and cultured under continuous tension in a collagen matrix, form myofibres that hypertrophied in response to the addition of IGF-I.

In vivo models: stretch-induced hypertrophy of mammalian skeletal muscle is associated with increased IGF-I mRNA expression (Schlechter et al., 1986; Czerwinski et al., 1994; Yang et al., 1996) and IGF-IEb mRNA appeared to be induced only in the stretched muscle (Yang et al., 1996). Additionally, both isoforms of muscle IGF-I were markedly up regulated in skeletal muscle by stretch and stretch combined with electrical stimulation (McCoy et al., 1999).

Functional overload for 8 days in hypophysectomized rat skeletal muscle, via the removal of synergists, reported an increase in IGF-I mRNA independent of GH (DeVol et al. 1990). In a time-course study using functional overload in both hypophysectomized and normal rats, Adams and Haddad (1996) discovered that increased muscle IGF-I peptide and mRNA levels were observed during the early phase (3 to 7 days) and decreased rapidly 1 to 2 weeks later. This coincides with similar studies by Sakuma et al (1998) suggesting that IGF-I is involved in the hypertrophic adaptation of muscle in response to increased mechanical loading.
1.4.18.1. Molecular mechanisms of IGF-I induced-muscle hypertrophy

It has been suggested that the Ca\(^{2+}\)/calmodulin-dependent serine phosphatase, calcineurin, which mediates activation of B and T lymphocytes, is involved in the induction of cardiac hypertrophy (Molkentin et al., 1998). Calcineurin is a heterotrimeric complex consisting of a catalytic subunit CnA, a tightly associated regulatory subunit CnB, and calmodulin, a calcium sensor. When Ca\(^{2+}\) binds to a calmodulin-calcineurin complex, it stimulates calcineurin, the major substrates of which are nuclear factors of activated T cells (NF-AT) transcription factors.

A model was proposed by Semsarian et al., (1999b), where skeletal muscle hypertrophy and a switch in myofibre phenotype was initiated by a stimulus that causes Ca\(^{2+}\) mobilization. This activates calcineurin and results in NF-ATc1 (isoform of NF-AT) dephosphorylation and nuclear translocation. The resulting transcriptionally competent NF-ATc1 species, initiate a programme of gene expression that leads to hypertrophy. After the pathway is activated, it becomes resistant to following calcineurin inhibition. One possible mechanism by which NF-AT is thought to alter fibre-type specific gene expression is by interaction with MEF2 proteins. Either IGF-I or activated calcineurin induces expression of the transcription factor GATA-2, which accumulates in a subset of myocyte nuclei, associated with calcineurin and NF-ATc1 (Musaro et al., 1999). Calcineurin inhibitors, cyclosporin A and FK506 suppressed the muscle hypertrophy induced by IGF-I. The activation of calcineurin by IGF-I observed in vitro was confirmed by in vivo injection of a plasmid encoding IGF-I in rat lattissimus dorsi muscle. Therefore, it was shown that IGF-I induces calcineurin-mediated signalling and activation of GATA-2, which cooperates with selected NF-ATc isoforms to activate gene expression.

Dunn et al. (2000) also demonstrated that overloading resulted in dephosphorylation of NF-ATc1 and in expression of GATA-2, both of which mediate calcineurin-dependent muscle hypertrophy. Also the administration of cyclosporin and FK506 to mice with the overloaded plantaris muscle prevented the increase in muscle mass and number of fibres, which is a natural outcome of this condition. Although calcineurin is required for muscle hypertrophy, its expression in transgenic mice is not sufficient. Consequently, it does not induce expression of GATA-2 and muscle hypertrophy is not generated even though NF-ATc1 is de-phosphorylated. In contrast, activation of satellite cells necessary for muscle hypertrophy seems independent of calcineurin (Dunn et al., 1999)
1.4.19. Expression of an autocrine growth factor (MGF) in muscle in response to stretch and overload

Skeletal muscle has been shown to increase in mass in response to passive stretch. Several studies have shown that increased mass is associated with production and addition of new sarcomeres at the ends of the fibres (Tabary et al., 1972; Williams and Goldspink, 1973; Goldspink et al., 1986; Goldspink et al., 1992). Increased IGF-I mRNA in response to stretch is associated with increasing muscle protein synthesis (Goldspink et al., 1995). Using a probe derived from exons 3 and 4 of IGF-I gene, Yang et al. (1996) isolated the isoform IGF-IEb, as already described before, that was expressed after 2 hours in rabbit stretched muscle, whereas IGF-IEa isoform was found in resting muscle. Due to the conditions used, IGF-IEb was termed mechano-growth factor (MGF), yet its characteristics are as defined on page 49. Its E domain differs from the liver IGF-IEa by the presence of a 52bp insert. Hence, this changes the reading frame of the derived carboxyl-terminal resulting in a different precursor IGF-I isoform.

Using RNase protection assays with a probe that distinguishes between the differently spliced forms of IGF-I, McKoy et al. (1999) showed that both were up regulated in response to stretch and stretch combined with electrical stimulation but not by electrical stimulation alone. MGF has also been detected in cardiac muscle (Skarli et al., 1998). Unlike the muscle L.IGF-I (IGF-IEa) and the main liver isoforms, MGF is not glycosylated and is therefore smaller with a short half-life and has been suggested to have an autocrine / paracrine mode of action. Recent findings by Owino et al. (2001) determined the functional differences between the two muscle isoforms that were cloned in our laboratory from rabbit stretched muscle by Yang et al. (1996). It was found that MGF and IGF-IEa are differentially regulated in response to mechanical overload and age difference. Five days after tenotomy, there was a significant increase in MGF levels in 3, 12 and 24month-old rats in the overloaded muscle compared to the contralateral (Owino et al., 2001). However, MGF levels in the 3month-old rats were markedly higher than those in the 24months-old rats after 24 hours.

The early induction of MGF following mechanical overload, indicates that this may play a role in the increase in protein synthesis and activation of satellite cells, which are the precursor cells associated with muscle regeneration. Additionally, the significant decrease of MGF in old animals could be explained by the reduction in the
functional return of regenerating muscles due to incomplete re-innervation of certain regions of muscle, which has been proved to be a significant factor for complete regeneration (Carlson and Faulkner, 1998).

Goldspink et al., (1996) also showed that MGF mRNA was not detectable in dystrophic murine muscle even when subjected to stretch, in contrast to normal muscle. However, the systemic muscle IGF-I (muscle L.IGF-I) was detectable in dystrophic muscle.

1.4.20. IGF-I and satellite cells

Satellite cells seem to have an important role not only in muscle fibre regeneration (Schultz et al., 1985) but in hypertrophy as well, since the prevention of satellite cell division by gamma irradiation hampers fibre hypertrophy following functional overload (Rosenblatt and Parry, 1993). IGF-I expressed in the satellite cells of regenerating muscle (Jennische et al., 1993) has been reported to stimulate satellite cell proliferation and fusion in primary rat satellite cell culture (Allen and Boxhorn, 1989). Therefore, these findings give rise to the belief that IGF-I plays an important role in hypertrophic adaptation of muscle via activation of satellite cells. Satellite cells have a limited capacity to divide, and after a finite number of cell divisions, they enter a state of irreversible growth arrest termed replicative senescence. Thus, when satellite cells exhaust their finite proliferative reserves, the effectiveness of regeneration, hypertrophy and myoblast-mediated gene therapy for the reconstruction of muscle is inhibited. Therefore, as already mentioned, overexpression of IGF-I in satellite cells for a period of around 4 months, delayed cellular senescence by extending their in vitro replicative life span (Chakravarthy et al., 2000). However, recent findings by Carlson et al. (2001) demonstrated that under favourable conditions such as cross-age transplantation into young hosts or marcaine injection without significant damage to the nerve supply, rat EDL muscles of very old rats still possessed a normal capacity for regeneration. Therefore, advanced age in itself does not appear to be a factor that limits the regenerative potential of rat muscle fibres. Given the results of the physiological studies and the background data present in the literature on aging, a reasonable conclusion appears to be that despite some potential losses in both numbers and in proliferative potential, there remains sufficient cellular reserve in extremely old muscle to allow regeneration to occur equally well as young muscle in vivo, as long as the satellite cells are regenerating in similar environments.
1.4.21. IGF-I and muscle atrophy

In growing rats subjected to decreased muscle loading via hind-limb suspension, it was reported that soleus muscle growth retardation was accompanied by cessation of mitotic activity in muscle (Schultz, 1989). Criswell et al. (1998) using transgenic mice, showed that overexpression of IGF-I in tibialis anterior and gastrocnemius muscles did not prevent the atrophy induced by unloading. Also, Lalani and co-workers (2000) reported that 17 days of space flight induced a 20% loss of tibialis anterior muscle mass without significant changes in muscle IGF-I mRNA content. Awede et al., (1999b) showed that 2 days of unloading induced a significant decrease of 30% in IGF-I mRNA levels in slow-twitch soleus muscle. After 8 days, IGF-I mRNA levels decreased to reach similar to control values, implying that IGF-I could play a role in myonuclear apoptosis associated with unloading-induced atrophy, given that IGF-I was reported to protect myonuclei against apoptosis in unloaded muscles (Allen et al., 1997). In view of these results, decrease of IGF-I levels in muscle may be relevant to the atrophy of slow-twitch muscles but not to that of fast-twitch muscles.

1.4.22. Expression of IGFBPs during regeneration of skeletal muscle

Following denervation, transcripts of IGFBP-4 and IGFBP-5 were up regulated by approximately three-fold in gastrocnemius muscle (Bayol et al., 2000). These results confirm previous findings, where increase of IGFBP-5 transcript was observed in a rat model of hind limb unloading where atrophy occurs, while with overload hypertrophy IGFBP-5 mRNA decreased (Awede et al., 1999b). Therefore, this could imply a role of IGFBP-5 in the changes in fibre size occurring in models of atrophy and hypertrophy. On the other hand, because IGFBP-4 is thought to inhibit IGF-I action on skeletal muscle, its increased expression in overloaded muscles could be interpreted as a mechanism by which IGFBP-4 limits the extent of hypertrophy by preventing the proliferating effect of IGF-I on satellite cells. During post-ischaemic regeneration, the expression of IGFBP-3, -4, -5 and -6 increased in different time patterns (Jennische and Hall, 2000). IGFBP-3 increased transiently during the early phase of regeneration, whereas IGFBP-4, -5 and -6 increased later on during regeneration. IGFBP-5 was the only one to be expressed in regenerating muscle, by in situ hybridisation. Ligand blotting also showed that IGFBPs examined above, were
expressed comparatively at low levels in normal adult muscle, however the levels
were up regulated during regeneration reaching similar values to those seen in
neonatal muscle (Jennische and Hall, 2000).

1.4.23. Expression of MRFs during muscle regeneration

The pattern of expression of the MRFs has been analysed during muscle regeneration
of mammals after different types of injury and using different methods of detection
(Füchtbauer and Westphal, 1992; Grounds et al., 1992; Marsh et al., 1997; Mendler et
al., 1998; Sakuma et al., 1999). Denervation of murine muscle increased both MyoD
and MRF4 transcripts 10-fold and myogenin 30-fold in contrast to innervating muscle
(Duclert et al., 1991). Adams et al. (1995) showed that long-term denervation induced
a long-term up regulation of MyoD and Myf5 transcripts and MyoD protein was up
regulated in rat regenerating muscle in response to denervation (Koishi et al., 1995).
During soleus muscle regeneration, after cardiotoxin injury, Myf5, MyoD and MRF4
transcripts are detected in proliferating myoblasts but not in quiescent satellite cells
(Launay et al., 2001). Moreover, in vitro culture of satellite cells isolated from rat,
demonstrated that MyoD was initially expressed, before proliferation commenced,
accompanied by Myf5 and MRF4 with myogenin being expressed at the start of
differentiation. Cooper et al. (1999) showed that adult activated satellite cells initially
express MyoD, or Myf5 or both MRF proteins.

Using a MyoD (-/-) mutant, Megeney et al. (1996) showed that MyoD plays a crucial
role in satellite cell function; satellite cells in mice lacking MyoD delay their process
from proliferation to differentiation, even if Myf5 was up regulated. Cornelison et al.
(2000) highlighted a requirement for MyoD to support proper MRF4 expression in
activated satellite cells. The relationship and involvement of MyoD and MRF4 in the
satellite cell differentiation pathway is different from that during embryogenesis
because at birth mice lacking MyoD are phenotypically normal.
1.4.24. Expression of myostatin in regenerating muscle

Myostatin is a member of the TGF-β superfamily and is known as the negative regulator during skeletal muscle development. Carlson et al. (1999) also showed that the expression of myostatin is inversely related to changes in skeletal muscle during processes like atrophy. Recent findings have demonstrated that myostatin mRNA expression is induced in regenerating area where activated satellite cells are proliferating (Yamanouchi et al., 2000) but the mechanism of its targeting satellite cells is still unknown.

1.5. Aims

Muscle is a mechanical tissue in which cells will become damaged in response to various types of injury. As cell replacement is not a mechanism via which the damaged cells are replaced, it is important that there is an effective local repair mechanism so that the tissue can regenerate following injury. There is currently a widespread interest in IGF-I isoforms and IGF binding proteins and their role in the regulation of compensatory muscle growth and regeneration of muscle tissue following damage. This selective review focused on the current state of our knowledge about extrinsic and intrinsic factors, myogenic and growth factors involved affecting muscle regeneration and hypertrophy. The purpose of this work was to determine how IGF-I splice variants might influence local tissue repair. In addition, to study their temporal expression pattern in response to different stimuli i.e. induction of damage and further regeneration, together with the temporal and spatial expression pattern of IGFBPs involved under such conditions.
CHAPTER II
General Materials and Methods
Introduction and Description of mRNA Quantification by Light Cycler Technology

For the studies described in this thesis, it was necessary to use a sensitive molecular biology technique that enabled an accurate amplification and quantification of gene expression.

2.1. PCR and other methods of quantifying low abundant genes

The Polymerase Chain Reaction (PCR) was originally developed to amplify defined regions of DNA in vitro from minute amounts of starting material, for the analysis of low abundance gene sequences (Mullis and Faloona, 1987). Since the introduction of the original PCR, the basic protocol has been further developed and applied to study mRNA (Imnis et al, 1990). PCR can be used along with reverse transcription (RT-PCR) to amplify specific region of mRNA. The mRNA is first transcribed into single stranded complementary DNA (first strand cDNA) by primer extension, using an oligonucleotide specifically designed to copy the mRNA or oligo-dT to prime at the poly-A tail or by using random hexamers (random priming). If the two ends of the mRNA sequence to be amplified are known, specific primers can be used to amplify from the first strand cDNA.

The intensive development of technologies for quantification of nucleic acids over the last decade reflects its importance in scientific research, diagnostics and biomedical research. Despite the fact that a number of methods are available for this purpose such as Northern Blotting, RNase protection assay (RPA) and competitive conventional PCR using several “control” gene sequences, the procedures involved are rather cumbersome and very time consuming. In Hybridisation methods such as Northern Blotting and RPA, the reaction kinetics are easier to determine, but the sensitivity is not sufficient for most practical applications. A non-linear response and saturation can also be a problem; like in Northern Blotting where a phosphoimager is used to determine the density of the bands. In contrast, due to the amplifying effect PCR-based methods are more sensitive. However, the kinetics of PCR are more complex and hence conditions for the use of PCR as a quantitative method has to be established for each gene sequence.
Real-Time RT-PCR is highly sensitive and allows quantification of rare transcripts and offers important insights into the local expression of transcripts present in low abundance. As well as this, it is easy to perform, provides the necessary accuracy and produces reliable as well as rapid quantification results. The application of fluorescent techniques, such as the one used in this study enables the reaction to be continuously monitored as Real-Time PCR combines the PCR amplification, product detection and quantification of newly synthesized DNA, as well as verification in the melting curve analysis.

### 2.2. Real-Time PCR compared to Conventional PCR

Real-Time PCR is a relatively new technique that offers several advantages compared to the traditional PCR methods (Table 2.2.).

<table>
<thead>
<tr>
<th></th>
<th>REAL-TIME PCR</th>
<th>CONVENTIONAL PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR run (30-40</td>
<td>In 30-40 minutes</td>
<td>3-4 hours</td>
</tr>
<tr>
<td>amplification cycles)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detection via gel</td>
<td>Not necessary; On-line visibility of PCR products</td>
<td>Necessary to visualize PCR products</td>
</tr>
<tr>
<td>electrophoresis/ gel staining</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantitative analysis</td>
<td>Accurate quantification of PCR products using standards of known concentration</td>
<td>Semi-quantitative analysis through band intensity in gel electrophoresis</td>
</tr>
<tr>
<td>Risk of contamination</td>
<td>Ready-to-use reaction mix (Taq DNA polymerase, reaction buffer, dNTPs and SYBR Green I dye) minimising risk of contamination during handling</td>
<td>Each reagent is added individually increasing contamination risk</td>
</tr>
</tbody>
</table>

**Table 2.2.** Comparison of Real-Time PCR using SYBR Green I with Conventional PCR
2.3. Real-Time PCR

The Light Cycler™ (Roche Diagnostics), one of the commercially available real-time PCR systems is a rapid thermocycler with online fluorescence detection in which the PCR is carried out in glass capillaries. Heating and cooling are by temperature-controlled airflow. The capillaries are placed in a rotation-symmetric chamber to ensure homogeneous temperature distribution as seen in figure 2.3 above.

The Light CyclerSystem can use several fluorescence formats for associating the amount of PCR product with fluorescence signals. The use of a double stranded DNA binding dye SYBR Green I for monitoring PCR, in contrast to the conventional method of gel separation and staining of PCR products with ethidium bromide, is one format.

Figure 2.3. The Real-Time PCR apparatus by Roche Diagnostics. The picture on the right shows the carousel with the borosilicate glass capillaries.
Hybridization probes is another format where two specially designed, sequence-specific oligonucleotides labelled with two different fluorescent dyes are used.

2.3.1. Monitoring Real-Time PCR using SYBR Green I fluorescence method

SYBR Green I dye binds to the minor groove of dsDNA and therefore different intensities of fluorescence signals can be detected during the various stages of PCR (figure 2.3.1) depending on the amount of dsDNA present. During denaturation of the dsDNA to ssDNA, SYBR Green I will not bind (a). During annealing (b), PCR specific primers hybridise to the target sequence resulting in small parts of dsDNA that SYBR Green I dye binds to, and fluorescent intensity increases (c). Finally, in the elongation phase (d), the PCR primers are extended, the entire DNA has become double-stranded, and a maximum amount of dye is bound. The fluorescence is recorded at the end of the elongation phase and increasing amounts of PCR products can be monitored from cycle to cycle, which are available to see in real-time as they occur.

**Figure 2.3.1.** SYBR Green I binding to DNA during Light Cycler amplification. The four steps are: a) during denaturation SYBR Green I (green “dumb-bell-shaped” structure) will not bind, b) specific primers will hybridise to the target sequence forming small parts of dsDNA that SYBR Green I binds to and c) fluorescent intensity increases. During elongation, d) the entire DNA has become double-stranded and a maximum amount of dye is bound (taken from Roche catalogue).
However, a signal can also be detected when SYBR Green I binds to double stranded by-products such as primer-dimers, that result from non-specific annealing and primer elongation events. These events occur as soon as PCR reagents are combined. The formation of primer-dimers competes with formation of PCR product, leading to reduced amplification efficiency and a less successful PCR. In this case, a “Hot Start” method can be used where an anti-\textit{Taq} DNA polymerase antibody is added to the reaction mixture to inactivate the enzyme until the temperature increases above 70°C. Heat-inactivation of the antibody is done simultaneously with heat denaturation of the target DNA. The maximum excitation of SYBR Green I dye occurs at 497 nm. Maximal emission of DNA stained with SYBR Green I occurs at 521 nm.

2.3.2. \textit{Experimental set-up and accurate quantification}

Once the programming screen used to define the cycle numbers for an experimental protocol has been set-up, the Light Cycler is ready to run. A typical experimental protocol contains four programmes, as seen in figure 2.3.2:

1) Initial denaturation programme,
2) Amplification programme,
3) Melting programme and
4) Cooling
Figure 2.3.2. Programming screen for setting up the Light Cycler experimental protocol (A). Panel B shows the Cycle Program that contains several Temperature Segments, each of which defines the time and temperature parameters that will be used for denaturation, annealing, extension and/or melting, cooling as well as the fluorescence acquisition mode used to monitor the amplification signal. Each experimental protocol has four programs a) denaturation, b) amplification, c) melting and d) cooling as shown in panel C. The graphic stimulation window in panel (D) is the temperature profile displayed as a red line, while the fluorescence acquisition profile is graphed in green (panel C).

Identifying the cycle, through which the log-linear signal can be singled out from the background, makes it possible to compare the target concentrations in samples. In a single run, serially diluted plasmid DNA of known concentration for the gene of interest, are used as external standards alongside the target sequences needed to be quantified for this particular gene. This allows measurement over a wide range of concentrations to be analysed. After PCR has been completed, a noise band on a fluorescent level is set, at
which the fluorescent signal development indicates that the PCR is in a log-linear phase (figure 2.3.3).

![Figure 2.3.3. Noise band screen of the Light Cycler program; both graphs on the noise band screen show fluorescence values on a log scale, Log Fluorescence vs. Cycle Number. A red noise band appears on the upper graph. This band is moved up or down to define which data points will be included in the analysis. All data points that fall below the noise band will be excluded. The data selected for analysis (i.e. above the noise band) will be displayed in the lower graph. A straight line is inserted through a user-defined number of data points above the noise band and the software determines the points of intersection (crossing points-cycle numbers) with the noise band for all of the standards, as seen in figure 2.3.4. These crossing points are then plotted against the logarithm of the concentration. By comparing the crossing points for the samples with the crossing points of standards, the concentrations of both standards and samples with unknown concentrations can be obtained.](image-url)
Figure 2.3.4. Analysis screen using the Fit Point method in a Light Cycler protocol. The software uses the selected Number of Points to calculate a “best fit” log-linear fluorescence curve for each sample. The intersection (marked with a red cross) of this line with the Crossing Line is the so-called Crossing Point (expressed as a fractional cycle number, i.e. a cycle number that is calculated to two decimal places). This Crossing Point value is displayed in the table column immediately to the left of the graph. All the Crossing Points of the standards are used to plot a graph of Cycle Number vs. Log Concentration. This standard curve, in turn, is used to estimate the concentration of each sample.

2.3.3. Melting curve analysis with SYBR Green I detection

Each dsDNA product has its own specific melting temperature (Tm), which is defined as the temperature at which 50% of the DNA becomes single-stranded and the rest remains double-stranded and can be affected by the length and the G+C content of that fragment. Sequence confirmation for the amplified product once the amplification cycles have been completed, is achieved by melting curve analysis. During melting curve analysis,
products are denatured at 95°C, annealed at a specific temperature, usually 5-10 degrees higher than that of the PCR annealing temperature and then slowly heated to 95°C where fluorescence is measured at every 0.2°C. As soon as the double-stranded amplicon starts to denature, the SYBR Green I dye is released from the fragment and fluorescence is dropped sharply. The Tm of the fragment can be easily visualized by taking the first negative derivative (-dF/dT) of the melting curve and a peak can be seen when plotting it, which shows the specific Tm of the fragment (figure 2.3.5).

Figure 2.3.5. The Melting Peaks Screen of a typical Light Cycler protocol displays two graphs. The upper graph, which is carried over from the Light Cycler Data Analysis Front Screen, shows run data as Temperature vs. Fluorescence. The calculated first derivative of the run data is shown in the lower graph as Temperature versus -dF/dT. The data in the lower graph displays "melting peaks" for each of the amplified products.
Using a positive standard as a control during the PCR run, the melting temperatures of the unknown samples can be compared with that of the control to confirm the PCR product identity from the non-specific products (figure 2.3.5). The melting curve analysis will differentiate signals coming from the by-products since their Tm will be lower than that of the specific product. Knowing the Tm of these by-products can help increase the specificity by increasing the temperature at which the Light Cycler instrument measures the fluorescence. This can be done by including an additional step in each PCR cycle, in which the Light Cycler instrument is programmed to measure the fluorescence at the specific temperature that it has been set to.

Small amounts of specific product can also be identified through the sensitivity that melting curve analysis provides.

**Materials and Methods:**

2.4. Total RNA isolation

The single-step method of RNA isolation using acid guanidinium thiocyanate-phenol-chloroform after Chomczynski and Sacchi (1987) was used to isolate total RNA. Precautions were taken to prevent the inadvertent introduction and contamination by RNases, by wearing disposable gloves at all times. Additionally, all glassware was baked at 200°C overnight. Eppendorf tubes and pipette tips were autoclaved and baked overnight at 80°C. Solutions, except those containing primary amines, were treated with 0.1% v/v DEPC (Sigma).

Frozen samples of rat TA muscle, weighing approximately 0.3g, were homogenized in 5mls of denaturing solution containing 4M guanidinium thiocyanate, 25mM sodium citrate (pH 7.0), 0.5% sarcosyl and 0.1M 2-mercaptoethanol in a 50ml falcon tube, using an Ultra-Tek homogenizer. Afterwards, 330μl of 3M sodium acetate pH 4.2 (DEPC-treated), 5mls of phenol and 1ml chloroform-isoamyl alcohol (24:1) were sequentially added to the homogenate, with thorough mixing after the addition of each reagent. The final suspension was vortexed vigorously and cooled on ice for 15 minutes. The homogenates were then transferred to 15ml falcon tubes and centrifuged in a GSA rotor (Sorvall RC-5B) at 14000rpm for 30 minutes at 4°C. After centrifugation, the upper
aqueous phase that contains the RNA was removed, taking care not to disturb the interphase (which contains DNA) and transferred to a fresh 15ml falcon tube. Two volumes of ice-cold absolute ethanol were added to the samples, which were placed at -20°C overnight to precipitate the RNA. The precipitated RNA was centrifuged at 14000rpm for 30 min. at 4°C. The supernatant was discarded and the resultant RNA pellet was dissolved in 0.5ml of denaturing solution completely and 1ml of cold absolute ethanol was added to each tube. The samples were stored at -70°C until required.

2.5. Measurement of total RNA concentration

To determine RNA purity and concentration, an aliquot of the RNA stock solution was taken in a 1.5ml eppendorf tube and spun down at 14000rpm at 4°C for 30 minutes. The RNA pellet was washed three times in the same volume of cold 70% ethanol in DEPC water. Finally, the RNA was left to air-dry and resuspended in 50-100µl of DEPC-treated water, heated at 65°C for 10 minutes to ensure the pellet is dissolved and rapidly cooled on ice to eliminate secondary structure prior to cDNA synthesis. Then, 2µl of RNA solution dissolved in 500µl of DEPC-treated water was scanned at 260nm using an UV-160A kinetics spectrometer (SHIMATZU). The RNA concentration was determined by its absorbency at 260nm where absorbency of 1 cm path length cuvette is equivalent to 40µg/ml RNA.

2.6. Electrophoresis of RNA

To analyse the RNA extracted from the experimental TA muscles, total RNA was separated under denaturing conditions using formaldehyde agarose gel electrophoresis. A 1.2% formaldehyde gel was prepared by boiling 3.6g of agarose in 231ml of DEPC-treated water to dissolve. After cooling to 55°C, 60 ml of 5 x MOPS buffer (0.1M MOPS, pH 7.0, 40mM sodium acetate, 5mM EDTA, pH 8.0) and 9 ml of 37% formaldehyde were added. The gel was poured and allowed to set. RNA samples were prepared for electrophoresis as follows: 10µg of total RNA was mixed with 4µl of 5 x MOPS buffer, 7µl of formaldehyde and 20µl of formamide. Samples were incubated at 65°C for 15 minutes and then chilled on ice. After brief centrifugation, 4µl of gel loading buffer (50% glycerol, 1mM EDTA pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol FF) was
added to each sample. Samples were then loaded onto the gel, which had pre-run for 5 minutes at 5 V/cm and electrophoresed in 1x MOPS buffer for 2-2.5 hours at 80-100V. To confirm the integrity of RNA samples, visual inspection of ethidium bromide stained 18s and 28s ribosomal RNA under ultra violet was done, following electrophoresis. All reagents were supplied by Sigma.

2.7. Primers for cDNA synthesis and PCR

Random hexamers (dN)_6 (Roche), which bind at non-specific points along the RNA template were used to prime all first strand cDNA synthesis. In order to facilitate the efficiency of reverse transcription of transcripts expressed at low levels such as MGF, a short specific primer MGF-RT was used. It anneals 106bp downstream of the polymerase chain reaction reverse primers IGF-IEar (reverse) and MGF-R (reverse), taken from the rat MGF cDNA sequence (S. Yang; personal communication) at exon 6.

For RT-PCR and Real-Time PCR all the forward and reverse primers were synthesized to be between 18-24 nucleotides long, with a GC content ranging from 40-60% and a melting T°C between 55°-65°C.

IGF-IEar forward primer corresponds to the last 20bp of exon 2 and IGF-IEar was designed to span the first 11bp of exon 6 and the last 11bp of exon 4 from the cDNA sequence of rat IGF-I (S. Yang; personal communication). For MGF, the forward primer was the same as for IGF-IEar (MGF-F) and MGF-R reverse primer corresponds to the first 11bp of exon 6 and the last 11bp of exon 5 from the cDNA sequence of rat MGF (S. Yang; personal communication).

IGFBP4-F forward primer and IGFBP4-R reverse primer were designed from Mus Musculus IGFBP-4 cDNA sequence (acc. number: X81582).

IGFBP5-F and IGFBP5-R were designed from Rattus norvegicus (acc. number: NM012817).

MyoDF and MyoDR were designed from Mus Musculus (acc. number: M84176).

M-cadF and M-cadR were designed from Mus Musculus (acc. number: M74541).

GAPDH-F and GAPDH-R were designed from Human GAPDH complete cDNA (accession number J04038). Primers used for PCR are listed in Table 3. All the PCR
primers were designed using Omega Version 2.0 (Oxford Molecular) and synthesized by Sigma Genosys.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5'-3'</th>
<th>Annealing T°C (Tα) for PCR</th>
<th>Tα for Real-time PCR</th>
<th>PCR-Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP4F</td>
<td>GACGCCAGAAAAGAAGCTGACC</td>
<td>62°C</td>
<td>60°C</td>
<td>282</td>
</tr>
<tr>
<td>IGFBP4R</td>
<td>GTCCACACACCAGCAGATGC</td>
<td>62°C</td>
<td>60°C</td>
<td>282</td>
</tr>
<tr>
<td>MyoDF</td>
<td>GAGACATCCTCAAGCGATGC</td>
<td>60°C</td>
<td>60°C</td>
<td>108</td>
</tr>
<tr>
<td>MyoDR</td>
<td>AGCACCTGTAAATCGGATTG</td>
<td>60°C</td>
<td>60°C</td>
<td>108</td>
</tr>
<tr>
<td>M-cadF</td>
<td>ATGTGCCACAGCCACATCG</td>
<td>60°C</td>
<td>60°C</td>
<td>256</td>
</tr>
<tr>
<td>M-cadR</td>
<td>TCCATACATGTCCGCCAGC</td>
<td>60°C</td>
<td>60°C</td>
<td>256</td>
</tr>
<tr>
<td>GAPDH F</td>
<td>CCACCCAAGTCCTAGCACC</td>
<td>60°C</td>
<td>60°C</td>
<td>452</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>GCCAAATTCGTGGTCACTACC</td>
<td>60°C</td>
<td>60°C</td>
<td>452</td>
</tr>
<tr>
<td>MGF-rt</td>
<td>TTGCAGGTTGCT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Primers used for RT-PCR and Real Time PCR with their annealing temperature in both amplification protocols and the expected size of the PCR products.

2.8. First strand cDNA synthesis

To prepare the RNA for cDNA synthesis, calculations were made to ensure the same amount of RNA (2µg) from each sample was used for RNA to be reverse transcribed into single-stranded DNA. Prior to cDNA synthesis reaction, RNA samples were denatured at 65°C for 15 minutes in order to eliminate possible secondary structure and then placed on ice for 5 minutes before adding it to the reaction. All products underwent identical reaction conditions during reverse transcription.
The cDNA synthesis reactions were assembled on ice in a 20μl volume containing 2μl of 10x Reaction buffer (100mM Tris-HCl, 500mM KCl pH 8.3), 4μl of 25mM MgCl₂, 2μl of dNTPs (10Mm of each), 2μl of 2μg/μl random primers p(dN)₆, 1μl of 50 units/μl RNase inhibitor, 2μg of total RNA and 0.8μl of 20 units of AMV reverse transcriptase. We used the 1st strand cDNA synthesis kit for RT-PCR from Roche Diagnostics. Control reactions were set up as above at the same time, but omitting the addition of Reverse transcriptase. The reactions were briefly vortexed and incubated at 25°C for 10 minutes where primers anneal to the RNA template. Afterwards, they were incubated at 42°C for 60 minutes where RNA is reverse transcribed, resulting in cDNA synthesis. Following the 42°C incubation, the AMV reverse transcriptase was denatured at 99°C for 5 minutes and then cooling to 4°C for 5 minutes. The cDNA samples were stored at -20°C until required.

In specific cDNA synthesis reactions, for MGF RT-PCR, 1μl of 25pmoles of the sequence-specific primer MGF-RT was used, in addition to the random primers.

2.9. RT-PCR

Initially, PCR was performed in a small number of samples to check that the gene of interest was present and thereafter, running the same sample with optimal MgCl₂ concentration and annealing temperature to create external standards for quantification analysis in the Light Cycler. Double stranded DNA was amplified from the first strand cDNA reaction in a PCR using the primers for the gene of interest, given above in Table 2. Reactions were performed in a 50μl volume containing 5μl of 10x PCR buffer (100 mM Tris-HCl, 500 mM KCl pH 8.3), 2μl of dNTPs (10mM of each), 1μl of 25pmoles of forward primer and 1μl of 25pmoles of reverse primer and 1μl of first strand cDNA. MgCl₂ concentration was optimised for each PCR reaction and ranged between 1.5mM to 2.5mM (The kit used was from Roche Diagnostics). The reaction components were gently mixed and briefly centrifuged. The samples were heat denatured at 94°C for 5 minutes and then 2.5units of Taq polymerase (Roche Diagnostics) were added. Amplification was then carried out by 35 cycles of denaturation at 94°C for 1 minute followed by annealing at specific T°C as stated above in Table 2, for 1 minute followed by elongation at 72°C for 1 minute. A final elongation step at 72°C for 10 minutes was
included to ensure all PCR products had 3' A overhangs for cloning. PCR products were analysed by electrophoresis of an aliquot of the reaction on a 1% agarose gel containing ethidium bromide (10mg/ml) and bands of interest were excised. These together with the remaining PCR product corresponding to the bands were purified using Wizard PCR Purification kit (Promega). The dsDNA concentration was determined by its absorbency at 260nm where absorbency of 1 cm path length cuvette at 260nm is equivalent to 50µg/ml DNA.

2.10. Cloning of PCR products

Amplification products can be efficiently cloned into specifically designed vectors like PGEM-T after Wizard PCR Preps DNA purification (Promega) so that sequence analysis can confirm the identity of the products and to be also used as standards for quantification in Real-Time PCR; 3µl of purified PCR product was ligated to 50ng of PGEM-T vector in a 10µl volume, in the presence of ligation buffer, T4 DNA ligase (Promega kit). Reactions were incubated at 4°C overnight.

Transformation of JM109 High Efficiency Competent Cells (Promega) was carried out as follows: 50µl of cells were pipetted directly into a fresh 1.5ml eppendorf tube with 2µl of ligation reaction in it. The vials were incubated on ice for 20 minutes and then heat-shocked for 45-50 seconds at 42°C, followed by incubation on ice for 2 minutes. 950µl of room temperature SOC medium was added to each vial and they were incubated in a rotary shaker for 1 hour at 37°C. Cells were spread on LB agar plates containing 50µg/ml ampicillin which had been spread with 40µl X-gal (40mg/ml) and 40µl IPTG. The plates were incubated at 37°C overnight.

Positive transformants were initially identified by blue/white colony selection. Small-scale plasmid preparation was then carried out from overnight cultures, using the Wizard Minipreps DNA Purification System (Promega). The presence of PCR product was verified by restriction digestion of plasmid DNA and electrophoresis on a 1% agarose gel containing ethidium bromide. 10µg of each mini-prep DNA was then sent off for sequencing analysis. After confirmation, the plasmid DNA prepared for each transcript was used as standards in a dilution series for quantifying the unknown samples.
2.11. Real-Time RT-PCR using DNA Master SYBR Green I

Reactions for both unknown samples and external standards, were performed as follows: 20μl volume glass capillaries contained 1μl of each forward and reverse primer at 10pmole concentration, optimised MgCl₂ ranging between 1.5-2mM concentration, 2μl of 10x DNA Master SYBR Green I (a ready-to-use reaction mix containing Taq DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye and 10mM MgCl₂), 1μl of cDNA and sterile water to adjust the final reaction volume. A negative control was present in each run where the template DNA was replaced with PCR-grade water. The DNA Master SYBR Green I kit used was by Roche Diagnostics. A typical Light Cycler protocol contains four programs:

**Program 1: Denaturation (1 cycle)**

Initial denaturation was performed at 95°C for 30sec or at 95°C for 10min when “Hot-Start” protocol was used.

**Program 2: Amplification (40-45 cycles)**

It consists of three segments: a) Denaturation at 95°C, b) Annealing performed at temperature specific for each primer, as shown at table 2, with an optimised incubation time of 3-7 sec according to the primers used, so as to increase the specificity of primer binding and c) Elongation at 72°C; the increase in incubation time was calculated as the length of the amplicon (bp) increases using the following formula, but optimised after the initial run representative of the gene studied: \[ t = \frac{bp}{25} \text{ sec} \]

An additional step was included where fluorescence acquisition of each PCR product was recorded above the specific temperature set, 87-89°C, in order to eliminate the non-specific fluorescence signal and to ensure accurate quantification of the desired product. Then the cycle is repeated.

**Program 3: Melting Curve (1 cycle)**

Following completion of Program 2, melting curve analysis was performed. It consists of three segments: a) a rapid denaturation at 95°C, b) a short hold at an optimum annealing temperature calculated approximately 10° higher than the primer annealing temperature
during amplification, with an optimised incubation time of 30-45 sec for each primer used and then a slow denaturation at 95°C. During the slow denaturation step, fluorescence is measured continuously to generate a high-resolution melting curve for every sample.

**Program 4: Cooling**

At the end of the melting curve, the instrument is cooled down to 40°C for 30 sec and quantification analysis can be performed, as described in section 2.3.3.

### 2.12. Computer-based analyses

Primer design and secondary structure predictions were obtained using the OMIGA v2.0 (Oxford Molecular). Sequence analyses were performed using BLAST searches on the EMBL database. Nucleotide sequence alignments and pair-wise alignments for sequence comparisons and primer positioning were performed using the CLUSTAL W programme, part of the EMBL genome analysis facilities.
CHAPTER III
Changes in gene expression of the IGF-I splice variants in myotoxin-damaged and regenerating rat skeletal muscle
3.1. Introduction

Experimental models of muscle growth and regeneration have implicated IGF-I as an important mediator of anabolic pathways in skeletal muscle cells. Although liver is the principle source of circulating IGF-I, targeted ablation of IGF-I demonstrated that liver-derived IGF-I is not required for post-natal body growth (Sjogren et al., 1999). Administration of recombinant IGF-I protein to cultured muscle cells elicits a biphasic response. Initially, it stimulates cell proliferation followed shortly by myogenic differentiation (Engert et al., 1996; Rosenthal and Cheng, 1995).

The important role of growth factors like IGF-I and bFGF has been based in studies involving injection of neutralizing antibodies that inhibit muscle regeneration (Lefaucheur and Sebille, 1995). It is also known that local IGF-I expression is consistently elevated to its highest levels during the proliferation of myoblasts and declining subsequently (Edwall et al., 1989; Grounds 1991) in the regenerating rat skeletal muscle, indicating that IGF-I is important for the regeneration process. Furthermore, there is a markedly higher binding of $^{125}$I-IGF-I to tissue sections from regenerating than normal muscle (Jennische and Matejka, 1992) demonstrating increased expression of either IGF receptors and/or IGFBPs. Muscle cells are known to produce and secrete IGFBPs (Funk et al., 1992; Jennische and Hall, 2000). IGFBPs are autocrine/paracrine regulators of the cellular effects of the IGFs’ actions (Bach et al., 1994; Clemons, 1990, 1993).

The aim of the experimental work in this chapter was to elucidate the role of locally produced IGF-I isoforms, IGF-IEa and MGF in normal young, fast-twitch rat tibialis anterior muscle in response to damage and regeneration via a myotoxic agent. The association of IGF-I mRNA expression with muscle regeneration following bupivacaine injection has been looked into before. However, the investigation of the alternatively spliced IGF-I variants and their role in the damage-regeneration process, with the aid of RealTime PCR to quantify levels has never been studied until now. Additionally, the potential role of IGFBPs in the physiological response to damage and regeneration.
process was investigated to evaluate the regulation of gene expression of both IGF-I isoforms and its main IGFBPs in this model. The expression pattern of a transcription factor MyoD, was also measured in order to clarify the functional implications of the muscle capability to express the two-locally produced IGF-I isoforms in response to the degeneration-regeneration method.
3.2. Materials and Methods

3.2.1. Experimental design

The following animal model was designed with two controls:

- Animals (4) receiving bupivacaine injection dissolved in saline (BI)
- Animals (4) receiving saline injection (SI) served as an injection control
- Animals (4) receiving no injection (NI) served as control

In all groups, the contralateral limb was also examined and served as a sub-control group. The inclusion of the SI and NI controls were necessary to obtain unbiased estimates of the effects of damage and recovery in muscle by bupivacaine, in relation to the IGF-I system. Effects of bupivacaine injection were examined at 5 time points: at 4, 7, 11, 14 and 24 days.

The SI group was also examined at the same time points and the NI group was only tested at 4 and 14 days.

3.2.2. Project License

All experiments were performed under the control of the Home Office Animal (Scientific Procedures) Act 1986 under the project license PPL 70/4916.

3.2.3. Animals and Bupivacaine protocol

A total of 60 Sprague-Dawley rats (250-300gr body weight, 10-12 weeks of age at the start of the experiment) were used. Young animals were studied because they have a greater potential for muscle regeneration than older animals (Schultz and Lipton, 1982).

Anaesthesia in animals was induced at a rate of approximately 3% halothane in oxygen at a flow rate of 2L/min and maintained at approximately 1-2%. The left hind quarter was shaved so that the TA was visible and an 0.3ml injection of either 0.5% bupivacaine hydrochloride (1-butyl-2', 6'-pipeloxylidide hydrochloride) (Sigma) in 0.9% sodium chloride, or 0.9% sodium chloride, were made into the middle of the TA muscle. A 26-gauge x13 mm length needle was introduced at the midpoint of the muscle, inserted at an angle and advanced proximally along the muscle’s longitudinal axis. The needle was then slowly withdrawn as the muscle expanded. Animals regained consciousness 10-15
minutes later. Regular check-ups of the animals were made to ensure that the rats were not in pain during the recovery period.

3.2.4. Tissue preparation

At the time points stated above, the animals were killed by appropriate methods in Schedule 1 of the Home Office Act using CO$_2$ in rising concentrations and death was ensured by cervical dislocation. The tibialis anterior (TA) muscle from both hind limbs was removed quickly, weighed under cold conditions and cut into two parts cross-sectionally. One part of TA was taken from the mid-belly region, covered in cryopreservative (Tissue-Tek II, O.C.T Compound) quickly-frozen by immersion in isopentane that had been cooled to liquid nitrogen and stored at -70°C until further processing. The rest of the TA muscle was packed into a 1.5ml eppendorf tube directly frozen in liquid nitrogen and stored at -70°C for total RNA isolation.

3.2.5. Total RNA isolation

The single-step method of RNA isolation using acid guanidinium thiocyanate-phenol-chloroform after Chomczynski and Sacchi (1987) was used to isolate total RNA, as described in section 2.4 in General Materials and Methods in Chapter II.

3.2.6. Measurement of total RNA concentration

RNA purity and concentration was performed, as described in section 2.5 in General Materials and Methods in Chapter II.

3.2.7. Electrophoresis of RNA

RNA integrity of BI, SI and NI groups’ TA muscles was performed, as described in section 2.6 in General Materials and Methods in Chapter II.
3.2.8. Primers for cDNA synthesis and PCR
The primers used in this study for both RT-PCR and Real-Time PCR are described in section 2.7 in General Materials and Methods in Chapter II.

3.2.9. First strand cDNA synthesis
Reactions for first strand cDNA synthesis was performed, as described in section 2.8 in General Materials and Methods in Chapter II.

3.2.10. RT-PCR
RT-PCR reactions were performed using the corresponding primers and conditions for the genes of interest, as described in section 2.9 in General Materials and Methods in Chapter II.

3.2.11. Cloning of PCR products
PCR products were cloned into a PGEM-T vector, as described in section 2.10 in General Materials and Methods in Chapter II.

3.2.12. Real-Time RT-PCR using DNA Master SYBR Green I
Reactions for both unknown samples and external standards were performed as described in section 2.11 in General Materials and Methods in Chapter II. A duplicate number of runs were performed for each sample and the mean of the values was used for statistical analysis.

3.2.13. Computer-based analyses
Analysis and confirmation of sequences was performed, as described in section 2.12 in General Materials and Methods in Chapter II.
3.3. Results

3.3.1. RNA extraction of experimental and control muscle

Total RNA was extracted from bupivacaine-injected, saline-injected and normal non-injected and their contralateral TA muscles from 10-12 weeks old Sprague-Dawley rats. The integrity of RNA was confirmed by running a 1.2% agarose formaldehyde gel and the results are shown in figure 3.3.1.

![Figure 3.3.1. Electrophoresis of total RNA extracted from rat TA muscle in order to check the integrity of the RNA samples. Lanes 1-3, 4 day BI muscle; lanes 4-6, 7 day BI muscle; lanes 7-9, 11 day BI muscle; lanes 10-12, 14 day BI muscle; lanes 13-15, 24 day BI muscle; lanes 16-17, normal muscle. Lanes 11 to 13 demonstrate that samples run faster than the rest.](image)

**Figure 3.3.1.** Electrophoresis of total RNA extracted from rat TA muscle in order to check the integrity of the RNA samples. Lanes 1-3, 4 day BI muscle; lanes 4-6, 7 day BI muscle; lanes 7-9, 11 day BI muscle; lanes 10-12, 14 day BI muscle; lanes 13-15, 24 day BI muscle; lanes 16-17, normal muscle. Lanes 11 to 13 demonstrate that samples run faster than the rest.

3.3.1.1. PCR amplification

First strand cDNA was synthesised from the RNA extracted, as described in section 3.2.9. One or two samples were used each time in RT-PCR to detect and amplify transcripts of IGF-1Ea, MGF, IGFBP-4, IGFBP-5, M-cadherin, and MyoD. Bands with optimal conditions for MgCl₂ and annealing temperature for each transcript were
then excised, gel purified and cloned for sequence analysis (MWG Biotech). The results of the amplification during optimisation of IGF-IEa for example, are shown in figure 3.3.2. It shows products amplified using the IGF-IEa and IGF-IEaR primers. The size of the PCR products observed, were approximately 250 base pairs for all muscle samples, which is the expected size for the IGF-IEa as calculated from previous data (S.Yang; personal communication). The PCR included positive controls from tissue that each transcript is highly expressed in and negative controls where there is no template

Following confirmation of sequencing analysis, RT-PCR was performed again in order to use the PCR products, following purification and cloning, as standards for quantification in Real-Time PCR.

Figure 3.3.2. PCR amplification of IGF-IEa transcript. Total RNA was extracted from tibialis anterior muscle and converted to cDNA. Amplification was carried out with 35 cycles of denaturation at 94° for 1 minute, annealing at 60°C for 1 minute and elongation at 72°C for 1 minute. The picture shows PCR products during MgCl₂ titration, obtained using IGF-IEa and IGF-IEaR primers: Lane 1 1Kb ladder marker; lane 2 1.5mM MgCl₂ cDNA; lane 3 2.5mM MgCl₂ cDNA; lane 4 3.5mM MgCl₂ cDNA; lane 5 no template control; lane 6 positive control for IGF-IEa.
3.3.2. Sequence analyses of IGF-IEa and MGF amplified products

Sequencing of the cloned 264bp and 313bp PCR products indicated that IGF-IEa and MGF respectively, had been specifically amplified from the experimental muscle samples. They were identified according to sequence comparisons and homologies to existing sequences for IGF-IEa and MGF (Yang et al., 1996 and personal communication) with additional BLAST confirmation. These sequences correspond to exons 2, 3, 4 and start of exon 6 for IGF-IEa and exons 2, 3, 4, 5, and a few bases from exon 6 (part of the primer) for MGF, as known from existing data for the IGF-I isoforms (S. Yang; personal communication).

The CLUSTAL W DNA programme was used to produce alignments of the cloned nucleotide sequence data, obtained for IGF-IEa and MGF as seen in figures 3.3.3-3.3.8 with the existing cDNA sequences for IGF-IEa and MGF as identified in rat, rabbit and human (S. Yang; personal communication). The nucleotide sequences coding for IGF-IEa and MGF are highly homologous to the existing IGF-I and MGF cDNA nucleotide sequences across human, rat and mouse. The difference between IGF-IEa and MGF is the extra 52bp in E domain of exon 5 for MGF that is not present in IGF-IEa, as shown in figure 3.3.3. Its presence alters the reading frame and hence the 3'-end of the MGF peptide.

The rat and human IGF-I sequences presented below, were taken from the published human IGF-I sequence (Chew et al., 1995) and the rat IGF-I sequence (Rotwein, 1986), and together with the rabbit sequence cloned by S. Yang et al. (1996) in order to compare our sequences with the existing ones, using specific primers for MGF and IGF-IEa.
Figure 3.3.3. Alignment of the cloned MGF PCR product to rat MGF cDNA. Character to show that a position in the alignment is perfectly conserved (*). Gaps indicated by (-) were inserted to maintain alignments. Underlined letters refer to primer sequences of MGF-F and MGF-R. An additional 52bp in exon 5 (shown as blue) is characterised by MGF, which IGF-IIa lacks. The percentage homology was 90%.
**Figure 3.3.4.** Alignment of the cloned MGF PCR product to rabbit MGF cDNA (Yang *et al.*, 1996). Character to show that a position in the alignment is perfectly conserved (*). Gaps indicated by (-) were inserted to maintain alignments. Underlined letters refer to primer sequence as mentioned above and exons are the same as shown above in figure 3.3.3. The percentage homology was 88%.
<table>
<thead>
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<tr>
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</tbody>
</table>

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**Figure 3.3.5.** Alignment of the cloned MGF PCR product to human MGF cDNA. Character to show that a position in the alignment is perfectly conserved (*). Gaps indicated by (-) were inserted to maintain alignments. Underlined letters refer to primer sequence as mentioned above and exons are the same as shown in figure 3.3.3. The percentage homology was 78%.
### Figure 3.3.6

Alignment of the cloned IGF-IEa PCR product from muscle sample to rat IGF-IEa cDNA. Character to show that a position in the alignment is perfectly conserved (*). Gaps indicated by (-) were inserted to maintain alignments. Underlined letters refer to primer sequences of IGF-IEa F and IGF-IEa R. Notice that exon 5 is not present as in MGF sequence. The percentage homology was 80%.
Figure 3.3.7. Alignment of the cloned IGF-IeA PCR product from muscle sample to rabbit IGF-IeA cDNA (Yang et al., 1996). Character to show that a position in the alignment is perfectly conserved (*). Gaps indicated by (-) were inserted to maintain alignments. Underlined letters refer to primer sequence as mentioned above in figure 3.3.6 and exons are as shown above. The percentage homology was 82%.
Figure 3.3.8. Alignment of the cloned IGF-Ie PCR product from muscle samples to human IGF-Ie. Character to show that a position in the alignment is perfectly conserved (*). Gaps indicated by (-) were inserted to maintain alignments. Underlined letters refer to primer sequences as mentioned in figure 3.3.6 and exons are as shown above. The percentage homology was 73%.
3.3.3. Optimisation of IGF-IEa and MGF amplification by Real-Time PCR

Following confirmation of sequence analysis of the plasmid DNA for the gene of interest, serial dilutions in nuclease-free water were prepared from the original concentration of the dsDNA standards. Optimisation of MgCl₂ and annealing temperature was performed for the standards in the Roche Light Cycler for each transcript, prior to the quantification runs including the samples. Specificity of Light Cycler RT-PCR products was documented with gel electrophoresis and resulted in a single product with the desired length (figure 3.3.9). Use of standards allowed measurement of the unknown samples over a wide range of concentration, where the specificity of PCR product identity of each sample was confirmed by its amplification under identical conditions to the standard. Specificity of the MGF products was documented with melting curve analysis (Light Cycler Software 3.39). The melting temperatures of the products are template dependent (Table 4). Unspecific products and primer-dimers have melting temperatures lower than 82°C (figure 3.3.10).

To confirm accuracy and reproducibility of Real-Time PCR, each sample was run in duplicates in a single Light Cycler run. Additionally, in order to test the inter-variation amongst kits, the same experimental run was carried out using different premix cups of DNA Master SYBR Green I kit (Roche Diagnostics).

Figure 3.3.9. Confirmation of MGF specific Light Cycler PCR products after 45 cycles: Lane 1, 100bp ladder; lane 2-4, calibration curve with 313bp product (12ng, 2.4ng, 0.12ng dilution series of plasmid DNA); lane 5-8 MGF specific muscle PCR products.
Table 4. Different melting temperatures of PCR products for each different transcript.

<table>
<thead>
<tr>
<th>mRNA template</th>
<th>Observed Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-IEa</td>
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</tr>
<tr>
<td>MGF</td>
<td>89.63°C</td>
</tr>
<tr>
<td>IGFBP-4</td>
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</tr>
<tr>
<td>IGFBP-5</td>
<td>89.66°C</td>
</tr>
<tr>
<td>MyoD</td>
<td>87.95°C</td>
</tr>
<tr>
<td>M-cadherin</td>
<td>89.85°C</td>
</tr>
</tbody>
</table>

Figure 3.3.10. Melting Curves of MGF Light Cycler PCR products: the melting temperature of MGF PCR products was at 89.7°C (blue line) and for primer-dimers was lower than 82°C (yellow and red line). The fourth segment during the amplification program melted the unspecific Light Cycler products at 89°C and eliminated any non-specific fluorescence signals.
Regulation of investigated gene transcripts

Relative quantification is always based on a reference transcript. Normalisation of the target genes with an endogenous standard was done via the reference gene expression to compensate for inter-PCR variations. The housekeeping gene that was chosen was glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that remained constant, independent of the conditions imposed in this study. Therefore, all results were normalised against GAPDH and results are shown below.

3.3.3.1. Quantification of IGF-I isoforms in damaged, regenerating and normal muscle

IGF-Iεα and MGF (or IGF-Iεβ in the rat), which are locally produced in muscle, were quantified under conditions of myotoxic damage and regeneration for a period of 24 days. Both transcript levels in the BI group were compared to their contralateral TA muscle, the SI and NI groups and values were normalised against the internal standard GAPDH. IGF-Iεα and MGF were expressed in all the groups used in this study, as shown in figures 3.3.11 and 3.3.12. The expression of both IGF-Iεα and MGF was significantly higher (**P<0.001) in the damaged muscle from 4 to 24 days than in the control groups’ levels, i.e. the contralateral muscle to the BI ones, the SI and NI groups. The SI and NI groups showed no significant changes for any of the transcripts. Statistical analysis was performed using two-way ANOVA.

The maximal and temporal expression of IGF-Iεα and MGF mRNA differed during the 24 days post bupivacaine injection. Four days after myotoxic damage, both IGF-Iεα and MGF mRNA levels showed a dramatic increase in the bupivacaine treated muscles, with the IGF-Iεα expressed at levels about 35-fold higher than those seen in MGF. Expression of IGF-Iεα kept increasing until 11 days after injection, whereas MGF expression gradually decreased until day 24. On the other hand, IGF-Iεα levels in the bupivacaine treated muscle dropped suddenly at day 14 towards control levels, which seemed to increase again at day 24.
Figure 3.3.11. Quantification of MGF levels in damaged and control muscle groups normalised against GAPDH. There is highly significant increase (**\(P < 0.001\)) in MGF levels in the BI group compared to their contralateral, SI and NI groups from 4 to 14 days. Significant increase (*\(P < 0.05\)) of the BI group was also observed at day 24. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. For all groups \(n = 4\). Values are expressed as normalised mean ± normalised standard error of the mean (S.E.M).

Figure 3.3.12. Quantification of IGF-IEa levels in damaged muscle and control groups normalised against GAPDH. Highly significant increase (**\(P < 0.001\)) was observed in IGF-IEa levels in the BI muscles compared to their contralateral, SI and NI groups at 4, 7, 11 and 24 days. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. For all groups \(n = 4\). Values are expressed as normalised mean ± normalised standard error of the mean (S.E.M).
MGF expression shows a noticeable 10-fold increase in the damaged muscle in comparison to the MGF levels in the normal muscle from day 0 to day 4, whereas IGF-IIEa levels of damaged muscle increased only 3.5 times compared to those in normal muscle. The percentage decrease of MGF levels from day 4 to day 7 was higher, compared to that at following days. On the other hand, the percentage increase of IGF-IIEa levels was maximal between days 4 and 7 compared to that between days 7 to 11.

3.3.3.2. Expression of MRF regulators during satellite cell activation

In order to relate the expression of IGF-IIEa and MGF to the de novo myogenesis associated with the regeneration process of muscle, the myogenic regulatory factor MyoD was studied.

The expression of MyoD was significantly higher (**P<0.001) in the BI TA muscle than in the control muscle groups until day 11 (figure 3.3.13). MyoD levels of the BI group gradually decreased from day 4 until day 14 and levels started to increase again thereafter. However, MyoD expression of the BI group was significantly lower (**P<0.001) at day 14 compared to that observed in the control groups.

Values remained similar within the same control group throughout the 24 days. MyoD levels of the contralateral muscle to the BI group were significantly higher than MyoD levels of the other control groups. All values were normalised against GAPDH.
Figure 3.3.13. Quantification of MyoD levels in damaged TA and control groups normalised against GAPDH. There is a gradual decrease of MyoD mRNA levels in the bupivacaine-injected TA muscle from day 4 to day 14 with significant differences to control values (**P<0.001). Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. For all groups n = 4. Values are expressed as normalised mean ± normalised standard error of the mean (S.E.M).

The satellite cell molecular marker M-cadherin was quantified during the 24-day muscle regeneration. M-cadherin levels in the BI group were significantly higher (**P<0.001) than those in the control groups, until day 14. M-cadherin showed maximal expression at day 4-post bupivacaine injection with a gradual decrease reaching control levels by day 24 (figure 3.3.14). Expression of M-cadherin in SI and NI groups was unaltered during the 24 days.

A marked up-regulation of M-cadherin expression was observed in the contralateral to bupivacaine-injected muscle at days 4 and 7 compared to the other days. The expression pattern of M-cadherin in the BI group seemed to follow a similar trend as seen with MGF expression in figure 3.3.11.
Figure 3.3.14. Quantification of M-cadherin levels in damaged and control groups normalised against GAPDH. M-cadherin levels in the BI group were significantly higher (**P<0.001) compared to the control M-cadherin levels from day 4 and then reached similar values at day 24. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. For all groups n = 4. Values are expressed as normalised mean ± normalised standard error of the mean (S.E.M).

3.3.3.3. Involvement of IGFBP-4 and IGFBP-5 in muscle damage and regeneration

IGFBP-4 expression in the BI group was maximal (**P<0.001) at day 7 compared to that in the control groups. After 7 days, IGFBP-4 levels declined to reach similar values to control groups by day 24 (figure 3.3.15). A marked down-regulation of IGFBP-4 expression in the BI group was observed at day 4 and 14 compared to that seen in their contralateral muscles. IGFBP-4 levels of the contralateral muscle to the BI group were higher than those of the other control groups at all time points.

On the other hand, the expression of IGFBP-5 was significantly higher (**P<0.001) in BI group than in SI and NI groups (figure 3.3.16). Four days following damage, a striking increase of 7-fold in IGFBP-5 levels was seen in comparison to those in control groups. IGFBP-5 levels decreased thereafter until day 11 and increased again until day 24, reaching similar values to those at day 7. IGFBP-5 expression of the BI group showed a similar trend to that of MGF. Values for both transcripts were normalised against GAPDH.
Figure 3.3.15. Quantification of IGFBP-4 levels in damaged and control groups normalised against GAPDH. There was a significant increase (**$P<0.001$) in the BI group compared to the control groups at days 7 and 11. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. For all groups $n=4$. Values are expressed as normalised mean ± normalised standard error of the mean (S.E.M).

Figure 3.3.16. Quantification of IGFBP-5 levels in damaged and control groups normalised against GAPDH. IGFBP-5 levels were significantly higher (**$P<0.001$) in the BI group compared to the control levels at all time points. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. For all groups $n=4$. Values are expressed as normalised mean ± normalised standard error of the mean (S.E.M).
3.4. Discussion

The aim of this study was to assess the temporal expression pattern of the two IGF-I isoforms that are locally produced in skeletal muscle in response to damage by the sensitivity and accuracy of the Real-Time PCR technology. Furthermore, any possible connection amongst the local IGF-I system, its IGFBPs and other factors that play a significant role in muscle damage and regeneration was examined.

Advantages and disadvantages of the Real-Time RT-PCR technique

The use of Real-Time PCR method has been shown to have several advantages and disadvantages. The availability of diverse applications such as the “Hot-start” system where amplification is delayed until a higher temperature is reached by combining a polymerase with an anti-Taq antibody facilitates the amplification of the desired product and minimizes amplification of non-specific products. In this study, Real-Time PCR of IGF-IeA, IGFBP-4, MyoD and M-cadherin transcripts was performed using this system.

In general, one drawback of Real-Time PCR is that multiple dye molecules bind to a single amplified molecule and consequently the amount of signal generated after irradiation is dependent on the mass of dsDNA produced in the reaction. Assuming the same amplification efficiencies, amplification of a longer product will generate more signal than a shorter one. If amplification efficiencies are different, quantification will be even more inaccurate. In this study, even though statistical analysis was not performed, samples in the same group showed relatively similar amplifications.

Primer design is essential for successful PCR quantification. The use of mRNA specific primers decreases background priming, whereas the use of random and oligo-dT primers maximises the number of mRNA molecules that can be analysed from a small sample of RNA. Primers can cause marked variation in calculated mRNA copy numbers and compared with specific downstream primers, random hexamers can overestimate mRNA copy numbers by up to 19-fold (Zhang & Byrne, 1999). RNA samples prepared for MGF Real-Time PCR were the only batch transcribed into cDNA using a specific RT-primer to MGF, whereas cDNA synthesis of the same samples for quantitative analysis of the other transcripts was done using random hexamers. Therefore, the use of random hexamers in
cDNA synthesis of transcripts apart from MGF, could explain why overall an approximate 100-fold difference in values was observed amongst these and the values seen for MGF.

A lot of research has shown that there are several points to be taken into account for precise quantitative Real-Time PCR. Quantification results depend on the positions of reaction tubes in the thermocycler (Wilhelm et al., 2000). It was shown that amplification efficiencies, especially in the first cycles, depend not only on the priming efficiencies of the primers and the melting temperature of the amplicon, but also on the melting behaviour of the amplicon’s genomic vicinity. Complete denaturation of genomic DNA was necessary to maximize precision of Real-Time PCR. Boiling DNA before PCR commences or higher denaturation temperatures in the initial cycles can improve the accuracy of quantification in some cases (Wilhelm et al., 2000).

RT-PCR-specific errors in the quantification of mRNA transcripts are easily compounded by any variation in the amount of starting material between samples. The accepted method for minimising these errors and correcting sample-to-sample variations is to amplify simultaneously with the target, a cellular RNA that serves as an internal reference, against which other RNA values can be normalised (Karge et al., 1998). The ideal internal standard should be expressed at a constant level among different tissues of an organism, at all stages of development and should be unaffected by the experimental treatment. GAPDH is a ubiquitously expressed, moderately abundant message. It was used to normalise values quantified in this study and to correct any inter-assay variation between samples, since it remained constant throughout the 24day recovery in response to injury. It has been used as an endogenous control for quantitative RT-PCR analysis because its expression is constant at different times and after experimental manipulation (Edwards and Denhardt, 1985; Winer et al., 1999).

Besides, the accuracy of the quantification will be critically dependent on the accuracy of the initial RNA measurement. The major disadvantages of the A$_{260}$ method are the large relative contribution that proteins and free nucleotides make to the signal, the inability to
distinguish between DNA and RNA, the interference caused by contaminants commonly found in nucleic acid preparations and the relative insensitivity of the assay.

**IGF-IEa and MGF expression in myotoxin-damaged and regenerating muscle**

This study showed for the first time that in muscle, IGF-I mRNA isoforms are differentially regulated during the regenerative process after myotoxin-induced damage. The MGF splice variant was maximally expressed before IGF-IEa. The different kinetics of expression therefore provides an alternative regulation of IGF-I according to specific conditions. IGF-IEa mRNA levels of the BI group peaked at day 11 and were 8 times higher than those from their contralateral group and about 11 times higher than those in the SI and NI groups. IGF-IEa levels were reduced to control levels by 14 days. These results are similar to previous reports where liver IGF-I mRNA expression was elevated six-fold at 5 and 10 days post bupivacaine injection in the TA muscle; by 15 days IGF-I levels decreased to control levels in the 2-month old rats (Marsh et al., 1997). Other methods of damage causing ischaemic injury by glycogen depletion also showed highest levels of IGF-I, three days after injury (Edwall et al., 1989). The major difference between our study and others is that previously no distinction has been made between the different isoforms of IGF-I locally produced in muscle in response to damage.

In this study, a transient surge of MGF mRNA occurred by 4 days post bupivacaine-damage showing a 6-fold increase over MGF levels of the contralateral group and a 10-fold increase compared to those of the NI group. By day 14, MGF levels had reached similar levels with the control groups. This is the first time that this IGF-I splice variant has been investigated in response to damage and the regenerative process subsequently. Indeed, the previously reported early increase in IGF-I gene expression in damaged muscle (Edwall et al. 1989; Caroni and Schneider, 1994; Marsh et al. 1997) may have been a reflection of the increase in MGF rather than IGF-IEa mRNA. However, the down regulation of MGF expression in contrast to IGF-IEa up regulation in response to damage, could be associated with their stability at a transcriptional, post-transcriptional level. The process of mRNA decay is a major control point for gene expression (Brawerman, 1993). Each alternative IGF-I mRNA has a particular half-life indicating a
distinct biological function. The presence of two N-linked glycosylation sites present in the Ea form of the peptide but not in the Eb, raises the question as to whether glycosylation of the Ea version is involved in the processing or biological function of this pro-hormone (Bach et al., 1990). A short half-life for IGF-IEb (MGF) might be necessary to accommodate its changing roles within the regenerating process of the damaged muscle, assuming an autocrine role. Yet, this speculation can be contradicted by the marked 100-fold increase in IGF-IEa levels compared to MGF, observed with Real-Time PCR. One possible explanation for such difference could be that the use of the specific RT primer for MGF cDNA synthesis resulted in an inhibition of the amplification of MGF mRNA by the Real-Time PCR method, or the use of random hexamers might have overvalued the amount of IGF-IEa transcript, as mentioned before (Zhang and Byrne, 1999). However, the data indicates that despite the use of random primers, the estimated levels of M-cadherin mRNA are comparable to those for MGF and maybe there are other reasons to suspect that M-cadherin expression is relatively very low. Therefore, despite this observation, it is feasible that the data is actually correct and MGF might be a very minor IGF-I transcript even in the challenged muscle.

**Is MGF expression associated with satellite cells present in response to damage?**

The early induction of MGF indicates that this may play a significant role in the increase in protein synthesis, proliferation and differentiation of satellite cells in response to damage. The satellite cell molecular marker M-cadherin in the damaged muscle demonstrated the same expression pattern as MGF, with a peak at 4 days and decreasing during muscle regeneration until 24 days after, to reach similar levels to the control groups. M-cadherin has been detected by *in-situ* hybridisation (Moore and Walsh, 1993) and immunostaining (Cifuentes-Diaz et al., 1995; Irintchev et al., 1994) in tissue sections taken from regenerating mouse skeletal muscle. Over a 96-hr time course in which fibre-associated satellite cells were activated to divide and differentiate, M-cadherin increased gradually and was 100% at 96 hr (Cornelison and Wold, 1997), which overlaps with the expression levels of the M-cadherin present in the damaged muscle in the quantitative analysis in this study. M-cadherin is present in the quiescent satellite cells in normal muscle (Irintchev et al., 1994), as demonstrated by its expression in the normal muscle in
this study; therefore, the up regulation of M-cadherin in the BI group suggests that a subpopulation i.e. activated or replicating satellite cells or all satellite cells express M-cadherin. However, the data presented here cannot distinguish whether increased M-cadherin mRNA levels represent activated or quiescent satellite cells, since BrDU (bromodeoxyuridine) that stains cells that have entered the S phase of the cell cycle, was not used. Since injection of bupivacaine into the muscle appeared to affect the ability of the animal to fully weight bear on the injected limb, it is suggested that extra work load was imposed on the contralateral leg. Therefore, the experimental protocol could explain the altered use of the contralateral muscle with consequent up regulation of M-cadherin at 4 and 7 days after damage. The reports so far are somewhat conflicting over the incidence of M-cadherin mRNA and protein. Moore and Walsh (1993) found that there was no M-cadherin mRNA at all in healthy adult muscle and Cornelison and Wold (1997) found mRNA only in 20% of satellite-like cells on isolated muscle fibres in vitro. Quantitative analysis of M-cadherin in this study, showed a relatively low amount of mRNA present in damaged and normal muscle.

**MyoD expression in response to damage**

The mechanisms of differentiation and rapid growth of regenerating muscle depend on a variety of growth and myogenic factors. In order to relate the timing of expression of IGF-I splice variants with the *de novo* myogenesis associated with the regenerative process, we studied the time course of expression of MyoD. Knockout experiments in mice have demonstrated that MyoD has a role in myoblast determination (Koishi *et al*., 1995). Maximal MyoD levels in the BI group were present at day 4 with a gradual decrease to reach levels lower than those seen in the control groups at day 14. MGF expression seemed to be associated with the early events of the regeneration process indicated by the MyoD results, yet MyoD levels were also increasingly present in the contralateral muscle. Using an *in vitro* system of cardiotoxin injury-induced regeneration experiments on soleus muscle of adult mice, Launay *et al.* (2001) revealed that MyoD mRNA was still detected in multinucleated myotubes from 5 to 30 days. However the *in-situ* hybridisation signal strength decreased progressively. This coincides with the results presented here, even though the muscle studied was fast and not the slow-type. Moreover,
a peak of MyoD expression was seen 3 days after cold injury in TA muscle which gradually decreased until 12 days after, where muscle architecture was back to normal (Pavlath et al., 1998).

**The temporal expression pattern of IGFBPs in damaged and regenerating muscle**

The IGFBPs show a tissue-specific distribution, although many tissues express more than one IGFBP. IGFBPs are important in modifying the availability and biological effects of IGFs (Jones and Clemmons, 1995). This study showed for the first time that expression of IGFBP-4 and IGFBP-5 is acutely regulated during adaptive changes induced in skeletal muscle by damage via a myotoxic agent. Quantitative RT-PCR demonstrated that IGFBP-4 and IGFBP-5 were expressed at a comparatively low level in normal muscle but at 4 and 14 days after bupivacaine, control levels were higher than damaged muscle for IGFBP-4. IGFBP-4 is considered to be an inhibitor of IGF-action (Jones and Clemmons, 1995). Previous studies demonstrated that in the regenerating muscle, IGFBP-4 was mostly expressed by connective tissue cells with maximal expression 4 days after injury by glycogen depletion and reduced by 7 days (Jenniche and Hall, 2000). Maximal IGFBP-4 mRNA levels was at day 7 and its expression pattern coincided with the trend of IGF-IEa even though its values were twice higher than IGF-IEa as shown in the graphs. Expression on an mRNA level cannot distinguish between muscle cells connective tissue or inflammatory cells present due to damaged muscle, therefore high expression of BP 4 at such specific time point could be a means of preventing connective tissue overgrowth, allowing time for the more slow process of regeneration of muscle fibres. Further investigation is discussed in chapter 4 where immunohistochemistry of IGFBP-4 was performed.

On the other hand, IGFBP-5 showed a transient increase at day 4 with a decline following by day 11 and increasing to similar levels with day 7 after 24 days of bupivacaine-induced damage. Expression of IGFBP-5 seemed to follow MGF expression pattern. Free IGFBP-5 is susceptible to degradation, but binding to IGFs appears to protect IGFBP-5 from proteolytic cleavage (Camacho-Hubner, et al., 1992). IGFBP-5 is also protected from degradation, by binding to several components of the extracellular matrix (Jones et
IGFs bound to such immobilised IGFBP can be slowly released, creating a high local concentration of IGFs. The 52bp insert in the E domain of MGF isoform results in the reading frame being altered and hence the carboxy-terminal end of the MGF peptide (Yang et al., 1996). The carboxy terminus of IGF-I is important in determining the affinity of the peptide for a particular receptor and/or binding protein. Data in this present study showed an acute surge of both IGFBP-5 and MGF four days after damage with a consequent decrease during regeneration. Ligand blotting of MGF and IGFBP-5 in muscle protein extracts with a specific antibody to MGF, could possibly answer whether MGF binds to IGFBP-5.

**Conclusions**
The results of the present study support the idea that the two splice variants of IGF-I have different kinetics and apparently there is a mechanism for using IGF-I in a different manner under conditions of damage and regeneration, as stem cell activation might seem to be associated with MGF expression. However, stem cell activation was also seen in the contralateral muscle, as indicated by the M-cadherin data, yet MGF was not seen. The temporal expression patterns of IGF-I\(E^a\) and MGF splice variants in damaged and/or regenerating muscle seem to be related with that of certain IGFBPs. In order to ensure an optimal regeneration process, a strict control of IGF actions is necessary and this is probably best achieved by an ordered and cell-specific expression of their binding proteins.
CHAPTER IV

Localization of general IGF-I and IGF Binding Proteins in rat TA muscle following damage and regeneration
4.1. Introduction

A stereotyped sequence of repair following skeletal muscle damage is observed in experimental models, which have certain features in common. Fibre degeneration with subsequent influx of leukocytes into the damaged area predominates in the first few days. Regeneration begins once the phagocytic inflammatory cells clear necrotic tissue. This phase of muscle remodelling is characterised by the activation of undifferentiated skeletal muscle precursor cells or satellite cells. Cell adhesion molecules, i.e. M-cadherin, N-CAM have been previously found on activated satellite cells (myoblasts) and on myotubes during regeneration therefore, they serve as molecular markers of regenerating muscle fibres. IGF-I and other growth factors have been implicated in satellite cell activity regulation, yet it was not known what type of IGF-I might be involved. In addition, it has been revealed that macrophages also produce growth factors. Macrophage-derived IGF-I could play important autocrine, paracrine roles in events such as macrophage differentiation, expansion of responsive B and T cell clones after wound healing and influence the mobility and proliferation of satellite cells (Arkins et al., 1993). Later studies demonstrated that murine macrophages are a source of a single 25-kDa secreted protein that binds IGF-I and the molecular identity of this protein is IGFBP-4 with a 97% rat homology. This binding protein may antagonize the extracellular effects of IGF-I (Li, et al., 1996).

In recent years, it has become increasingly apparent that autocrine and paracrine actions of the IGF system within skeletal muscle may play a major role not only in myogenesis but also in the maintenance of muscle fibre growth and integrity in the postnatal state (Jones and Clemmons, 1995; Florini et al., 1996). Although it has been appreciated that IGFBPs may modulate the endocrine and the local autocrine/paracrine influences of IGF-I in tissues like skeletal muscle (Mohan and Baylink, 1996), the precise actions of IGFBPs at the tissue level are unknown. However, it has been suggested that they act to modulate local IGF-I action by augmenting local IGF-I bioavailability and/or providing an IGF-I depot (Jones and Clemmons, 1995; Mohan and Baylink, 1996; Clemmons, 1998).
Previous findings have shown that regenerating skeletal muscle cells following myotoxic snake venom, express IGF-I (Jennische and Hansson, 1987) and the time sequence expression during the various stages of the regeneration process was very similar to that previously reported after ischaemic skeletal muscle injury in rats (Jennische et al., 1986). The most intense IGF-I immunoreactivity was seen in myoblasts and myotubes with a significantly lower immunoreactivity in satellite cells and immature muscle cells but no immunoreactivity in surviving muscle cells (Jennische and Hansson, 1987).

Both mRNA and protein levels of IGF-I and IGFBPs have been shown to be up regulated during regeneration after ischaemic injury (Jennische and Hall, 2000). In situ hybridisation showed IGFBP-5 restricted to the regenerating muscle cells, whereas connective tissue cells expressed IGFBP-4. Developmental expression and location of IGFBP-4 and IGFBP-5 mRNA and peptides was shown to be down-regulated with increasing age in porcine skeletal muscle whereas immunoreactivity was located in developing muscle cells with little to connective tissue, except at later stages of development (Gerrard et al., 1999).

Immunohistochemical and in-situ hybridisation studies have been reported on IGF-I localization to non- or /and myofibrillar structures. However, a methodical analysis of the IGF-I system immunoreactivity in a well-established model of degeneration-regeneration on skeletal muscle was vital in order to establish the spatial and temporal expression of this system. Therefore, we tested the following hypotheses on the model used in this study: 1) significant IGF-I immunoreactivity would be associated with invading inflammatory cells and small regenerating muscle fibres, 2) IGF-I protein localization on myofibres would precede or be concurrent with histological (centrally located myonuclei) and/or developmental markers (embryonic myosin heavy chain), 3) localization of IGFBP-5 protein would be on muscle fibres and related to the temporal localization of IGF-I protein whereas the occurrence of IGFBP-4 protein would only be associated to non-muscle cells.
4.2. Materials and Methods

The experimental protocol of bupivacaine-injected rat TA muscle and all the control groups are as described in Chapter III, Methods and Materials, section 3.2.1 and 3.2.3.

4.2.1. Tissue preparation

Bupivacaine was injected at the midpoint of the left TA muscle, as described on page 90. The TA muscle from both limbs were removed and weighed quickly before being cut as follows: a portion of the mid-belly part of the BI TA, where the damage area was evident, was taken, as described in section 3.2.4 of methods and materials in chapter III, so that transverse sections could be cut for histological and immunohistochemical analysis. The rest of the damaged part of the mid-belly and the tendon sites were processed for RNA extraction. Serial 10-μm-thick transverse sections were cut from all muscles on a cryostat microtome (LEICA CM3050) at -20°C and applied to super-frost premium plus-coated glass slides (BDH). All sections were allowed to air-dry at room temperature for at least 10 minutes and then at -70°C until further processing.

4.2.2. Morphological staining and Immunohistochemistry

Mayer’s haematoxylin was used to stain muscle fibre nuclei with eosin as a counter stain (H&E): Sections were brought to room temperature and allowed to air dry. Hydrated with successive alcohols of decreasing strength (100%, 95%, 80%, distilled water), 2 min each. Stained with Mayer’s Haematoxylin (50gr aluminium ammonium sulphate, 4gr haematoxylin, 0.3gr sodium iodate, 1.5gr citric acid and 75gr chloral hydrate in 1lt of dH20 and filtered) for 15 minutes and put slides under running tap water for at least 20 minutes. Counterstained with Eosin for 1 minute and dehydrated with 75% and 100% alcohols, respectively, 2 minutes each. Cleared with xylene for 2x5 minutes and mount with DPX medium.

IGF-I, IGFBP-4 and IGFBP-5 protein localization using immunohistochemistry

Duplicate serial sections were stained for IGF-I, IGFBP-4, IGFBP-5 protein localization. The same protocol was used for all three.
Sections were fixed in 4°C cold acetone for 10 minutes, washed twice in 0.01M Phosphate-buffered-saline pH 7.4 (PBS tablets by Sigma) for 5 minutes and endogenous peroxidase activity was quenched by immersing the slides into 0.5% hydrogen peroxide (H$_2$O$_2$) in Methanol for 20 minutes using a shaker. Following two times washing for 5 minutes in PBS, sections were blocked with a mixture of 1% bovine serum albumin-BSA- (Sigma), 5% defatted dried milk and 5% horse serum diluted in PBS, for an hour at room temperature. The blocking solution was aspirated from each section and following that, sections were incubated with the specific primary antibodies in a humidified chamber at 4°C overnight. Monoclonal mouse anti-human IGF-I (AB61 by AutogenBioclear, UK) was diluted 1:40 in 5% horse serum in PBS. Affinity-purified goat polyclonal IGFBP-4 (sc-6005) and IGFBP-5 (sc-6006 both by AutogenBioclear) were also diluted 1:40 in 5% horse serum in PBS.

Following overnight incubation, slides were washed three times for 5 minutes in PBS and incubated with the corresponding secondary antibodies. Horse anti-mouse IgG antibody rat absorbed (Vector Laboratories) was used for IGF-I, diluted 1:200 in 5% horse serum in PBS, and left for 1½ hour at room temperature in a humidified chamber. Horse anti-goat IgG secondary antibody to IGFBP-4 and IGFBP-5 was diluted 1:150 in 5% horse serum in PBS and applied on sections for 1½ hour under the same conditions as IGF-I.

Slides were washed three times for 5 minutes each in PBS, then incubated with Vector Laboratories ABC peroxidase reagent kit for 30 minutes at room temperature, washed three times for 5 minutes and the immunocomplex was visualised by incubating the sections with the DAB substrate kit (Vector Laboratories) for 5 minutes. The colour reaction was stopped by washing the sections in water and following dehydration in ethanol washes (50%, 75% and 90%-100%), the sections were mounted using DPX mountant medium (BDH).

Controls:

Sections incubated in PBS in the absence of primary antibody served as controls. As additional controls, some tissue sections were incubated with the IGF-I, IGFBP-4, or IGFBP-5 antibody, which had been pre-adsorbed overnight at room temperature with recombinant human IGF-I (rhIGF-I) or blocking peptides for IGFBP-4 and IGFBP-5.
(AutogenBioclear, UK) at 50 µg of peptide per mL of 1:200 or 1:150 dilution of antibodies, respectively.

**Embryonic MyHC Immunohistochemistry used for identification of regenerating muscle fibres**

The primary monoclonal antibody (MyHc330) and secondary antibodies were a gift from A.F.M Moorman (Anatomy and Embryology Department, AMC, Amsterdam). The established protocol was performed on gelatin embedded single fibres and detection was based on indirect unconjugated immunoperoxidase technique (PAP) according to Moorman et al. (1984). However, the protocol was modified to use a detection method based on biotin-streptavidin technique: sections were fixed for 5 minutes in a 4% (w/v) paraformaldehyde in 100mM phosphate buffer (pH 7.4), and washed twice in 150mM NaCl, 50mM Tris/HCl, pH 7.6 (TBS). Endogenous peroxidase activity was quenched by immersing the slides in 0.3% hydrogen peroxide (H₂O₂) in methanol for 20 minutes, using a shaker. The sections were washed twice for 5 minutes and in order to optimise specific binding of the monoclonal antibody the sections were pre-incubated in a mixture of 5% horse serum, 0.5% Triton X-100 in TBS for an hour at room temperature in a humidified chamber. Sections were dried and incubated with the embryonic MyHC330 monoclonal antibody, in a humidified chamber at room temperature overnight.

Overnight incubation was followed by washing three times in TBS for 5 minutes each, dried and incubation with a horse anti-mouse secondary antibody rat adsorbed, diluted 1:200 in 5% horse serum in TBS for 1 ½ hour at room temperature. To visualise immunolabelling, the sections were then treated as described above in the IGF-I protocol, using the ABC reagent kit and dehydration procedure.
M-cadherin immuno-fluorescence to identify satellite cells

The M-cadherin rabbit polyclonal primary antibody and the established protocol according to Irintchev et al. (1994) were kindly supplied by Professor Wernig (Institute of Neurophysiology of University of Bonn, Germany).

Sections of 6µm thickness were fixed in methanol for 4 minutes at 4°C. Blocking solution of 20% normal goat serum (NGS) in PBS was applied for 30 minutes at room temperature in a humidified chamber. The solution was aspirated from sections and they were incubated with M-cadherin primary antibody diluted 1:50 in PBS containing 0.7% lambda carrageenan (Sigma) and 0.02% sodium azide, at 4°C overnight. Lambda carrageenan has a high viscosity and a stabilizing effect on antibodies therefore it leads to better binding. Following washing in PBS, sections were pre-incubated with 20% NGS diluted in PBS for 30 minutes to enhance specificity prior to incubation with a biotin-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories) diluted 1:200 in PBS-carrageenan solution for 1 hour at room temperature. After washes in PBS, a fluorescein (DTAF)–conjugated streptavidin (Jackson Immunoresearch) antibody diluted 1:200 in PBS was applied for 30 minutes at room temperature. Sections were washed in PBS. Double / triple staining: Additional staining of nuclei with bis-benzimide, blue fluorescence and for laminin to reveal the basal lamina was performed for unequivocal identification of the satellite cells.

Following the last wash from M-cadherin secondary fluorescein antibody, sections were incubated with mouse anti-human laminin monoclonal antibody (Chemicon) diluted 1:1000 in PBS-carrageenan solution, for an hour at room temperature. After three washes in PBS, 5 minutes each, a secondary anti-mouse Texas Red-labelled antibody (Molecular Probes) diluted 1:200 in PBS was incubated for 45 minutes at room temperature. Following washing in PBS, 1µg/ml of bis-benzimide (Hoechts, Sigma) diluted in PBS was incubated for 5 minutes for nuclei staining, washed and sections were mounted in Fluoromount (Agar Scientific).
4.2.3 Image Processing and Analysis of immuno-histochemical/-fluorescent preparations

Images were acquired on a Nikon TE300 inverted microscope with fluorescent attachment (Nikon, UK) and Photonic Science low light level, peltier-cooled, CCD camera (Photonic Science, UK), controlled by Kontron KS400 image analysis software (Zeiss microscience, UK).

In order to analyse the total staining (in %) of IGF-I, IGFBP-4, IGFBP-5 and embryonic MyHC on the whole muscle sections and the total damage/ regeneration (in %) with H&E staining, whole muscle sections were imaged under bright field conditions at 10x magnification using a motorised XY-stage (Prior, UK) mounted on the Nikon inverted microscope and controlled by the KS400 image analysis software. Therefore, multiple microscope fields were collected (up to 10x10 fields) using the montage macro of the KS400 image analysis software to produce large montages of the whole muscle section. For M-cadherin specific staining, a minimum of 5 random fields per section were acquired from 3 sections per slide, over 4 slides at 20x magnification using fluorescence illumination with standardised imaging conditions for all specimens.

Image analysis of IGF-I, IGFBP-4, IGFBP-5, embryonic MyHC and H&E staining was performed using a custom written KS400 macro that allowed the user to interactively draw around damaged muscle fibres and express the identified area as a percentage of the total muscle area. Image analysis of M-cadherin-positive staining was assessed by a semi-automatic segmentation macro, which allowed some limited interaction by the operator and expressed the results as AREA% of positive M-cadherin staining over the identified field.
4.3. Results

4.3.1. Animals and TA muscle weights

Muscle weight: After 4 days, the BI muscle was significantly less heavy (31%; *P<0.05) than the NI muscle group and remained significantly less heavy, 20%, 22%, 4% at 7, 11 and 14 days respectively. Following day 14, the wet weight of BI muscle increased significantly (9%; **P<0.001) compared to the NI muscle. However, the wet weight of NI muscles remained constant until day 14 and increased thereafter but significantly lighter than the BI muscle.

Figure 4.3. Wet weight of BI TA and NI TA muscles. There was significant decrease of BI muscle mass compared to NI muscle mass at 4 days (*P<0.05). After 24 days the BI muscle was significantly heavier than NI (**P<0.001). Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. N = 4. Values are expressed as mean ± standard error of the mean (S.E.M).
Muscle weight to body weight ratio:
The ratio of muscle wet weight to whole body weight was significantly smaller in BI animals from 4 to 24 days recovery compared with NI muscles. At 24 days, the muscle to whole body weight ratio in the BI muscles became significantly larger (*P<0.05) than the NI muscles. On the contrary, in the NI muscles this allometric relationship was stable throughout the study.

Figure 4.3b. Muscle weight to body weight ratio of bupivacaine-injected and non-injected control rat TA muscle. The muscle to body weight ratio was significantly smaller in BI muscles until day 14 compared to NI (*P<0.05). After 24 days the ratio in BI muscles was significantly larger than NI (*P<0.05). Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. N = 4. Values are expressed as mean ± standard error of the mean (S.E.M).
**4.3.2. Time course of morphological changes**

For the purpose of structural analysis on frozen sections, serial sections were stained for routine histological (H&E) and immunohistochemical (embryonic MyHC) examination. None of the saline-injected (SI) muscles underwent necrosis except for a small number of fibres along the needle track, as shown in the haematoxylin and eosin staining (figure 4.3.1). However, this was not seen in all muscle samples in this group. Additionally, SI group showed no differences in their normal muscle architecture when compared to the non-injected (NI) fibres and contralateral to bupivacaine-injected muscles, at all time points (figure 4.3.1). Fibres from NI muscles appeared normal at all recovery times.

Bupivacaine-treated TA muscles exhibited a sequence of degenerative and regenerative changes. At 4 days after bupivacaine injection, most of the muscle fibres had degenerated completely apart from the periphery where muscle fibres were intact and had escaped the bupivacaine insult. Macrophages filled the necrotic fibres and ghost-like remnants of the original fibres could occasionally be seen (figure 4.3.1b). The remaining fibres displayed features indicative of partial damage: circular shape and ‘moth-eaten’ appearance, hyaline cytoplasm and pyknotic (but peripheral) myonuclei. Dispersed colonies of regenerative fibres with central myonuclei could also be seen amongst the necrotic and normal fibres (figure 4.3.1b). The fibres reacted strongly with the embryonic MyHC antibody (figure 4.3.3).

At 7 days, a substantial fraction of the fibre population consisted of small regenerating fibres with peripheral nuclei migrating to the centre (defined as fibres that had an area less than 50 per cent of the average for control muscle fibres) and many with central myonuclei as seen with the H&E stain (figure 4.3.2). These fibres were larger than at day 4 and most of them reacted strongly with the embryonic MyHC antibody (figure 4.3.3). Some of them however, stained intermediately dark-to-dark or did not stain at all.

On day 11, regenerating fibres were larger and most of them with central myonuclei present (figure 4.3.2). The number of embryonic myosin-positive fibres was still large, (figure 4.3.3).
After 14 days, the regenerating fibres were larger compared to those at 7 and 11 days (figure 4.3.2). The myonuclei of most of the fibres had migrated to the periphery of the fibre. The reaction with embryonic MyHC antibody had decreased compared to 11 days (figure 4.3.4).

Finally, on day 24, the fibre differentiation and morphology appeared normal and most of the myonuclei were now located at the periphery. Muscle fibres were densely packed and polygonal in shape (figure 4.3.2). Some small fibres were still present with central myonuclei. Fibres did not react with embryonic MyHC antibody as seen in figure 4.3.4.
Figure 4.3.1 Transverse sections of rat TA muscle 4 days after injection: (a) bupivacaine injection (BI), (b) saline injection (SI) and (c) no injection (NI). The fibres in the NI and SI muscles were closely apposed, polygonally shaped and had peripherally located myonuclei. Some damage occurred near the injection (arrow in (b)). Bupivacaine caused massive muscle fibre degeneration and the necrotic debris was removed by macrophages. A small number of fibres in the periphery of the section survived the bupivacaine insult and some others usually exhibited some signs of damage including a circular appearance (asterisk). Mayer's Haematoxylin. Scale Bar= 50μm.
Figure 4.3.1b Transverse sections of rat TA muscle 4 days after bupivacaine injection. Massive muscle fibre degeneration with macrophage infiltration amongst fibres that survived the bupivacaine can be seen (asterisk). The presence of small degenerating fibres with peripheral myonuclei starting to regenerate (shown by arrows) with a few regenerating myofibres with central myonuclei (arrowheads) can be seen. Mayer’s Haematoxylin. Scale Bar= 50µm
Figure 4.3.2 Transverse sections of bupivacaine-injected rat TA muscle after 7 d recovery (a), 11 d (b), 14 d (c) and 24 d (d). Regenerating fibres with central myonuclei were more at day 11 from day 7. By day 14, the regenerating fibres were larger and on day 24 the muscle architecture appeared normal with the muscle fibres having a polygonal shape. Mayer's Haematoxylin. Scale Bar=50µm
Figure 4.3.3 Reaction of transverse sections of bupivacaine-injected rat TA muscle stained with embryonic MyHC antibody after: (a) 4 d, (b) 7 d and (c) 11 day recovery. The regenerating fibres (myotubes) were grouped into small fascicles and reacted with the embryonic MyHC antibody. Scale Bar=50μm
Figure 4.3.4 Reaction of transverse sections of bupivacaine-injected rat TA muscle stained with embryonic MyHC antibody after: (a) 14 days recovery and (b) 24 days recovery. At 24 days no staining was present with the embryonic MyHC antibody. Scale Bar= 50μm
4.3.3. Variation of damage and regeneration with time

Using the KS400 Image analysis, as described in section 4.2.3, the multiple microscope fields that were collected as a montage, enabled the assessment of damage/regeneration areas present in each sample at all time points, in response to the bupivacaine insult. All control groups; SI, NI and the contralateral to bupivacaine muscle samples were also analysed, however they showed no signs of damage at any time so the mean percentage of damage in relation to the whole muscle fibre area was zero.

As seen from figure 4.3.3.1 the percentage of damaged-regenerated area at day 4 was 67% and thereafter decreased gradually until day 24 where most of the muscle fibre architecture has returned back to normal and there was only a 5% regenerated area amongst the normal muscle fibres.

The two-way ANOVA revealed that there was significant interaction (*P<0.05) between the different time points and the duration of recovery of muscle fibres to normal, except between days 11 and 14.

Figure 4.3.3.1. Mean percentage of damaged-regenerating muscle fibre area in relation to the whole muscle section. There is a continuing decrease in the regenerating area from day 4 until day 24 where normal muscle fibres with peripheral myonuclei can be seen. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. N = 4. Values are expressed as mean ± standard error of the mean (S.E.M).
4.3.4. Embryonic MyHC as a marker for regeneration in relation to time

To detect the presence of regenerating fibres at each time point, embryonic MyHC antibody was used. Embryonic labelling was absent from all muscle fibres in the control groups: SI, NI and the contralateral muscle to bupivacaine-injected ones and the normal muscle fibres that survived the bupivacaine insult.

In agreement with the data from the mean percentage of damaged-regenerating area shown with the H&E analysis, the mean percentage of embryonic myosin-positive muscle fibres declined from day 4 to reach zero value at day 24 (figure 4.3.4.1).

The two-way ANOVA revealed that there was significant interaction (*P<0.05) between the different time points and the presence of regenerating fibres decreasing consecutively.

![Graph](image)

**Figure 4.3.4.1.** Mean percentage of regenerating muscle fibre area stained with embryonic MyHC antibody. Embryonic myosin-positive fibres decrease with time as the muscle fibre returned to normal musculature by day 24 where no embryonic MyHC staining could be seen. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. N = 4. Values are expressed as mean ± standard error of the mean (S.E.M).
4.3.5. Immunohistochemical localization of IGF-I, IGFBP-4 and IGFBP-5 proteins on bupivacaine-injected TA

In the TA muscle of control rats, no specific IGF-I immunoreactivity could be demonstrated in muscle cells or capillaries. Injection of saline did not result in any change of the IGF-I immunoreactivity.

4 days: Injection of bupivacaine resulted in a marked muscle fibre degeneration. A massive invasion of macrophages into injured muscle fibres was conspicuous in rats examined 4 days after injury. Moderate IGF-I immunoreactivity could be demonstrated in the small muscle fibres with large nuclei indicative of regeneration (figure 4.3.5.1, arrows) and in relatively normal looking muscle cells (arrowhead) adjacent to injured muscle cells. Capillaries (blood vessels) were numerous and in most cases showed increased IGF-I immunoreactivity (figure 4.3.5.1, V). Normal muscle fibres located peripherally and had escaped the bupivacaine insult (figure 4.3.5.2, a) also displayed IGF-I immunoreactivity.

Figure 4.3.5.1. Section from rat TA muscle 4 days after bupivacaine injection, showing moderate IGF-I immunoreactivity in small regenerating fibres with large nuclei (arrows), in capillaries (V) and large muscle fibres (arrowhead). Scale Bar= 50μm.
Comparing IGF-I (a) with IGFBP-5 (b) and IGFBP-4 immunoreactivity (c), as can be demonstrated in figure 4.3.5.2, the normal muscle fibres did not show any IGFBP-5 or IGFBP-4 immunoreactivity. However, in figure 4.3.5.2 (c) the small regenerating muscle fibres showed IGFBP-4 immunoreactivity (arrows) and so did a few others that seemed to demonstrate both IGFBP-5 and IGFBP-4 immunoreactivity, as seen from serial sections in figure 4.3.5.3 (b) and (c) pointed with arrowheads. Some non-specific staining was also seen in the blood vessels (V). Omission of primary antibody eliminated any staining (figure 4.3.5.3, c).

7 days: all the debris had been removed and clusters of myoblasts / mononucleated myotubes demonstrated intense immunoreactivity and co localization for all IGF-I, IGFBP-5 and IGFBP-4 (figure 4.3.5.4, a, b, c), amongst the surviving muscle fibres that were negative (figure 4.3.5.4, a). Overall, stronger IGF-I immunoreactivity was observed compared to that seen at day 4.

11 days: most of the regenerating fibres had central myonuclei. IGF-I immunoreactivity was evidently dispersed amongst the mononucleated myotubes. Some of these showed IGF-I immunoreactivity whereas others were negative (figure 4.3.5.5, a). This was also consistent with IGFBP-4 immunoreactivity (figure 4.3.5.5, c). No IGFBP-5 immunoreactivity was present, apart from some non-specific staining in some capillaries.

14 days: all the regenerating fibres had central myonuclei and were larger than those at day 11. IGF-I immunoreactivity was more intense in some fibres, intermediate or negative in others (figure 4.3.5.6, a), probably relatively to the fast or slow type of fibres. No muscle fibres indicated any IGFBP-5 or IGFBP-4 immunoreactivity.

24 days: normal muscle fibres could be seen with a polygonal shape and very little IGF-I immunoreactivity was demonstrated in a few muscle fibres. No IGFBP-5 or IGFBP-4 immunoreactivity was observed in any muscle fibres (figure 4.3.5.7).

Pre-adsorption of primary antibodies with the recombinant or blocking peptides eliminated staining that was present in the muscle fibres stained with the respective antibody, while the staining intensity was brought down to the level of non-specific background. The results are shown in figure 4.3.5.8.
Figure 4.3.5.2. Step sections from bupivacaine-injected rat TA muscle after 4 days, stained for IGF-1 (a), IGFBP-5 (b) and IGFBP-4 (c). Small muscle fibres with central nuclei indicative of regeneration showed IGFBP-4 immunoreactivity (arrow, (c)) whereas normal muscle fibres on the left did not show any immunoreactivity for either IGFBP-5 (b) or IGFBP-4 (c) as with IGF-1. Intra-muscular nerves also showed intense immunoreactivity for IGFBP-4 (arrowhead, (c)). Scale Bar=50µm.
Figure 4.3.5.3. Step sections from bupivacaine-injected rat TA muscle after 4 days, stained for IGFBP-4 (a), IGFBP-5 (b) and omission of IGFBP-5 antibody (c). Strong IGFBP-5 and IGFBP-4 immunoreactivity was present in the same small muscle fibres with large nuclei indicative of regeneration (arrowheads), whereas larger round fibres adjacent to the regenerating fibres (arrows) did not show any IGFBP-5 or IGFBP-4 immunoreactivity. The walls of blood vessels showed both IGFBP-5 and IGFBP-4 staining. Scale Bar= 50μm.
Figure 4.3.5.4. Step sections from bupivacaine-injected rat TA muscle after 7 days, stained for IGF-I (a), IGFBP-5 (b) and IGFBP-4 (c). Clusters of regenerating fibres with central nuclei (arrows) or with peripheral nuclei (arrowhead) showed intense IGFBP-4 immunoreactivity (c). IGF-I immunoreactivity was also seen in regenerating fibres with central nuclei (a, arrows) but few (a, arrowhead) did not stain for IGF-I. Normal-looking muscle fibres did not show any IGF-I immunoreactivity either. Scale Bar= 50μm.
Figure 4.3.5.5. Serial sections from bupivacaine-injected rat TA muscle after 11 days, stained for IGF-I (a), IGFBP-5 (b) and IGFBP-4 (c). Some fibres with central nuclei showed IGF-I and IGFBP-4 immunoreactivity (arrowheads) whereas others were negative (arrows). No IGFBP-5 immunoreactivity was seen in the fibres. Scale Bar= 50µm.
Figure 4.3.5.6. Serial sections from bupivacaine-injected rat TA muscle after 14 days, stained for IGF-I (a), IGFBP-5 (b) and IGFBP-4 (c). IGF-I immunoreactivity was not evenly distributed in the regenerating myofibres, some were stained more than others, whereas others did not stain at all (a). No IGFBP-5 or IGFBP-4 immunoreactivity was observed. Scale Bar= 50µm.
Figure 4.3.5.7. Step sections from bupivacaine-injected rat TA muscle after 24 days, stained for IGF-I (a), IGFBP-5 (b) and IGFBP-4 (c). IGF-I immunoreactivity was only apparent in very few muscle fibres, as shown in picture (a). All muscle fibres were negative for both IGFBP-5 and IGFBP-4. Scale Bar= 50µm.
Figure 4.3.5.8. Step sections of bupivacaine-injected muscle that had been pre-adsorbed overnight at room temperature with (a) recombinant human IGF-1 (rhlGF-1) on a 4-day section, (b) blocking peptide for IGFBP-5 on a 7-day section and (c) blocking peptide for IGFBP-4 on 11-day section at a concentration of 50 µg peptide per mL. Scale Bar= 50µm.
4.3.5.1. **Image processing**

Image analysis of all sections stained for each antibody, was performed as described in section 4.2.3. The mean percentage of muscle fibre area stained for IGF-I, IGFBP-4 and IGFBP-5 is shown in figure 4.3.5.9. Whereas the percentage of muscle fibre area stained for IGF-I was shown to increase until day 14 (40%) (**P<0.001) and then decrease, the percentage of muscle fibre area stained for IGFBP-4 was significantly (**P<0.001) the highest at day 4 and thereafter started to decline significantly until day 11. The fibre area stained for IGFBP-5 was not significantly different between 4 and 7 days that disappeared subsequently.

![Graph showing the percentage of muscle fibre area stained with IGF-I, IGFBP-4 and IGFBP-5.](image_url)

**Figure 4.3.5.9.** Mean percentage of muscle fibre area stained with IGF-I, IGFBP-4 and IGFBP-5. IGF-I immunoreactivity in muscle fibres was shown to be inversely proportional to IGFBP-4. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. N = 4. Values are expressed as mean ± standard error of the mean (S.E.M).
4.3.5.2. Centrally located myonuclei

There was significant interaction between the type of injection and the duration of recovery for the relative number of fibres with centrally located myonuclei.

Virtually all the fibres in both the NI and SI group muscles examined, exhibited peripherally located myonuclei. Muscle fibres in the BI muscles with central myonuclei were counted by eye, collecting at least five images under x20 magnification of all sections. An average was calculated as shown in figure 4.3.5.10a. In BI muscles, the number of centrally located myonuclei was highest after 7 days recovery. Thereafter, the number gradually fell and by 24 days a very small number of fibres exhibited any centrally located myonuclei.

Muscle fibres with central nuclei stained for IGF-I was also calculated under the same conditions. These IGF-I positive fibres usually exhibited indications of regeneration, i.e. concurrent expression of embryonic MyHC and / or centrally located nuclei. At 4 days after injury, 7% of the IGF-I positive fibres contained central nuclei and 67% expressed embryonic MyHC. By 7 days, 25% of IGF-positive fibres contained central nuclei and 59% expressed embryonic MyHC. After 11 days, 52% of IGF-positive fibres contained central nuclei and 42% expressed embryonic MyHC. At day 14, 42% of IGF-I positive fibres had central nuclei and 30% expressed embryonic MyHC (figure 4.3.5.10b).
Figure 4.3.5.10a. Average number of muscle fibres containing central nuclei in relation to those stained for IGF-I. There were significant differences (**$P<0.001$) between the average number of centrally located nuclei and those stained for IGF-I from 7 to 14 days. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. N = 4. Values are expressed as mean ± standard error of the mean (S.E.M).

Figure 4.3.5.10b. Percentage of muscle fibres containing central nuclei stained for IGF-I and those stained for eMyHC. There were significant differences (**$P<0.001$) between those at all time points except day 24 where no staining for IGF-I and eMyHC was present. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. N = 4. Values are expressed as mean ± standard error of the mean (S.E.M).
4.3.6. Expression pattern of M-cadherin in normal and regenerating rat TA muscle

Regeneration of adult muscle after necrosis involves proliferation and fusion of activated satellite cells. The orderly sequence of primary and secondary myotubes that occurs in fetal myogenesis is not repeated. Very immature and relatively mature muscle fibres are intermingled and different stages of myogenesis are found simultaneously.

M-cadherin antibody labelled small mononucleated cells in a ring-like shape (figure 4.3.6.2, a, b; 4.3.6.3, a, b) in bupivacaine-injected muscle sections. The reaction product was usually confined at the surface of the cells, i.e., at the plasma membrane and never in the cytoplasm. Double labelling with laminin, a major component of the basal lamina of blood vessels and muscle fibres was performed in order to identify the origin of these cells. This staining showed that M-cadherin positive cells were muscle satellite cells contained within the basal lamina of muscle fibres (figure 4.3.6.1).

The M-cadherin immunoreactivity in the regenerating muscle was dependent on the phase of regeneration: in muscle at four days after injury the general level of M-cadherin staining appeared increased in the small fibres probably the myoblasts, the progeny of satellite cells. Inflammatory cells in the damaged area 4 days after injury did not show any M-cadherin immunoreactivity.

Seven days following the bupivacaine insult, the central part where all the regenerating myofibres were located, M-cadherin staining was localized inside the laminin-positive basal lamina of the regenerating fibres but the overall staining was less compared to day 4. Many relatively mature young fibres with attached satellite cell-like cells were depicted and the reaction product was mainly at the interphase between the myoblast and muscle fibre (arrows, figure 4.3.6.2 b). Eleven days after, where more myoblasts have fused to myotubes, M-cadherin expression was down regulated. M-cadherin staining could also be seen in the satellite-like cells, however clusters of larger young myofibres with intense reaction at the sites of contacts (arrowheads, figure 4.3.6.3, a) could also be seen. A few myoblasts (asterisk, figure 4.3.6.3, a) attached to myotubes was still noticeable. M-cadherin expression was markedly down-regulated 14 and 24 days after injury.

In normal muscle, very few myofibres also demonstrated M-cadherin immunoreactivity (figure 4.3.6.4, b) and some capillaries as well (asterisks, figures 4.3.6.4, a; fig.4.3.6.5).
Figure 4.3.6.1. Triple fluorescent staining visualizing laminin (a) Texas Red-labelled antibody, (b) M-cadherin -DTAF and (c) nuclei with bis-benzimide on a cross-section from a regenerating area, 7 days after bupivacaine injection. The arrow points to M-cadherin positive ring-like structure (b), located under the basal lamina of the muscle fibre (a) but not containing any nuclear profile. Scale Bar= 50μm.
Figure 4.3.6.2. M-cadherin staining in cross-sections of bupivacaine-injected TA muscle 4 days (a) and 7 days (b) after recovery. M-cadherin positive rings can be seen in regenerating fibres (arrows) in both pictures. Scale Bar= 50μm.
Figure 4.3.6.3. M-cadherin staining in cross-sections of bupivacaine-injected TA muscle 11 days (a) and 14 days (b) after recovery where there is very light M-cadherin staining. M-cadherin positive rings can be seen (arrows) and some staining between myofibres (arrowheads, a). Possible few myoblasts attached to young myofibres could also be seen (asterisk, a). Some auto fluorescence can be seen at 14 day section. Scale Bar = 50μm.
Figure 4.3.6.4. M-cadherin staining in cross-sections of bupivacaine-injected TA muscle 24 days after recovery (a) and its contralateral non-injected muscle (b). Very few M-cadherin positive rings can be seen (arrows) in normal muscle fibres with some M-cadherin staining in capillaries (asterisk, a). Scale Bar= 50μm.
Figure 4.3.6.5. M-cadherin staining in the (a) contralateral to the bupivacaine-injected TA at 11 days and (b) omission of M-cadherin antibody on the same section. There were one or two normal myofibres (arrow) or capillaries (asterisk) that reacted to M-cadherin. Scale Bar= 50μm.
4.3.6.1. Image processing

Image analysis of all sections stained for M-cadherin was performed as described in section 4.2.3 of Methods and Materials. The mean percentage of the regenerating muscle fibre area labelled for M-cadherin compared to the normal muscle fibre area stained, is shown in figure 4.3.6.6. M-cadherin immunoreactivity decreased from day 4 with a steep decline after 11 days of recovery where larger mature myofibres were present.

Figure 4.3.6.6. Mean percentage of regenerating muscle fibre area stained for M-cadherin compared to the normal muscle fibre area. M-cadherin immunoreactivity in muscle fibres was down regulated after 4 days. Significant differences were observed between the regenerating and normal area stained for M-cadherin at day 4 (**P<0.001) compared to day 7. Significant difference was seen between day 11 and 14 (*P<0.05). Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. N = 4. Values are expressed as mean ± standard error of the mean (S.E.M).
4.4. Discussion

In the present study it was demonstrated that regeneration after myotoxic skeletal muscle injury in rats is accompanied by increased muscle local expression of IGF-I. IGF-I immunoreactivity during the stages of regeneration process was similar to that previously reported after snake venom toxin on mouse EDL muscle (Jennische and Hansson, 1987) and ischaemic skeletal muscle injury in rats (Jennische et al., 1986). The IGF-I antibody used in this study was a general antibody, therefore it was not possible to distinguish between IGF-I isoforms on the protein level.

Morphological analysis of damaged tissue by bupivacaine injection

Muscle fibre mass and phenotype is not fixed post-natally but can be changed by various external stimuli which lead to alterations in contractile, transport and metabolic proteins (Goldspink, 1999). Muscle hypertrophy caused by muscle regeneration in rats was accompanied by an increase in IGF-I mRNA (Edwall et al., 1989). In the present study, bupivacaine-injected muscle weight loss was significantly marked through the eleven days examined. However, the damaged muscle seen after 4 days, even with the poor detailed resolution of frozen sections of such thickness, could compensate for the loss in mass resulting from the dissolution of muscle fibres. The muscle wet weight started to show a 9% increase compared to NI control muscle only after 24 days. However, the present study did not examine further days of recovery when increased mass has been observed 40 days after bupivacaine injection on EDL muscle (Rosenblatt and Woods, 1992).

A bupivacaine injection typically damages 80% of the muscle, which is seen at about two days. This is accompanied with a massive invasion of macrophages into the injured muscle and is then regenerated from activated satellite cells that differentiate and fuse into myotubes (Rosenblatt and Woods, 1992). After 3 days, most of the debris has been removed and clusters of myoblasts showing high IGF-I immunoreactivity was reported (Jennische and Hansson, 1987). Taking into account this morphological sequel of events, it was decided that 4 days after injection would be an appropriate initial stage to examine the localization and effects of IGF-I peptide after damage and through the regeneration
process. However, as demonstrated by the histological staining, four days after bupivacaine injection an appreciable inflammatory response was still observed amongst some surviving muscle fibres and some regenerating ones, seen in all muscles in this group, indicating a delayed process compared to that reported for the EDL muscle (Rosenblatt and Woods, 1992).

Even though the same concentration and volume of bupivacaine was administered into the TA muscle, which is a bigger muscle compared to the EDL, it remains elusive why the onset of regeneration was delayed. One possible explanation could be that since muscle samples excised for analysis were taken from the injection site in the mid-belly region, the distribution of bupivacaine was not homogeneous and other areas away from the injection could have shown less damage and more regenerating phases. Different muscles and fibre types respond differently to various mechanisms of injury and may contain different concentrations of protein markers.

**Implications of IGFBPs regulation of IGF-I actions in the damaged muscle**

Under the conditions of a myotoxic anaesthetic-induced muscle injury employed in the present study, local expression of IGF-I occurred in small regenerating fibres and fibre clusters after 7 days. The most intense IGF-I immunoreactivity was observed in the regenerating fibres at day 14 and no immunoreactivity in normal muscle fibres, except for a few at day 4 located at the periphery of the muscle. However, the temporal protein expression of IGF-I was not concurrent with the appearance of markers of regeneration i.e. centrally located nuclei and embryonic MyHC as originally hypothesized. IGF-I is localised and stabilized by its IGF binding proteins. The local concentration of IGF-I is very much determined by the abundance of these IGFBPs. It was interesting to note that the temporal expression pattern of IGFBP-4 protein did correlate with that of regenerating markers instead. Strong IGFBP-4 immunoreactivity was observed in small regenerating fibres, distributed across the whole muscle at day 4, down regulated at day 7 and staining was significantly less at day 11.
As described in the introduction, several studies have shown that invading macrophages are involved in numerous aspects of muscle regeneration through production of soluble growth factors (Robertson et al., 1993; Cantini et al., 1994) and that murine macrophages are a source of a secreted protein, IGFBP-4, that binds to IGF-I and may antagonize the extracellular effects of IGF-I. Furthermore, IGF-I transcripts have been detected in murine bone marrow-derived macrophages (BMDM) treated with hyaluronic acid (Noble et al., 1993).

Additional studies on involvement of IGF-I and IGFBP-4 in proliferation and differentiation of BMDM, showed an increased IGF-I and IGFBP-4 mRNA, while the stable IGFBP-4 mRNA expression indicated a post-transcriptional regulation of its secretion and stability. Besides, the ability of IGF-I analogues (long R3 IGF-I and des [1-3] IGF-I) but not rhIGF-I to enhance BMDM differentiation on day 4 showed that accumulation of IGFBP-4 in BMDM development might have an inhibitory effect on IGF-I actions by sequestering free IGF-I (Long et al., 1998).

All these facts could support the increased IGFBP-4 immunoreactivity during the inflammatory response after four days, with a very weak IGF-I immunoreactivity in the damaged area. The accumulation of IGFBP-4 protein as early as 4 days following injury and its gradual decrease could imply regulating the requirements of tissue availability and bioactivity for IGF-I thereafter.

Studies using Western ligand blotting and in-situ hybridisation revealed an increased expression of IGFBPs during regeneration after ischaemia and muscle-specific localization of IGFBP-5 transcript, whereas mostly connective tissue and a few muscle cells expressing IGFBP-4 (Jennische and Hall, 2000). Bupivacaine-injected muscles showed IGFBP-5 immunoreactivity in small regenerating fibres after 4 and 7 days co-localized with IGF-I and IGFBP-4 in some areas. However the levels of protein expression were quite low compared to IGFBP-4. Free IGFBP-5 is susceptible to degradation but binding to IGFs or to several components of the extracellular matrix, appear to inhibit proteolytic cleavage of IGFBP-5 (Camacho-Hubner et al., 1992; Jones et al., 1993). IGFs bound to such immobilised IGFBP can be slowly released, creating a high local concentration of IGFs. High expression of IGFBP-5 during muscle
regeneration could suggest a mechanism for supplying optimal IGF concentration at the receptor level. In our observations, the presence of IGFBP-5 protein could sustain IGF-I peptide levels while IGFBP-4 may inhibit IGF-I actions.

mRNA and protein analysis of IGF-I and IGF-binding proteins

A few conclusions can be drawn from the mRNA and protein analysis together. As can be seen from the expression patterns in the figure below, not only IGF-I mRNA but also the corresponding total IGF-I protein is increased in damaged/regenerating muscle. An early response in damaged skeletal muscle is a persistent, continuous elevation of IGF-I mRNA and protein levels. The IGF-I mRNA response is a muscle fibre reaction, implying that an early response in damaged muscle fibres is the release of markedly elevated amounts of IGF-I protein sustained longer than the IGF-I gene expression. In addition, regulation of IGF-I is complex and evidence can support a variety of mechanisms whereby IGF-I protein could increase without changes in IGF-I mRNA (Yang et al., 1995). Therefore, the biological impact of IGF-I in damaged-regenerating muscle may be comparable to that displayed during development, that is at a time when IGF-I is expected to be major regulator of muscle growth, proliferation and of differentiation (Florini et al., 1991). On the other hand, even a quantitative determination of IGF-I protein in muscle may not be sufficient to assess the possible biological impact of the growth factor, as its effective concentration is further affected by the presence of specific IGF-binding proteins (Baxter and Martin, 1989). IGFBP-4 mRNA levels peaked at 7 days and started to decline thereafter, but were sustained in low levels until 24 days. However, IGFBP-4 protein expression was shown to decrease following a spurt at 4 days, when damage was more evident, as discussed earlier. The earlier increase of
IGFBP-4 mRNA than IGF-I associated to damage may suggest that in vivo, IGFBP-4 could act by a different mechanism on IGF-I action, or that it prevents the proliferating effect of IGF-I on satellite cells. This IGFBP-4 increase could explain the increase in IGF-I peptide independent of changes of its mRNA and this can be confirmed by the protein data shown in the figure. In contrast to IGF-I and IGFBP-4, IGFBP-5 mRNA was down regulated after 4 days and protein expression was only observed until day 7.

However, in view of the possible inhibitory effect of IGFBP-5 on myoblast proliferation induced by IGF-I (Ewton et al., 1998) the decrease of its gene expression might be a means of facilitating the activating effect of IGF-I on satellite cells for the complete regeneration of skeletal muscle.

Differences however, between mRNA and protein localization may reveal detection thresholds for either mRNA or protein, or secretion of the protein without binding to the target organ. Therefore the protein may be washed out during the immunohistochemical procedure such as in the case of IGFBP-5 protein immunoreactivity after 7 days. Whereas mRNA and protein expression of the different IGFBPs was clearly distinct, muscle tissue and other cells were found to express both IGFBPs examined in this study, suggesting an autocrine or paracrine role. This could be explained by the fact that IGFBPs have similar functions and each contribution is necessary for the functional status of an organ especially during or after harsh effects imposed on it.

**Presence of M-cadherin protein in regenerating and normal muscle**

The expression of M-cadherin protein in TA muscle is in total agreement with the detection of its mRNA expression during muscle damage and regeneration, as described in chapter III. Our results demonstrated that M-cadherin was present in satellite cells in both regenerating and normal muscle fibres in the same muscle, which coincides with previous studies (Keller et al., 1999; Cifuentes-Diaz et al., 1995; Irintchev et al., 1994) but no immunoreactivity was observed in NI control or SI groups. IGF-I stimulates proliferation only when satellite cells have already entered the cell cycle and is therefore considered as a progression factor (Bischoff, 1994). Double immunofluorescence was performed using IGF-I mouse monoclonal and M-cadherin rabbit polyclonal antibodies in order to co-localize IGF-I and M-cadherin proteins on regenerating myofibres. This was
done with the intention of a possible connection between IGF-I and activated and / or quiescent satellite cell proliferation and differentiation during muscle regeneration in response to a model of damage. However, no conclusion could be drawn since the protocol didn’t work. The appearance of M-cadherin protein expression in normal myofibres amongst the regenerating ones, may be consistent with the suggestion that the signal regulating satellite cell proliferation is a diffusible factor that spreads throughout muscle to exert an influence on the entire satellite cell population (Bischoff, 1994) that the population of macrophages might provoke (Cantini and Carraro, 1995).

Conclusions
In summary, the data identify IGF-I as a likely initiator of restorative reactions in damaged muscle. The IGF-I peptide was present mostly in regenerating myofibres with a delayed maximal expression. On the contrary, an early expression of IGFBP-4 protein in the damaged and regenerating fibres was significantly marked. The sustained expression of IGF-I protein supports its role as a major regulator of muscle regeneration and growth. Contrary to the original hypothesis, inflammatory cells did not appear to be a major source of IGF-I. Finally, IGFBP-5 immunoreactivity was also localized in regenerating muscle fibres and was relatively associated with the temporal expression of IGF-I protein but transient. The sustained protein expression of total IGF-I implies a possible role for the IGF-IEa isoform and its binding proteins in the regeneration process of skeletal muscle.
CHAPTER V
Expression of IGF-IEa and MGF splice variants in rat skeletal muscle induced by stretch and stimulation
5.1. Introduction

A number of cell types respond to mechanical signals. Known as mechanocytes these possess mechanisms for local control of growth, remodelling and repair. Skeletal muscle undergoes hypertrophy in response to stretch, increased load and exercise. Passive stretch induces muscle fibre hypertrophy partly by division of myoblasts that fuse with muscle fibres; both to increase muscle fibre length (Tabary et al., 1972) and to increase girth (Moss and Leblond, 1971). Recently, a novel murine transcript (SMPX-small muscle protein X chromosome) was shown to exhibit increased expression in response to 7 days of passive stretch imposed on TA muscle in vivo (Kemp et al., 2001).

Skeletal muscle growth during development is dependent on release of systemic growth hormones. In contrast, adult muscle mass regulation depends on selective hypertrophy of muscle fibres exposed to such mechanical force that promotes the local synthesis of growth factors as well as systemic growth factors (Brahm et al., 1997). Therefore, the systems sensing increased mechanical stimuli and those converting these signals into gene expression and protein synthesis alterations are intrinsic to the muscle and probably function at the level of the specific muscle fibres.

The manner by which a mechanical stimulus is detected by the individual muscle fibres and translated into biological signals leading to localized increases in muscle bulk is under investigation. Some key regulators of mechanically induced skeletal muscle hypertrophy have been identified; two splice variants of IGF-I cloned by Yang et al. (1997), the muscle L.IGF-I (IGF-IeA) very similar to the liver type of IGF-I and MGF or mechano-growth factor which was only detected in exercised or stretched muscle. Electrical stimulation combined with stretch after 4 days, resulted in a greater increase of both types of IGF-I transcript in comparison to electrical stimulation alone that resulted in no significant increase over that in muscle from sham-operated controls (McKoy et al., 1999).

When the hypertrophic response to stretch, load and exercise fails, muscle wasting (atrophy) follows. Atrophy also results from prolonged bed rest (Widrick et al., 1997), hind-limb suspension (Fitts et al., 1986) and zero gravity (Vandenburgh et al., 1999).
The reparation of damaged fibres requires the activation of satellite cells known to express the MRF genes (Smith et al., 1994; Cornelison and Wold, 1997). MRFs’ mRNA levels were elevated 3- to 6-fold above the normal level and were distributed evenly along the length of the stretched muscles (Zador et al., 1999). Therefore, the elevated transcript levels of MRFs represent most likely a pre-requisite for growth and local tissue repair.

The potential role of IGFBPs in the physiological response to changes in loading has been studied and gene expression of IGFBP-4 and IGFBP-5 was acutely regulated during adaptive changes induced in skeletal muscle by overloading and unloading (Awede et al., 1999b).

In an attempt to establish more precisely the time of onset of expression of the two IGF-I isoforms in the hypertrophy process, the present study employed passive stretch combined with low-frequency electrical stimulation model to produce mechanical overload. As you will see in chapter 6 however, the intervention resulted in muscle damage. Therefore, the effects of IGF-I isoforms and IGFBPs are interpreted in relation to damage. The use of Real-Time PCR was used to assess the temporal expression pattern of the IGF-I splice variants. In addition, the regulation of gene expression of the IGF-I isoforms and its main IGFBPs in skeletal muscle subjected to the stretch and stimulation regimen was investigated. Activated satellite cells express a member of muscle regulatory transcription factors, including MyoD. Therefore by measuring its expression levels it was thought some light may be shed on any relationship amongst the MRFs, MGF and IGF-IEa expression in response to a mechanical stimulus and damage.
5.2. Materials and Methods

5.2.1. Experimental design

- The tibialis anterior of 6 young Sprague-Dawley rats was subjected to continuous stretch in the extended position for 1, 5 and 7 days and 1 hour of stimulation at a frequency of 30Hz of the peroneal nerve at the beginning of the study.
- Sham-operated controls (6) where the electrode wires were inserted touching the peroneal nerve but the electrical stimulation circuit was not switched on were used.
- A group of (6) young rats subjected to no stretch or stimulation was also used as control.

In all groups, the contralateral limb was also examined and served as a sub-control group.

5.2.2. Project License

All experiments were performed under the control of the Home Office Animal (Scientific Procedures) Act 1986 under the project license PPL 70/4916.

5.2.3. Animals and Surgical Procedure

A total of 20 Sprague-Dawley rats (250-300gr body weight, 10-12 weeks of age at the start of the experiment) were used. Animals were induced at a rate of approximately 3% halothane in oxygen at a flow rate of 2 litre / min and maintained at approximately 1-2%.

The left hindquarter of the hind limb was immobilized in plantar flexion using a fibreglass cast. Care was taken to ensure that the blood flow of the foot was not compromised by omission of plaster cast around the ankle and by placing a cotton bud on the hind limb to ensure casting was not too tight. When oedematous conditions and swelling of the feet were observed, these animals were immediately sacrificed. Electrical stimulation involved implanting stainless steel electrodes on either side of the peroneal nerve and these were attached to a micro-stimulation circuit. According to the original protocol by McKoy et al. (1999), when activated, the stimulators delivered bi-directional pulses of 1-ms duration, supra-maximal intensity (3V) and a frequency of 10 Hz. In this study the protocol was altered and adjusted to the present model. At the end of the
stimulation, absorbable sutures (VICRYL 4/0) were used to close the incision and 0.3ml of an analgesic (Tamgesic) was given subcutaneously. Animals regained consciousness 10-15 minutes later. Regular check-ups of the animals were made to ensure that there was no swelling of the limb due to tight casting and that the sutures were still in place. After 1, 5 and 7 days, groups of rats subjected to stretch combined with stimulation together with sham-operated controls were killed by appropriate methods in Schedule 1 of the Home Office Act using CO$_2$, in a rising concentrations and ensured death by cervical dislocation.

5.2.4. Tissue preparation

The TA muscles from both hind limbs were removed quickly, weighed under cold conditions and cut into three parts transversely. One part was taken from the tendon site and the other from the mid-belly region covered in cryo-preservative (Tissue-Tek II O.C.T Compound) quickly-frozen by immersion in isopentane that had been cooled to liquid nitrogen and stored at -70°C until further histochemical and immunohistochemical analysis. The rest of the TA muscle was distributed equally and packed into 1.5ml eppendorf tubes directly frozen in liquid nitrogen and stored at -70°C for total RNA isolation.

5.2.5. Total RNA isolation

The single-step method of RNA isolation using acid guanidinium thiocyanate-phenol-chloroform after Chomczynski and Sacchi (1987) was used to isolate total RNA as described in section 2.4 in General Materials and Methods in Chapter II.

5.2.6. Measurement of total RNA concentration

Total RNA was measured as described in section 2.5 in General Materials and Methods in Chapter II.
5.2.7. Electrophoresis of RNA
Analysis of the RNA extracted from stretched and stimulated, sham controls and normal muscle was performed following the same protocol as described in section 2.6 in General Materials and Methods in Chapter II.

5.2.8. Primers for cDNA synthesis and PCR
The same primers were used as described in section 2.7 in General Materials and Methods in Chapter II.

5.2.9. First-strand cDNA synthesis
First strand cDNA synthesis from RNA was followed according to the same protocol described in section 2.8 in General Materials and Methods in Chapter II.

5.2.10. RT-PCR
cDNA samples were used in PCR reaction using the appropriate primers for the gene of interest and this was performed as explained in section 2.9 in General Materials and Methods in Chapter II.

5.2.11. Cloning of PCR products
Cloning of PCR products was carried out in order to analyse the sequencing of the products and to further construct internal standards for the Real-Time PCR in the Light Cycler for quantification of the unknown samples. The method is described in section 2.10 in General Materials and Methods in Chapter II.

5.2.12. Real-Time RT-PCR using DNA SYBR Green I detection method
Quantification of cDNA samples from stretched and stimulated TA muscle, sham controls and normal TA muscle was performed according to the protocols described in 2.11 in General Materials and Methods in Chapter II.
A duplicate number of runs were performed for each sample and the mean of the values was used for further statistical analysis.

5.2.13. Computer-based analyses
Analysis of sequences was performed as described in section 2.12 in General Materials and Methods in Chapter II.

5.2.14. In-situ Hybridisation probes and protocol

A. Specimen preparation: 10µm transverse sections of the TA mid-belly from 1, 5 and 7 day samples applied on super-frost premium plus-coated glass slides (BDH) were used, as described in section 6.2.1 of Materials and Methods in chapter VI. The sections were fixed in DEPC-treated PBS containing 4% paraformaldehyde pH 7.5 for 5 minutes at 4°C, washed with DEPC-treated PBS pH 7.4 and stored at -70°C until further processing.

B. Probe preparation: Primers were synthesized (Sigma-Genosys) using T7 and SP6 promoter sequences with part of the MGF 52bp sequence from exon 5 of IGF-I, for each primer. The sense (forward) primer used was SP6 + MGF part of sequence:

5’TGATTTAGGTGACACTATAGAATCTAGATCCCAGCCCCTATCGACACA3’

and the antisense (reverse) primer used was T7 + MGF part of sequence:

5’ TGTAATACGACTCAGATAGGGAAGCTTCTTTCTTCTTCTTGCAGC 3’.

RT-PCR was performed in order to amplify MGF flanked by T7 and SP6 promoter sequences. 1µl of purified MGF plasmid already prepared as described in section 3.2.11, was amplified using the primers above in 2x50 µl reactions, as described in section 3.2.10 in Methods and Materials in chapter III. Amplification was carried out by 35 cycles of denaturation at 94°C for 1 minute followed by annealing at 60°C for 1 minute and elongation at 72°C for 1 minute. Electrophoresis of 2µl PCR products on a 1.2% agarose gel was performed to check the size of the bands (figure 5.1). Following electrophoresis, PCR products were purified (Wizard PCR Preps DNA purification, Promega) and the concentration was determined by its absorbency at 260nm.
Figure 5.1. Electrophoresis on a 1.2% agarose gel of MGF purified plasmid flanked by T7 and SP6 promoter sequences, following PCR amplification. Amplification was carried out with 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and elongation at 72°C for 1 minute. The picture shows PCR products at around 110bp, obtained using T7 and SP6 primers with part of MGF sequence included: M=100bp; ladder; lane 1 and 2 PCR products.

C. RNA labelling with DIG-UTP by in vitro transcription with SP6 and T7 RNA polymerases: PCR-fragments with RNA polymerase promoters ligated at their 5’ terminus act as templates for transcription. DIG-11-UTP is incorporated by SP6, T7 at approximately every 20-25th nucleotide of the transcript. The standard labelling reactions for SP6 (sense) and T7 (antisense) digoxigenin-labelled RNA probes accordingly, were as follows: to a microfuge tube on ice the following were added: 200ng of PCR product, 2μl of 10xDIG RNA Labelling mix, 2μl of 10x transcription buffer (400mM Tris-HCl, pH 8.0; 60mM MgCl₂, 100mM DTT, 20mM spermidin), sterile RNase-free water to a final volume of 18 μl and 2 μl RNA polymerase (SP6 or T7 respectively). Following a brief mix and centrifuge, the reactions were incubated for 2 hours at 37°C. The reactions were stopped by adding 2 μl 0.2M EDTA-solution, pH 8.0 on ice. The protocol and reagents used were by Boehringer Mannheim.
D. In-situ hybridisation standard procedure (by Boehringer Mannheim):

Pre-hybridisation:

- Sections were washed 2x5 minutes with DEPC-treated PBS, pH 7.4 and then washed further 2x5 minutes with DEPC-treated PBS containing 100mM glycine.
- Sections were treated for 15 minutes with DEPC-treated PBS containing 0.3% Triton-X-100 and then washed 2x5 minutes with DEPC-treated PBS.
- Permeabilization of sections was performed for 30 minutes at 37°C with TE buffer (100mM Tris-HCl, 50mM EDTA, pH 8.0) containing 1µg/ml RNase-free Proteinase K (Sigma).
- Sections were post-fixed for 5 minutes at 4°C with DEPC-treated PBS containing 4% PFA and washed 2x5 minutes with DEPC-treated PBS.
- To acetylate sections, slides were placed in containers on a rocking platform and incubated with 0.1M triethanolamine (TEA) buffer (Sigma), pH 8.0 containing 0.25% (v/v) acetic anhydride for 2x5 minutes. Acetic anhydride (Sigma) is highly unstable so it was added just before incubation.
- Sections were incubated at 37°C for an hour with pre-hybridisation buffer (4xSSC (1xSSC= 150mM NaCl, 15mM sodium citrate, pH 7.2- Sigma) containing 50% v/v deionised formamide (Sigma)).
- Pre-hybridisation buffer was drained from the slides and hybridisation buffer was applied containing 10µg per 100ml of hybridisation solution of the DIG-labelled SP6 and T7 RNA probes. Sections were covered with coverslips (BDH) and incubated at 42°C overnight in a humid chamber.

Hybridisation buffer: 40% deionised formamide, 10% dextran sulfate, 1x Denhardt’s solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 10mg.ml RNase-free bovine serum albumin), 4x SSC, 10mM DTT, 1mg/ml yeast tRNA and 1mg/ml denatured salmon sperm DNA that is added shortly before hybridisation. All reagents were provided by Sigma.
Post-hybridisation:
- Coverslips were removed by immersing the slides in 2xSSC for 5-10 minutes in different containers for the two RNA probes.
- Sections were washed in a shaking water bath at 37°C 2x15 minutes with 2xSSC and 2x15 minutes with 1xSSC.
- To digest any single-stranded (unbound) RNA probe, sections were incubated for 30 minutes at 37°C in NTE buffer (500mM NaCl, 10mM Tris, 1mM EDTA, pH 8.0) containing 20μg/ml RNase A (Sigma).
- Sections were washed 2x30 minutes in a shaking water bath at 37°C with 0.1xSSC.

Immunological Detection:
- Sections were washed 2x10 minutes in a shaking platform with Buffer 1 (100mM Tris-HCl, pH7.5 and 150mM NaCl).
- Sections were incubated for 30 minutes with blocking solution (Buffer 1 containing 0.1% Triton X-100 and 2% normal sheep serum (Sigma)).
- The Blocking solution was taken off and sections were incubated for 2 hours in a humid chamber with Buffer 1 containing 0.1% Triton X-100, 1% normal sheep serum and 1 in 500 dilution of sheep anti-DIG-alkaline phosphatase (Fab fragments) (Boehringer Mannheim).
- Sections were washed 2x10 minutes with Buffer 1 in a shaking platform.
- They were incubated for 10 minutes with Buffer 2 (100mM Tris-HCl, pH 9.5, 100mM NaCl and 50mM MgCl₂).
- Sections were covered with colour solution containing 10ml of Buffer 2, 45μl nitroblue tetrazolium (NBT), 35μl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP or X-phosphate), both by Boehringer Mannheim and 1mM levamisole (Sigma) and incubated for 6 hours (following optimisation) in a humid chamber in the dark.
• The colour reaction was stopped by incubating the slides in Buffer 3 (10mM Tris-HCl, pH8.1 and 1mM EDTA) and then washed in distilled water. Sections were mounted with an aqueous mounting solution.

All solutions were prepared with DEPC-treated distilled water (0.1% DEPC, Sigma). Different glassware was used for pre- and post-hybridisation steps and baked at 180°C to avoid RNase contamination. Gloves were used throughout the procedure.

Controls that gave negative results were: 1) tissue mRNA was digested with RNase prior to in-situ hybridisation, 2) hybridisation was also performed with SP6 sense probe and 3) the anti-DIG antibody was omitted.
5.3. Results

5.3.1. RNA extraction of muscle samples and first strand cDNA synthesis

Total RNA was extracted from stretched and stimulated, sham-operated and normal controls and their contralateral rat TA muscles. The integrity of RNA was confirmed by running a 1.2% agarose formaldehyde gel and the results are shown in figure 5.3.1.

![Figure 5.3.1](image)

**Figure 5.3.1.** Electrophoresis of total RNA extracted from rat TA muscle to check the integrity of the RNA samples. Lanes 1-4, one day stretched and stimulated (s/s) samples; lanes 5-8, five day s/s samples; lanes 9-12, seven day s/s samples; lanes 13-14, sham control samples; lanes 15-16, normal control samples. In cases like sample 10 that didn’t show any ribosomal bands the samples were re-extracted and run again before the absorbance was measured and used further.

First strand cDNA was synthesised from these RNAs. RT-PCR was performed on IGF-IeA, MGF, IGFBP-4, IGFBP-5, M-cad and MyoD transcripts to check expression in stretched and stimulated TA muscle in relation to the control groups.

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5.3.2. Sequence analyses

Sequencing of the cloned PCR products for each different transcript, indicated homologies to existing sequences through BLAST database as described in section 3.3.2 of chapter III. Following confirmation of sequencing analysis, RT-PCR was performed again and the bands were excised and together with their respective remaining PCR products were purified (Wizard PCR Purification kit, Promega), cloned into PGEM-T vector and used as standards for quantification in Real-Time PCR.

5.3.3. Real-Time RT-PCR analysis

Serial dilutions in nuclease-free water from the original concentration of the dsDNA standards were prepared. Optimisation of MgCl$_2$ and annealing temperature on the standards was also performed in the Roche Light Cycler for each transcript, to allow measurement of the unknown samples. The specificity of the PCR product identity of each sample was confirmed by its amplification under identical conditions to the standard. Specificity of the desired PCR products was documented with melting curve analysis (Light Cycler Software 3.39). The melting temperatures of the products are template dependent (Table 4 of sub-section 3.3.3 in Chapter III).

GAPDH gene transcript levels

GAPDH expression was tested in all experimental and control TA muscle samples following 1, 5 and 7 days post stretch and stimulation (s/s). As GAPDH levels remained constant in response to bupivacaine injection, described in chapter 3, this was also the case between the left and right TA muscles in normal and 1day (s/s) groups. Although similar GAPDH levels were observed in both hind limbs in the sham control group, they were significantly ($P<0.001$) lower (21%) than those in the normal group.

However, GAPDH mRNA levels at 5day showed a significant ($**P<0.001$) 28% decrease compared to those at 1day s/s group and 32% to normal muscle. Additionally, GAPDH levels of the experimental muscle at 7 days were insignificantly lower (16%) to 1day GAPDH levels, whereas those of the contralateral muscle at 7 days were significantly ($**P<0.001$) lower (38%) than those at 1day. Significant increase (26%)
(**P<0.001) of GAPDH levels was also observed in the experimental muscle at 5 days compared to its contralateral muscle (figure 5.3.2).

One possible explanation for these changes may be that GAPDH was susceptible to the protocol induced. Additionally, the measurement of RNA concentration from each sample may not have been as accurate therefore, unequal volumes of RNA were used for first strand cDNA synthesis and RT-PCR. Nevertheless, electrophoresis of equal volumes (1μg) of cDNA samples on 1.2% agarose gel with the intention of checking the integrity of first strand cDNA synthesis, did not illustrate any intensity differences amongst them, as can be seen in figure 5.3.3. Due to all these parameters, transcript values from Real-Time PCR could not be normalised against GAPDH.

**Figure 5.3.2.** Quantification of GAPDH mRNA levels during 1, 5 and 7 days post stretch and stimulation of TA muscle, the sham-operated control group and normal muscle. There were significant (**P<0.001) changes amongst 1d s/s group and normal group with the 5day and sham groups for both experimental and contralateral muscles. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. For all groups n = 6. Values are expressed as mean ± standard error of the mean (S.E.M).
Figure 5.3.3. Electrophoresis of first strand cDNA samples on 1.2% agarose gel, to check the intensity of the smears run using equal volumes (1μg) from each sample. Lane 1, 1Kb marker; lanes 2-5, one day stretched and stimulated (s/s) samples; lanes 6-9, five day s/s samples; lanes 10-13, seven day s/s samples; lanes 14-15 sham control samples; lanes 16-17 normal control samples.

5.3.3.1. Quantification of IGF-I isoforms in stretched and stimulated TA muscle
IGF-IEa and MGF transcript levels were quantified in response to s/s, after 1, 5 and 7 days. Both transcript levels in the stretched and stimulated muscle were compared to their contralateral TA muscle, the sham-operated and normal control groups.

IGF-IEa and MGF transcripts were expressed in all three different groups, as shown in figures 5.3.4 and 5.3.5. IGF-IEa levels of s/s TA showed an 82%, 95% and 97% significant increase (**p<0.001) at 1, 5 and 7 days respectively, compared to control groups levels. MGF mRNA levels of s/s TA exhibited significant increases of 92%, 64% and 46% at 1, 5 and 7 days respectively, in comparison to those of the control groups' i.e. the sham-operated and the normal control group. While IGF-IEa expression was up regulated from day 1 and peaked at day 7-post s/s, MGF expression showed a marked increase at day 1 and decreased significantly (80%) thereafter. Nevertheless, IGF-IEa levels were nearly four times higher than MGF levels at day 1, 22-fold higher at day 5 and 132-fold at day 7. At 5 and 7 days post s/s IGF-IEa levels of the contralateral TA muscle exhibited a 8-fold increase in comparison to those at 1day. In contrast, MGF levels in the contralateral muscle remained similar at all time points. Sham-operated and normal control groups showed no significant changes in any of the two transcripts.
Figure 5.3.4. Quantification of IGF-I\(\text{Ea}\) levels in picograms (pg) per µg of total RNA per sample: there was a significant increase (**\(P<0.001\)) in IGF-I\(\text{Ea}\) levels in stretched and stimulated muscle compared to the sham-operated and normal control muscle group. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. For all groups \(n = 6\). Values are expressed as mean ± standard error of the mean (S.E.M).

Figure 5.3.5. Quantification of MGF levels in pg, per µg of total RNA per sample. There was a significant increase (**\(P<0.001\)) in MGF levels in the stretched and stimulated muscle in relation to the sham and normal control groups. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. For all groups \(n = 6\). Values are expressed as mean ± standard error of the mean (S.E.M).
5.3.3.2. Expression of MyoD in response to mechanical activity

The expression of the transcription factor MyoD in response to mechanical activity was studied. The maximal expression of MyoD was at day 7 progressively increasing from day 1 (figure 5.3.6). MyoD levels were significantly higher (**P<0.001) in the stretched and stimulated TA compared to the control groups at all time points. An 88%, 91% and 93% increase was observed at 1, 5 and 7 day respectively in the s/s TA MyoD levels compared to those of the normal TA. MyoD mRNA levels of the contralateral muscles to the s/s ones and the sham controls were relatively similar at all time points, yet significantly higher (61-76%) compared to the MyoD levels of the normal muscle.

![Graph showing MyoD levels](image)

**Figure 5.3.6.** Quantification of MyoD levels in pg, per µg of total RNA per sample. There was a significant increase (**P<0.001) in MyoD levels in the stretched and stimulated TA muscle at all time points compared to the sham and normal control groups. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. For all groups n = 6. Values are expressed as mean ± standard error of the mean (S.E.M).
5.3.3.3. Expression of M-cadherin in stretched and stimulated TA muscle

M-cadherin mRNA levels of the s/s TA showed significant increase of 82% (*P<0.05), 90% and 75% (**P<0.001) compared to those from normal TA control group, as seen in figure 5.3.7. M-cadherin expression in stretched and stimulated muscle peaked at day 5 with a 49% increase (***P<0.001) from day 1 and decreased 59% (**P<0.001) at day 7, thereafter. M-cadherin levels remained similar amongst the control groups at all time points.

![Graph showing M-cadherin levels](image)

**Figure 5.3.7.** Quantification of M-cadherin levels in pg, per µg of total RNA per sample. There is a significant increase (*P<0.05) at day 1 and highly significant up-regulation (**P<0.001) in M-cad expression in the stretched and stimulated TA muscle compared to the sham control and normal groups at 5 and 7 days and. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. For all groups n = 6. Values are expressed as mean ± standard error of the mean (S.E.M).
5.3.3.4. IGFBP-5 transcript levels in response to mechanical stimulus

The expression of IGFBP-5 was significantly higher (**P<0.001) in stretched and stimulated TA muscle compared to the normal control and the sham control group at all time points (figure 5.3.8). IGFBP-5 levels of the s/s TA showed a 71%, 70% and 53% increase at 1, 5 and 7 days respectively compared to the normal control group’s levels. In the challenged TA muscle, IGFBP-5 levels were maximal at 1 day post s/s but showed non-significant differences from those at 5 days and levels decreased (29%) at day 7. However, IGFBP-5 mRNA of the contralateral TA to the experimental ones seemed to increase after one day, reaching similar insignificant values to those of the stretched and stimulated at day 7. IGFBP-5 expression showed a similar trend as the one seen for MGF at figure 5.3.5. IGFBP-5 levels of the sham and normal control groups remained relatively lower and constant.

Figure 5.3.8. Quantification of IGFBP-5 levels in pg, per μg of total RNA per sample. There was a significant increase (**P<0.001) in IGFBP-5 levels in the stretched and stimulated TA muscle compared to the sham control and normal groups. There was also an increase in IGFBP-5 levels in the contralateral TA to the stretched/stimulated at days 5 and 7. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. For all groups n = 6. Values are expressed as mean ± standard error of the mean (S.E.M).
5.3.3.5. Detection of IGFBP-4 transcript on s/s muscle with conventional RT-PCR

IGFBP-4 mRNA was amplified on stretched and stimulated and their contralateral TA muscles for all time points. Amplification was carried out at 62°C using 1μl of cDNA from each sample and PCR products were shown at the expected size, as seen in figure 5.3.9. Semi-quantitative analysis can be drawn from band intensity which showed that IGFBP-4 mRNA of experimental TA was up regulated, compared to that shown from their contralateral at specific days. However, there was some inter-variation in band intensity amongst experimental or contralateral group samples. Some could not be amplified, maybe due to the sensitivity of RT-PCR method or the amount of cDNA was not enough to detect any IGFBP-4.

Figure 5.3.9. PCR amplification of IGFBP-4 transcript in non- and stretched and stimulated muscle. Amplification was carried out with 35 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute and elongation at 72°C using 1μl of cDNA. Electrophoresis was carried out on a 2% agarose gel. PCR products were at the expected size, around 240 bp. Lane 1, 100bp ladder; lane 2-3 and 5, contralateral TA at day 5; lane 4 and 6, 5 days s/s TA; lanes 7-9, 1 day s/s TA; lanes 10-11, contralateral muscle at 1 day; lane 12, sham experimental muscle; lanes 13-14, contralateral muscle at day 7; lanes 15-17, 7 day stretched and stimulated muscle; lane 18, no template.
Conditions, i.e. annealing temperature and MgCl₂ concentration, for Real-Time PCR had to be optimised following conventional RT-PCR. In spite of this, the same primers that were used as in the bupivacaine study, could not only amplify IGFBP-4 transcript at the correct melting temperature but several other products at lower temperatures, as shown in melting curve analysis in figure 5.3.10 and electrophoresis of the PCR products in figure 5.3.11. Modification of several parameters, like trying higher or lower annealing temperature with less time to amplify for more specificity were tried and different IGFBP-4 primers were used, yet we were unable to amplify a single product and to quantify subsequently.

![Figure 5.3.10. Melting Curves of specific and non-specific IGFBP-4 PCR products, amplified at 61°C. Melting temperature of IGFP-4 PCR products was at 91.74°C (blue line) and melting temperatures lower, 87°C (green), 82°C(yellow) and 78°C (red) were non-specific products.](image-url)
Figure 5.3.11. Confirmation of IGFBP-4 Light Cycler PCR products after 40 cycles, amplified at 61°C. Lane 1, 100bp ladder; lane 2-4, serial dilutions of IGFBP-4 plasmid standards (1pg, 0.1pg, 0.001pg) at the expected size, 240bp; lane 5, 1day s/s TA showing a band lower than the 240 bp; lane 6, 5 day s/s TA; lane 7, 7day s/s TA; lane 8, contralateral muscle to experimental at day 7.

5.3.4. Localization of MGF mRNA in s/s muscle

Expression of MGF mRNA within the muscle fibres studied by in situ hybridisation using antisense and sense RNA probe is shown in figure 5.3.12. In situ hybridisation results confirmed the Real Time PCR data that MGF is produced in response to a mechanical stimulus at the muscle fibre level. This work showed that MGF gene expression was confined in the muscle fibres themselves, even though signal was also detected in satellite cells (pointed by the arrowhead, c). In the transverse sections at 1 and 5-day post stretch and stimulation, the MGF message was localised to normal muscle fibres but tended to be expressed strongly in the smaller regenerating fibres too as can be seen in the histological analysis in chapter 6. However, the preliminary results from the in situ hybridisation study showed that with the use of this model, the up regulation of MGF expression occurred in undamaged fibres and not in the damaged area as seen in day 5 (chapter 6). Due to the high background, the signal of MGF message in the 7-day stretched and stimulated TA was faint and indistinct in the few muscle fibres present and in the contralateral TA muscles at the three time points.

All the controls that were used, i.e. 1) tissue mRNA digested with RNase prior to in-situ hybridisation, 2) hybridisation performed with SP6 sense probe and 3) anti-DIG antibody omitted, gave negative results.
Figure 5.3.12. Localisation of MGF mRNA in the mid-belly region of s/s TA muscle after 1 day (a) and 5 day (c). The sense RNA probe was used on the same TA muscles at 1 (b) and 5 day (d) respectively and gave negative results. Scale bar= 50μm.
5.4. Discussion
In this study, the acute effects of the mechanical stimulus on the temporal expression of the two IGF-I isoforms on a fast-twitch rat skeletal muscle were investigated.

Evaluation of the applied model
The method of stretch and stimulation employed was chosen as the most appropriate type of mechanical signal to what happens in real life. A similar system by McKoy et al. (1999) has previously demonstrated that when stretch and stimulation was induced on rabbit EDL muscle, an even greater increase of MGF and IGF-IEa mRNA was expressed than that shown by stretch or stimulation alone. However, in this study the time course of their expression was not measured.

Most of the studies using stretch combined with low-frequency stimulation (usually at 10Hz) have been carried out on rabbits where stimulation of the sciatic nerve or peroneal nerve of the hind leg was delivered for short time periods. This was done through wires implanted on the animal subcutaneously, using a small stimulator circuit between the shoulders. In an animal model like the rat when not under anaesthetic, it is difficult to ensure that wires protruding out of the skin or a plaster cast around the hind leg will stay in place. In contrast to the rabbit, rats constantly move around their cage and other rats in the same cage try to take off anything that obstructs their hind legs i.e. fibreglass cast in the present model. In order to restrain the animals from chewing the cast, picric acid was sprayed on the surface of the fibreglass. In addition, we carried out some preliminary experiments on low-frequency stimulation protocol that involved stimulating the peroneal nerve of the hind leg from 4 to 8 hours continuously, while the animal was under anaesthetic, however the mortality rate was quite high possibly due to the long hours of the anaesthesia.

Although every effort was made to keep inter-animal variation minimal, variation seen in the results within the same group in both experimental and control samples could reflect the differences in the tolerance levels of individual animals to the experimental procedure. Although none of the animals appeared visibly distressed at any point, their levels of tolerance may have diverged as the anaesthetic wore off. Also, there was clearly
increased use of the non-treated leg and this presumably explains the increase in IGF-IEa and IGFBP-5 expression in the contralateral muscle.

In this study, the outcome of stimulation at 30 Hz for an hour only in contrast to the constant low-frequency stimulation (10Hz) combined with continuous stretch in the other studies would probably be not as efficient; yet the combination of stretch and stimulation resulted in obvious morphological damage, as described in chapter 6.

Some well characterized protocols appropriate for a rat animal model have been established, i.e. the high-frequency electrical stimulation (HFES) shown to induce skeletal muscle hypertrophy (Baar and Esser, 1999) and the low-frequency electrical stimulation (LFES) shown to induce endurance-like adaptations (Patel et al., 1998). However, these studies did not include muscle stretch combined with electrical stimulation.

**Reliability on housekeeping genes used for normalization**

RT-PCR-specific errors in the quantification of mRNA transcripts are compounded by variation in the amount of starting material between samples. The accepted method for minimising these errors and correcting sample-to-sample variation is to amplify simultaneously with the target, a cellular RNA that serves as an internal reference against which other RNA values can be normalised (Karge et al., 1998). The ideal internal standard should be expressed at a constant level in different tissues, at all stages of development and should be unaffected by the experimental treatment.

GAPDH is a ubiquitously expressed, moderately abundant message and has been used as a control for quantitative RT-PCR analysis. In some experimental systems its expression is apparently constant at different times following experimental manipulation (Edwards and Denhardt, 1985; Winer et al., 1999). This was also demonstrated in the bupivacaine study, in chapter 3. However, other reports state that GAPDH concentrations vary significantly with developmental stage (Puissant et al., 1994) and during the cell cycle (Mansur et al., 1993). Growth hormone (Freyschuss et al., 1994), oxidative stress (Ito et al., 1996), hypoxia (Graven et al., 1994) have all been shown to activate its transcription.

It has been shown that denervation (Saitoh et al., 1993), chronic stimulation (Cresci et
al., 1996) and overload-induced hypertrophy (Tsika et al., 1995) cause GAPDH mRNA levels in skeletal muscles to decline, whereas hind-limb suspension results in increases (McCarthy et al., 1997). However, the occurrence of these changes may depend upon the muscle being studied. For example, Mozdiak and colleagues (1998) reported no change in GAPDH mRNA levels in overloaded rat soleus muscle, whereas Tsika et al., (1995) reported a 200% decrease in GAPDH mRNA levels in overloaded rat plantaris muscle. Variations in GAPDH levels were found between time points in this study, therefore GAPDH could not be used as a control gene.

Other housekeeping genes used for normalization include β-actin and rRNA. Transcription levels of β-actin that encodes a ubiquitous cytoskeleton protein, has been shown to vary widely in response to experimental manipulation (Carlyle et al., 1996; Foss et al., 1998; McKoy et al., 1999). In addition, the presence of pseudogenes interferes with the interpretation of results (Raff et al., 1997) and primers, commonly used for detecting β-actin mRNA, amplify DNA also (Dakhama et al., 1996). The rRNA, which constitutes 85% of total cellular RNA, is the less likely to vary under conditions that affect mRNA expression. However, rRNA transcription can be affected by biological factors and drugs (Spanakis, 1993) and levels of transcription between samples taken from different individuals can be variable. In view of the model applied here, the internal standards described above, were proved to be unsuitable as well.

**Temporal expression pattern of IGF-I system and its role in the applied model**

This study showed that in muscle, IGF-IEa and MGF transcripts have different kinetics and that their expression was up regulated in response to a mechanical stimulus. MGF expression rapidly peaked at 1 day, whilst IGF-IEa peaked at 7 days after muscle damage was induced. Additionally, increased expression of IGF-IEa was observed in the contralateral muscle peaking at 5 days and staying constant thereafter. The continuous use of this limb could point towards the induction and increase of this splice variant. The difference in expression kinetics therefore, indicates a mechanism for using IGF-I in a different manner according to certain conditions.

Real-Time PCR analysis demonstrated considerable differences in IGF-IEa and MGF mRNA values of a 130-fold increase of IGF-IEa to MGF after 7 days of stretch and
stimulation. These changes were also present in the bupivacaine study since the same technical parameters were applied and possible explanations to these differences are described i.e. use of random hexamers for IGF-IEa cDNA synthesis and half-life of MGF. The earlier induction of MGF expression that peaked at day 1 is noteworthy, compared to the existing data of significantly increased MGF levels 3 days after surgical overload (Owino et al., 2001). This could be attributed to a more sizeable effect that the present protocol generated, compared to the tendon ablation procedure. The Real-Time PCR data agree with the preliminary in-situ results, showing that the MGF isoform is expressed in the muscle fibres themselves and not in non-muscle cells involved in the damage process. However, the MGF message seemed to be localised in the undamaged muscle fibres and in some small regenerating fibres. Yang et al., (1996) also showed localisation of the two IGF-I transcripts (7.5kb and 1.2kb mRNA) in small muscle fibres after stretch that also expressed the neonatal MyHC.

In contrast, IGF-IEa expression was up regulated but more slowly with significant increase at 5 days and maximal values at 7 days post stretch and stimulation. Elevated IGF-IEa mRNA was also demonstrated five days after surgical overload in a recent study (Owino et al., 2001). The results described in this study are in accordance with the elevated IGF-I expression at 3 and 7 days of overloading in normal and hypophysectomized rats (Addams and Haddad, 1996). Furthermore, increase in muscle IGF-I mRNA has been observed during work-induced compensatory hypertrophy (DeVol et al., 1990) and passive stretch-induced muscle growth in chicken (Czerwinski et al., 1994). Furthermore, the elevated IGF-IEa levels shown in the contralateral muscle to the stretched and stimulated muscles at 5 and 7 days are striking, which could be explained by the compensatory work that these muscles did due to the hind limb immobilization. On the contrary, MGF levels in the contralateral muscle remained relatively similar throughout the procedure. Therefore, MGF is expressed in normal and in contralateral to experimental muscle but its up-regulation was observed only in the stretched and stimulated muscle, as opposed to the IGF-IEa levels that was up-regulated in both non- and experimental muscle. This suggests that MGF has a role as a transient and damage-sensitive local growth factor.
Role of IGF-Ie and MGF in satellite cell expression and MyoD in response to stretch and stimulation

Mature myofibre nuclei are thought to be mitotically inactive (Stockdale and Holtzer, 1961) therefore, increased DNA content in skeletal muscle suggests satellite cell activation. Other studies point out that satellite cell proliferation may be required for compensatory hypertrophy to proceed (Schiaffino et al., 1976; Rosenblatt et al., 1994; Allen, et al., 1995). Because up regulation of myogenic regulatory factors is temporally consistent with satellite cell proliferation, it has been concluded that MRFs increases occur in the nuclei of activated satellite cells or newly formed myotubes (Koishi et al., 1995; Lowe et al., 1998). In the present study, we imposed a mechanical stimulus on rat skeletal muscle in order to establish the temporal relationship amongst M-cadherin, expressed by satellite cells, MyoD message that is involved in the proliferative programme, with the expression of IGF-I isoforms. IGF-I has been shown to stimulate satellite cell proliferation and to increase myonuclei number and myofibre size in tissue culture (Vandenburgh et al., 1991). M-cadherin mRNA was significantly increased up to 5 days post stretch/stimulation and then decreased to levels lower than those at day 1. Muscle RNA activity and protein synthesis rates are elevated within 1 day following a hypertrophy stimulus (Laurent et al., 1978) although satellite cells would not be activated quickly enough to account for these early increases.

MyoD expression was increasing until day 7 in response to the mechanical stimulus employed in this study. In contrast, surgical overload demonstrated maximal MyoD expression after 1 day (Owino et al. 2001). A recent study by Beauchamp et al. (2000) showed that in differentiating cultures after 48hr, the vast majority of CD34^"^ and Myf5^"^ satellite cells were strongly MyoD^"^ suggesting that most adult skeletal muscle satellite cells are quiescent but express these markers on activation. Sporadic expression of MyoD has been reported and attributed to satellite cell activation, presumably in response to local stimuli (Grounds et al., 1992; Creuzet et al., 1998). Therefore, the continuously increasing levels of MyoD up to 7 days in conjunction with the maximal levels of M-cadherin at 5 days could be explained by the delayed response of satellite cell
activation in response to the stimulus induced here. The concurrent temporal expression patterns of IGF-IEa and MyoD in this study indicate that IGF-IEa may be working via the induction of transcription factors and is required for the mechanism of activation and differentiation events necessary for satellite cells in response to a local stimulus and damage.

On the other hand, the MGF transcript is expressed as an early acute response prior to the rise in IGF-IEa, when damage is induced. Although both IGF-IEa and MGF levels were both elevated at 1 day, it might be that the response and activation of satellite cells to the mechanical stimulus is related to MGF induction.

Regulation of IGF-IEa and MGF by IGFBP-5

As discussed earlier, muscle development and maintenance involves systemic as well as autocrine isoforms of IGF-I. Therefore, it was of interest to see if any of the IGF binding proteins was up regulated following a mechanical stimulus, as the one employed in this study. Previous reports have shown that the IGFBPs are expressed to different extents in different muscle cell lines and changes in their expression correlate with those of the IGFs during myoblast proliferation and fusion (Ewton and Florini, 1995).

In the present work, we were concerned with investigating the expression of IGFBP-5 and IGFBP-4. IGFBP-5 expression peaked at day 1 and was down regulated subsequently. The early induction of IGFBP-5 transcript seemed to be concomittant with that of MGF but inversely related to IGF-IEa post stretch and stimulation. IGFBP-5 was reported to be up-regulated following denervation in the gastrocnemius muscle (Bayol et al., 2000) and following rat hind-limb unloading (atrophy) conditions (Awede et al., 1999). This up-regulation is compatible with its potentiating effects described in vitro (Jones and Clemmons, 1995; Florini et al., 1996). In view of the possible inhibitory effect of IGFBP-5 on myoblast proliferation induced by IGF-I (Ewton et al., 1998), its early onset in our study is of intriguing interest in relation to the acute MGF response to the mechanical activity, while its succeeding decrease could facilitate the continuous up regulation of IGF-IEa.

On the other hand, even though amplification and detection of IGFBP-4 PCR products were successful with conventional RT-PCR, we were unable to amplify a single product
from the same samples using Real-Time PCR. The same primers that were used in the bupivacaine study were also used in this. Moreover, the design of different primers yielded non-specific amplification. A number of parameters could account for this: 1) something in the preparation of cDNA synthesis could have hampered the amplification in this highly sensitive amplification method, 2) there was a substantial kit-to-kit variation that generated different results from the same samples, 3) amplification efficiency depend not only on the priming efficiency of the primers but on the position of the reaction tubes in the thermocycler and on the melting behaviour of the amplicon’s genomic vicinity (Wilhelm et al., 2000), 4) the constant freeze-thaw of the samples could have hindered their amplification, however when fresh new cDNA samples were prepared the same results were seen, 5) there could be an alternatively-spliced isoform in IGFBP-4 amplification in response to stretch and stimulation since a band close to the real product could be detected when the amplified products were run under electrophoresis. A qualitative analysis from the conventional RT-PCR could show an increase of IGFBP-4 expression at day 5 compared to 1 and 7 days post stretch and stimulation but no conclusive data can be drawn from this.

**Conclusions**

The results of the present study support the idea that IGF-IIEa and MGF have integrated yet distinct functional roles in response to damage, as presented with the damage model in chapter 3. The present findings support the idea that the temporal appearance of the MGF isoform is acute and suggests a potential role as a damage-sensitive growth factor in activating the satellite cell compartment. On the other hand, the continuous increase of IGF-IIEa expression may indicate a role in muscle mass regulation and in maintenance of the satellite cell compartment in response to a mechanical stimulus. The early expression of the MGF isoform seems to be facilitated by the up-regulation of IGFBP-5 and makes way for the more sustained effects of IGF-IIEa on muscle reparation by localizing it in the tissue. The ability to unmask the mechanism involved in the regulation of the local IGF-I system in damaged muscle, will serve in understanding better the processes involved in the restoration of skeletal muscle from injuries and atrophy, especially in elderly and people with muscle diseases.

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CHAPTER VI

Localization of general IGF-I protein and IGF Binding Proteins in stretched and stimulated rat skeletal muscle
6.1. Introduction

Immunohistochemistry relies upon the specific binding of an antibody to the antigen in the tissue, the reaction being localized with respect to cell structure by attaching a microscopically dense marker to the antigen-antibody complex. Unfortunately, it is possible to achieve 'credible' non-specific labelling on a specimen and the absence of immunolabelling does not always signify the absence of antigen. Therefore, methodological controls are essential to assure the quality of the immunolabelling technique. Methodological non-specificity can be assessed by immunolabelling a specimen where the antigen is known to be absent or immunolabelling the test specimen with a pre-immune or negative serum. Additionally, the antibody should be pre-adsorbed with excess antigen and used for immunolabelling the test specimen.

Immunofluorescence on fixed samples forms a vital part of most investigations into protein function, since it provides an idea of the location of a protein within the cell. In addition, immunofluorescence can tell us whether this location varies through the cell cycle or development and whether this is consistent in cells from other tissues or species. It should be stressed that selecting the right parameters and conditions will often make the difference between success and failure in obtaining a valid result. However, it could be that a particular antibody will never work for immunofluorescence.

In the present study we applied histological, immunohistochemical and immunofluorescent methods in order to investigate the location and function of the proteins of interest. Although localization of IGF-I protein has been extensively investigated in regeneration studies, not enough data exist on the whereabouts of IGF-I protein expressed in muscle under conditions of hind-limb immobilization and stimulation combined, using however a general IGF-I antibody. The IGFBPs have been examined at a protein level in smooth muscle, colon tissue, osteoblasts and pancreas. Therefore it was felt important to look into the localization of IGFBP-4 and IGFBP-5 proteins expressed in stretched skeletal muscle in conjunction with the emergence of
IGF-I peptide on the same muscle and their role in relation to time following the damage induced.

Furthermore, protein analysis as a follow-up to the mRNA data presented in the previous chapter, would enable us to clarify the overall functions and behavioural role of IGF-I and its binding proteins in response to damage that the model applied induced.

6.2. Materials and Methods

The experimental protocol of stretched and stimulated rat TA muscle and all the control groups are discussed and described in Chapter 5, Materials and Methods, section 5.2.1 and 5.2.3.

6.2.1. Tissue preparation

The TA from both limbs was removed and weighed quickly before being cut accordingly, for histological and immunohistochemical analysis, as described in section 5.2.4 of chapter 5. Serial 10-μm-thick transverse sections of the tendon and mid-belly muscle sections were cut on a cryostat microtome (LEICA CM3050) at -20°C and applied to super-frost premium plus-coated glass slides (BDH). All sections were allowed to air-dry for at least 10 minutes and then at -70°C until further processing.

6.2.2. Morphological staining and Immunohistochemistry

Mayer’s Haematoxylin was applied in order to stain muscle nuclei. IGF-I, IGFBP-4 and IGFBP-5 antibodies were supplied by Autogen Bioclear UK and SantaCruz. Embryonic MyHC antibody was a gift from A.F.M Moorman (Anatomy and Embryology Department, AMC, Amsterdam). M-cadherin antibody was kindly supplied by Professor Wernig (Institute of Neurophysiology of University of Bonn, Germany). Immunohistochemical / Immunofluorescence procedures were performed as described in sections 4.2.2 of chapter 4.

6.2.3. Image Processing and Analysis of Immuno-histochemical / -fluorescent preparations

Image analysis was performed as described in section 4.2.3 in chapter 4.
6.3. Results

6.3.1. Muscle wet weight post stretch and stimulation

*Muscle weight:* After one day of induced damage by stretch and stimulation the TA muscle weight of both experimental and contralateral muscles remained the same, as in the sham and normal control groups. Following 5 days the TA muscle wet weight of the experimental limb was significantly less heavy, 11.6% (**P<0.001), compared to the right contralateral TA and the 1 day group as seen in figure 6.3.1. Seven days following stretch and stimulation, the weight of the experimental TA muscle was 8% less heavy from the 1-day group but insignificantly heavier than that of 5 day experimental TA muscle.

![Figure 6.3.1](image)

**Figure 6.3.1.** Wet weight of both experimental and contralateral TA muscles following 1, 5 and 7 days after stretch and stimulation, sham and normal control groups. There was a significant decrease of the stretched and stimulated TA muscle at 5 days compared to its contralateral, the sham and the normal muscle groups (**P<0.001). Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. N=6. Values are expressed as means ± standard error of the mean (S.E.M).
6.3.2. Time course of morphological changes
Serial sections were stained for routine histological (H&E) and immunohistochemical (embryonic MyHC) examination for structural analysis. None of the sham control muscles and contralateral muscle to the stretched and stimulated ones showed any damage and morphological analysis was similar to the normal muscle group.

Stretching the rat hind limb in the extended position and exposing the sciatic nerve in the thigh for one hour of indirect (nerve) stimulation of the muscle exhibited a sequence of damaged and regenerating changes. One day following stretch and stimulation, the TA muscle in both tendon and mid-belly regions appeared reasonably normal with only a few small damaged areas as can be seen in the H&E stain (figure 6.3.2.1, b- arrowheads) and did not react with embryonic MyHC (figure 6.3.2.2, a).

However after 5 days, a massive inflammatory response was strongly evident in the tendon area with central myonuclei and less in the mid-belly region with some hypertrophied or normal muscle fibres that survived the damage (indicated by star, c) amongst the damaged area and some centrally located myonuclei (figure 6.3.2.1, c, d). The small fibres reacted strongly with the embryonic MyHC-330 antibody (figure 6.3.2.2, b, c) in both regions.

At 7 days, a fraction of the fibre population close to the tendon consisted of small regenerating fibres with central myonuclei (figure 6.3.2.1, e) and less in the mid-belly region. These fibres reacted with the embryonic MyHC-330 antibody (figure 6.3.2.2, d, e) whereas normal-looking muscle fibres did not react with the embryonic MyHC antibody.
Figure 6.3.2.1 Transverse sections of rat TA muscle near the tendon and mid-belly region following 1 (a-tendon, b-mid-belly) day, 5 day (c-tendon, d-mid-belly) and 7 day (e-tendon, f-mid-belly) of stretch and stimulation. A few damaged areas were seen on day 1 (b, arrowheads) and some central myonuclei next to the tendon site after 7 days (e-arrow). Mayer’s H&E. Scale bar = 50μm
Figure 6.3.2.2 Reaction of transverse sections of stretched and stimulated rat TA muscle with embryonic MyHC antibody after: (a) 1 day, (b-tendon; e-mid-belly) 5 day and (d-tendon; e-mid-belly) 7 days. Scale bar = 50μm.
6.3.3. Variation of damage following stretch and stimulation

Image analysis of a montage of H& E staining enabled the assessment of damage in response to stretch and stimulation of the hind limb at 1, 5 and 7 days. The mean percentage of damage in relation to the whole muscle fibre area was zero in all control groups.

The cross sectional area (CSA) of the tendon site section was visually smaller than the mid-belly region CSA. In general, significantly more damage was demonstrated in the muscle fibre area close to the tendon than in the mid-belly region at 5 days compared to day 7 where some damage—regenerating muscle fibres were seen near the tendon site and in comparison to some small areas of damage in the mid-belly region at day 1.

No damage was marked in the tendon site after 1 day, the mid-belly region at day 7 and the contralateral muscles at all time points.

Figure 6.3.3.1. Mean percentage of damaged-muscle fibre area in relation to the whole muscle section. Significant damage (\(** P<0.001\)) was obvious in the tendon site five days post stretch and stimulation, compared to the mid-belly site and in relation to the other time points and the contralateral group. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. N = 6. Values are expressed as mean ± standard error of the mean (S.E.M).
6.3.4. Embryonic MyHC as a marker of regeneration in response to stretch and stimulation

The presence of regenerating fibres following damage induced by the procedure used in this study could be assessed using the embryonic MyHC antibody. In relation to the qualitative microscopic view of the sections, image analysis showed a highly significant increase of regeneration in the tendon site compared to the mid-belly region at 5 days. Significant differences were apparent between 5 and 7 days also at both sites. However, there was no significant variation in the regenerating muscle fibre area amongst the tendon and mid-belly sites at 7 days. The two-way Anova revealed that there was significant interaction between the mean percentage of regenerating muscle fibre area with embryonic MyHC antibody in both sites of muscle examined and time.

![Graph showing the mean percentage of regenerating muscle fibre area stained for embryonic MyHC in relation to the whole muscle section. A highly significant increase \((**P<0.001)\) of regenerating muscle fibre area was seen in the tendon site compared to the mid-belly at 5 days and significant difference \((*P<0.05)\) in the regeneration area in the mid-belly region between 5 and 7 days. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. \(N=6\). Values are expressed as mean ± standard error of the mean (S.E.M).]

Figure 6.3.4.1
6.3.5. Localization of IGF-I, IGFBP-4 and IGFBP-5 on stretched and stimulated TA muscle

1 day following stretch and stimulation of the hind limb: (figure 6.3.5.1, a, b, c)
IGF-I immunoreactivity was observed localised only in the few damaged areas as observed in the H&E stain, shown with an arrow in figure 6.3.5.1, (a), on sections cut from the mid-belly region of the whole TA muscle. Generally, weak immunoreactivity of IGFBP-4 and IGFBP-5 was demonstrated on a few scattered muscle fibres. No specific immunoreactivity was detected in any of the antibodies in sections from the tendon site.

5 days post stretch and stimulation at the tendon region: (figure 6.3.5.2, a, b, c)
IGF-I immunoreactivity was strongly detected in a cluster of round small regenerating muscle fibres only amongst the inflammatory influx due to damage. Strong IGFBP-5 immunoreactivity was also evident in the same population of muscle fibres as IGF-I and additionally it was evidently dispersed amongst the myoblast / myotube population that the embryonic MyHC antibody have shown to stain (figure 6.3.2.2, b).
IGFBP-4 immunoreactivity was not identified in the same clusters of muscle fibres as the ones mentioned above. Generally however, IGFBP-4 showed a similar distribution as seen with IGFBP-5 immunoreactivity, distributed in small round-like muscle fibres.

5 days post stretch and stimulation at the mid-belly region: (figure 6.3.5.3, a, b, c)
No detection of IGF-I immunoreactivity could be seen in the mid-belly muscle section site. IGFBP-5 and IGFBP-4 immunoreactivity was dispersed amongst the damaged area in small round muscle fibres, similar to the IGFBP-4 and IGFBP-5 staining pattern in the tendon region.

7 days post stretch and stimulation at the tendon region: (figure 6.3.5.4, a, b, c)
A few small regenerating fibres with central nuclei around the tendon were more strongly stained for IGF-I than others. Similar IGFBP-5 staining pattern was shown on some dispersed small regenerating muscle fibres but not co-localizing with IGF-1 staining.
IGFBP-4 immunoreactivity seemed to stain peripherally around the muscle fibre, in the plasma membrane.

*7 days post stretch and stimulation at the mid-belly region:* (figure 6.3.5.5, a, b, c)

A substantial number of normal-looking muscle fibres larger than those in the tendon region were strongly stained for IGF-I in one side of the section. No co-localisation was demonstrated between IGF-I and IGFBP-5 since the muscle fibres stained for IGFBP-5 were located in a different part of the section. Some specific staining for IGFBP-4 was observed on a few muscle fibres although high background was evident.

IGF-I, IGFBP-4 and IGFBP-5 immunoreactivity could not be detected in the contralateral TA muscle to the stretched and stimulated ones, neither in the sham control and normal groups. Non-specific staining of IGFBP-4 and IGFBP-5 was present in some blood vessels.

Pre-adsorption of primary antibodies with the recombinant or blocking peptides for IGF-I, IGFBP-4 and IGFBP-5 eliminated staining that was present in the muscle fibres stained with the respective antibodies, while the staining intensity was brought down to the level of non-specific background.
Figure 6.3.5.1. Step sections from the mid-belly part of stretched and stimulated rat TA muscle at 1 day stained for IGF-1 (a), IGFBP-5 (b) and IGFBP-4 (c). A few scattered muscle fibres were stained for IGF-1, shown with arrows, in small areas of damage where nuclei were present with the H&E stain. Similar staining pattern was seen with the IGFBP-4 antibody but not with IGFBP-5. Scale bar = 50μm.
Figure 6.3.5.2. Serial sections from the tendon region of stretched and stimulated rat TA muscle at 5 days stained for IGF-I (a), IGFBP-5 (b) and IGFBP-4 (c). IGF-I immunoreactivity was present only in a cluster of small round-like fibres inside the damaged area where IGFBP-5 seemed to be localized as well. High staining of IGFBP-4 was evident within the damaged – regenerating muscle fibres. Scale bar = 50μm.
Figure 6.3.5.3. Step sections from the mid-belly region of stretched and stimulated rat TA muscle at 5 days stained for IGF-I (a), IGFBP-5 (b) and IGFBP-4 (c). No IGF-1 staining was demonstrated, while IGFBP-4 and IGFBP-5 showed similar staining patterns inside the damaged regenerating areas, as with the tendon region. Scale bar = 50μm.
Figure 6.3.5.4. Serial sections from the tendon region of stretched and stimulated rat TA muscle at 7 days stained for IGF-I (a), IGFBP-5 (b) and IGFBP-4 (c). Small regenerating muscle fibres with central nuclei were stained for IGF-I (arrows, a) with intensity variation. Some of these fibres were also stained for IGFBP-5, whereas IGFBP-4 did not specifically stain the whole muscle fibre but around the plasma membrane of the muscle fibres. Scale bar = 50μm.
Figure 6.3.5.5. Serial sections from the mid-belly region of stretched and stimulated rat TA muscle at 7 days stained for IGF-I (a), IGFBP-5 (b) and IGFBP-4 (c). IGF-I immunoreactivity was dispersed around the top half of the section whereas IGFBP-5 immunoreactivity was more distinctively evident in the bottom half of the section. Non-specific staining of IGFBP-4 was present apart from the high background. Scale bar = 50μm.
63.5.1. Image analysis of total IGF-I, IGFBP-4 and IGFBP-5 in the tendon and mid-belly regions of stretched and stimulated TA muscle

Image analysis on three sections for each specific antibody was performed as described in section 4.2.3 in chapter 4. The mean percentage area stained for all three antibodies in the tendon and mid-belly regions are shown in figure 6.3.5.6 and 6.3.5.7 respectively.

The mean percentage of muscle area stained for IGF-I was similar for both tendon and mid-belly regions and increased significantly until day 7 compared to day 1. The mean percentage of IGFBP-4 staining of the muscle area seemed to peak at day 5 reaching 90% at the tendon site compared to the 50% in the mid-belly region and decreased thereafter in both sites. Image analysis for IGFBP-5 showed similar percentages of staining in both tendon and mid-belly regions at day 5, yet higher in percentage of staining in relation to IGFBP-4 in the mid-belly region at day 5. The mean percentage of IGFBP-5 staining muscle area reached similar values with IGF-I at day 7 in the mid-belly region, but was lower in the tendon site.

Figure 6.3.5.6. Mean percentage of muscle area stained for IGF-I, IGFBP-4 and IGFBP-5 in the tendon site at 1, 5 and 7 days following stretch and stimulation. Significant increase (**P<0.001) was seen in all three antibodies staining the muscle area at day 5, in relation to the other time points. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. N = 6. Values are expressed as mean ± standard error of the mean (S.E.M).
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Figure 6.3.5.7. Mean percentage of muscle area stained for IGF-I, IGFBP-4 and IGFBP-5 in the mid-belly site at 1, 5 and 7 days following stretch and stimulation. Significant increase (\( * * P < 0.001 \)) was seen in IGFBP-4 and IGFBP-5 mean percentage of staining the muscle area at day 5, in relation to the other time points. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. N = 6. Values are expressed as mean ± standard error of the mean (S.E.M).

6.3.6. Expression of M-cadherin in stretched and stimulated TA muscle

M-cadherin antibody labelled small muscle fibres in a ring-like shape (figure 6.3.6.1, a; 6.3.6.2, a) in damaged-regenerated muscle sections in response to stretch and stimulation. The reaction product was confined to the plasma membrane and never in the cytoplasm. Double labelling with laminin, a major component of the basal lamina of blood vessels and muscle fibres was performed to identify the origin of these cells. This identified the M-cadherin positive cells deemed to be muscle satellite cells contained within the basal lamina of muscle fibres.

The M-cadherin immunoreactivity in the stretched and stimulated muscle was relative to the phase of damage-regeneration generated from the injury induced by the protocol. One day following stretch and stimulation, no significant changes in the morphology of muscle were observed, apart from a few very small damaged areas in the mid-belly region. Therefore, no M-cadherin staining appeared in any of these muscle sections (figure 6.3.6.3, a).
However, after five days the muscle sections in which there was marked inflammatory response and regeneration, showed strong M-cadherin staining beneath the basal lamina of small regenerating fibres. This was clearly noticeable at the tendon region (6.3.6.1, a), whereas in the mid-belly region M-cadherin immunoreactivity was also present in larger normal-looking muscle fibres.

Seven days after injury, regenerating fibres close to the tendon demonstrated strong M-cadherin staining localized inside the laminin-positive fibres but the overall staining was less compared to day 5. Similar overview of M-cadherin immunoreactivity was observed in the mid-belly region at day 7, where large hypertrophied fibres showed ring-like structures of M-cadherin staining around some smaller fibres (figure 6.3.6.2, b).

In sham-operated control and normal muscle, no M-cadherin staining was evident (figure 6.3.6.3, b).
Figure 6.3.6.1. M-cadherin staining in 6μm cross-sections of stretched and stimulated muscle after 5 days (a) in the tendon region and (b) in the mid-belly region. Small regenerating fibres and normal-looking muscle fibres show similar ring-like structures of M-cadherin staining inside the basal lamina of the fibres (arrows and arrowheads). Scale bar= 50μm.
Figure 6.3.6.2. M-cadherin staining in 6μm cross-sections of stretched and stimulated muscle after 7 days (a) in the tendon region and (b) in the mid-belly region. Regenerating fibres in the tendon area and large hypertrophied muscle fibres showed similar ring-like structures of M-cadherin staining inside the basal lamina of the fibres. Scale bar = 50μm.
Figure 6.3.6.3. M-cadherin staining in 6μm cross-sections of (a) 1 day stretched and stimulated muscle, (b) sham-operated experimental muscle and (c) normal rat TA. No damage or regeneration was observed in any of these muscle sections, with no M-cadherin staining being demonstrated. Scale bar = 50μm.
6.3.6.1. Image analysis of M-cadherin staining in the tendon and mid-belly regions

The mean percentage area of the stretch and stimulated TA muscle stained for M-cadherin are represented in the figure below in the tendon and mid-belly regions.

M-cadherin immunoreactivity was shown to peak significantly (*P<0.05) at day 5 in both regions of TA that was examined, compared to day 7. No M-cadherin immunoreactivity was observed at day 1 even in the normal muscle fibres, yet a substantial number of apparently normal muscle fibres were stained for M-cadherin at day 7 compared to the number of regenerating fibres in the tendon and mid-belly regions.

![Figure 6.3.6.4](image)

**Figure 6.3.6.4.** Mean percentage of damaged- regenerating and normal muscle area stained for M-cadherin following 1, 5, and 7 day of stretch and stimulation. Significantly (*P<0.05) increased M-cad immunoreactivity was demonstrated in both tendon and mid-belly regions at day 5 compared to day 7. Two-way analysis of variance (ANOVA) was used to determine significant differences among the means. N = 6. Values are expressed as mean ± standard error of the mean (S.E.M).
6.4. Discussion

In the present study, a stretch and stimulation model in which the TA muscle was immobilized in its lengthened position and stimulated, was used to investigate how it alters the pattern of expression of both IGF-I and its binding proteins. This relates to the work described in the previous chapter in which expression of the different IGF-I splice variants and IGFBPs was carried out at the mRNA level. In addition, the satellite cell marker M-cadherin was used to determine how these cells are activated following a mechanical stimulus.

Assessment of the protocol applied and muscle mass changes

It has been shown that muscle stretch is an important stimulus for growth, resulting in increased protein synthesis and fibre diameter (Goldspink, 1985). However, inappropriate stretch may result in muscle fibre damage leading to muscle cell death. The current model was applied in order to reproduce increased muscle mass by as much as 35%, as shown in studies of continuous stretch and stimulation after 3 days (D. Goldspink, et al., 1995).

However, the combination of low-frequency stimulation distributed for an hour just during the anaesthetic stage and continuous passive stretch illustrated, by morphological and histological analysis, that it caused a decrease in muscle wet weight by nearly 12% associated with muscle degeneration after 5 days. By 7 days, the experimental muscle wet weight was non-significantly lighter compared to the contralateral muscle weight and showed a pattern of regeneration. From these results we can conclude that the protocol generated atrophy due to the damage response instead of the increased muscle growth expected due to the non-adaptive response of the muscle. The effect seen from this protocol was probably a result of the immobilization technique of the hind limb rather than with the stimulation combined. However, the results of passive stretch came in contrast to previous studies of 13% increase in muscle wet weight of continuously stretched muscle after 3 days (D. Goldspink, et al., 1995) and 33% increase in TA wet mass after 6 days of passive stretch (Yang et al., 1997).
Other cast immobilization techniques where the knee of a rabbit was placed in an extended position for various periods of time, showed that muscle damage was caused by 12-48 h of vastus intermedius (VIP) immobilization and was still present until 14 days after (Kauhanen, et al., 1998). Additionally, immobilization models produced by pinning the knee and ankle at 90°C flexion have shown that disuse reduces muscle mass (Fischbach and Robbins, 1969; Ibebunjo and Martyn, 1999). Hind limb immobilization in the rat is known to cause skeletal muscle atrophy (Heslinga et al., 1995). In the present work the most marked morphological changes of muscle damage were at 5 days. Therefore, since the fibre-glass cast immobilized the hind leg from the knee downwards but not the ankle, we might have prevented stretch of the affected muscle which justifies the decreased muscle wet mass and necrotic pattern after 5 days.

**Total IGF-I peptide in response to atrophy**

Both regenerating and normal muscle fibres at 7 days expressed maximal total IGF-I protein with no significant differences between the tendon and mid-belly areas of the TA muscle. As the expression of IGF-IEa exceeded that of MGF splice variant over the 7 days following injury as seen in Chapter 5, most of the IGF-I peptide detected by the general antibody in this study should represent IGF-IEa. The IGF-I protein expression seen in damaged muscle in response to immobilization after 5 days could be associated with the release of the IGF-I induced as part of a repair process. The finding that the IGF-I immunoreactivity in the cytosol is low in the 5-day stretched muscle supports this. This could be due to the inflammation or the loss of IGF-I into the extracellular fluid since the plasma membranes would be torn in the stretched-stimulated muscle. One day following immobilization, IGF-I immunoreactivity was seen only in few damaged muscle fibres. Therefore, either IGF-I is only produced as a result of damage or there is a mechano-transduction system in the muscle fibres, which results in the production of IGF-I even before damage occurs.

Additionally, IGFBPs expressed in response to damage might be retaining IGF-I in those fibres. The pattern of increasing IGF-IEa at an mRNA level and of total IGF-I at a protein level in the model applied, could indicate a potential role and requirement for IGF-IEa to be sustained in conditions of muscle atrophy in order to initiate regeneration and muscle growth.
growth. However, observations by Criswell et al. (1998) and Suliman et al. (2001) showed that the levels of IGF-I in skeletal muscle was not directly related to the degree of atrophy in TA muscle over an 8-week immobilization protocol. The use of animal models to elucidate the role of IGF-I in atrophy has produced contradictory results. In denervation models of muscle atrophy, an increase in IGF-I has been observed, whereas in hind-limb suspension models IGF-I mRNA decreased and yet in other studies IGF-I expression levels were unchanged (Czerwinski et al., 1994; Caroni and Schneider, 1995; Yang et al., 1997; Awede et al., 1999b). The discrepancy of the protocol employed in the present study compared to other studies of passive stretch, suggests that different types of protocols and muscles studied, could lead to a different time course and pattern of IGF-I mRNA splice variants and peptide expression thereafter.

**Possible roles of IGFBP-4 and IGFBP-5 on IGF-I in response to atrophy**

To our knowledge, there are no data correlating the mRNA levels and protein localization of IGFBP-4 and -5 in response to mechanical damage in muscle. Therefore, in order to investigate the effect of external stimuli such as stretch or disuse on the peptide levels of the various IGFBPs in skeletal muscles and the consequences for IGF-I activity, IGFBP-4 and IGFBP-5 protein expression was examined. An overall analysis of the IGF-I system on the mRNA and protein levels from the previous and this chapter is demonstrated in the figure in the next page.

Although the protein expression of IGFBP-4 was maximal and higher than that of IGFBP-5 in the tendon region at 5 day and decreasing at 7 days, the opposite was observed in the mid-belly region of the TA muscle, with IGFBP-5 showing similar value (in % of area stained) to that seen with IGF-I at 7 days. While no significant changes were observed after 1 day of the protocol applied, significant degeneration and inflammatory response at 5 days was accompanied with considerable IGFBP-4 and IGFBP-5 protein expression. The precedence of maximal IGFBP-4 peptide expression at 5 days and low IGF-I immunoreactivity is quite noteworthy, since it has been shown that macrophages are a source of a secreted protein identified as IGFBP-4 that binds IGF-I and may antagonize the extracellular effects of IGF-I (Li et al., 1996) or by sequestering free IGF-I and inhibiting its actions (Long et al., 1998). This could also explain why the
temporal expression of IGF-I protein did not correspond with the appearance of marker of regeneration i.e. embryonic MyHC. If we bear in mind the qualitative RT-PCR data on IGFBP-4 mRNA levels, these seem to correlate with the IGFBP-4 peptide expression seen here.

On the other hand, cumulative IGFBP-5 protein expression was also found in conjunction with the increasing IGFBP-4 peptide in the damaged muscle at 5 days. Although, IGFBP-5 has been shown to have stimulating effects on IGF-I, the finding that the increase in IGFBP-5 message at 1 day preceded that of the peptide associated with the low levels of IGF-I protein, could suggest that there was a decreased translational efficiency or that IGFBP-5 is up regulated in response to a decreased mechanical loading which promotes muscle atrophy. The latter proposal is supported by the study of Awede et al. (1999b) in which a similar increase in IGFBP-5 transcript levels was observed in a rat model of hind limb unloading, whereas a decrease was observed with overload hypertrophy. Therefore, since IGFBP-5 immunoreactivity was observed in specific muscle fibres in this study, IGFBP-5 could be involved in the changes in fibre size and phenotype that occur in these models of atrophy as presented here and consequent hypertrophy. In addition, this indication can be substantiated with the observation that IGFBP-5 message was expressed in the contralateral muscles of the stretched muscles at all time points but no IGFBP-5 immunoreactivity was observed maybe due to lack of mRNA translation. MGF isoform was shown from its mRNA expression to have an acute response to the damage induced from the model applied, as also seen with the IGFBP-5 expression. A possible link to the role of IGFBP-5 with the MGF splice variant and their early induction in response to damage could be elucidated with the production of a specific to MGF antibody to investigate possible correlation of expression.
Satellite cells expressing M-cadherin protein in response to damage

The decrease of both IGFBP-5 message and protein levels could indicate a means of facilitating the activating increased effect of IGF-I on satellite cells. On the contrary, IGFBP-4 could prevent the proliferating effect of IGF-I on satellite cells, thus limiting the increase in muscle mass as shown at 5 days. The latter could explain the delayed activation of satellite cells expressing M-cadherin in this study. The M-cadherin protein was found to be present on satellite cells of normal, regenerating or denervated muscle in vivo (Bornemann and Schmalbruch, 1994; Irintchev et al., 1994).

In the present work, M-cadherin protein was significantly increased on satellite cells of damaged and regenerating muscle at 5 days decreased and expressed on satellite cells of normal muscle fibres by 7 days but not present at 1 day. These results seem to coincide partly with the M-cadherin mRNA levels presented in chapter 5, since in contrast to the protein M-cadherin message was expressed at 1 day. This finding may be justified by either that satellite cells were present but failed to synthesize M-cadherin and / or that M-cadherin mRNA and protein expression are differentially regulated. The protein appears 1-2 days later than its mRNA in mouse embryos (Rose et al., 1994). Functional assays performed in cell culture have implied a role for M-cadherin during fusion of
mononucleated myoblasts into multinucleated myotubes (Zeschnigk et al., 1995). In line with this, an up-regulation of M-cadherin expression during myotube formation was shown. This then declined after completion of this process (Pouliot et al., 1994; Kuch et al., 1997). M-cadherin is involved in cell-to-cell attachment and so when cells are fused M-cadherin is no longer needed. This could explain the decreased M-cadherin protein at 7 days, in the present study. The presence of M-cadherin protein in normal muscle fibres may be due to the suggestion that the signal regulating satellite cell proliferation is a diffusible factor that spreads throughout muscle to exert an influence on the entire satellite cell population (Bischoff, 1994).

Conclusions
Taken together, the present findings demonstrate that IGF-I message and protein are increased in response to damage in order to promote local growth. It remains unclear whether IGF-I is stimulated as part of a repair process or is part of an existing mechano-transduction mechanism in the muscle fibres that ensures IGF-I availability even before damage occurs. When the effects of stretch and stimulation on IGF-I, IGFBP-4 and IGFBP-5 mRNA and protein are taken together, it appears that the occurrence of IGFBP-4 and IGFBP-5 is differentially regulated and that significant effects of IGFBPs mRNA and protein can precede that of IGF-I. Therefore, these effects are not caused by alterations in IGF-I expression and are probably directly induced by the injury outcome. This indicates that IGFBPs are probably important in modulating the role of IGF-I in the adaptation of skeletal muscle subjected to a mechanical activity.
CHAPTER VII

General Discussion
The aim of the work described in this thesis was to investigate the role of the two IGF-I splice variants under conditions of damage and further regeneration of skeletal muscle. The application of a highly sensitive PCR technology enabled amplification of low-abundant transcripts for a qualitative analysis of the locally produced insulin-like growth factors in muscle. A number of studies have shown IGF-I expression associated with myofibre damage and regeneration. During regeneration of skeletal muscle in young rats following ischemia- or myotoxin-induced damage, elevated expression of IGF-I has been reported (Jennische and Hansson, 1987; Jennische et al., 1987; Edwall et al., 1989) which was diminished by the 15th day of recovery (Marsh et al., 1997). This is the first time however, that the two distinct IGF-I isoforms, IGF-IEa and MGF have been looked into under such conditions.

The results of the first study (chapter 3), where damage was induced by bupivacaine, demonstrated a surge of IGF-IEa mRNA expression that was maximal at 11 days and diminished thereafter to similar levels as those in the non-injected animals (fig. 3.3.12). On the other hand, MGF transcript showed a transient response at 4 days post bupivacaine injection and decreased thereafter (fig. 3.3.11). Similar patterns of expression at the mRNA level were observed in the stretch and stimulation study in chapter 5 where damage was induced after continuous stretching of the hind leg in the lengthened position following electrical stimulation for an hour. Following mechanical damage, IGF-IEa showed increasing levels until 7 days post stretch and stimulation (fig. 5.3.4) whereas MGF expression was induced as early as 1 day and declined subsequently (fig. 5.3.5). Interestingly, IGF-IEa levels of the contralateral muscle were also shown to increase up to 7 days post stretch and stimulation (fig. 5.3.4) in contrast to MGF levels that remained low, indicating that the mechanical stimulus for MGF differs from IGF-IEa expression and that the increase in the contralateral muscles is related to the compensatory work that these muscles were engaged in, following muscle damage in the experimental limb. It seems that in both myotoxin- and mechanical activity-induced damage models, the temporal expression pattern for each IGF-I splice variant was similar with MGF peaking before IGF-IEa. It is possible that MGF could have been induced
earlier than 4 days in the bupivacaine study as was revealed in the stretch and stimulation one. Therefore, it would be interesting as a future work to look at earlier time points of bupivacaine-induced damage in order to correlate its expression pattern as close as possible to that seen in the stretch and stimulation study. Preliminary experiments on the localisation of MGF mRNA on the stretched and stimulated damaged muscles by in situ hybridisation seemed to coincide with the quantitative mRNA data (fig. 5.3.12). Due to lack of time however, in situ hybridisation was not performed on the bupivacaine-injected muscles. Now, it would be interesting to carry out the same technique on these muscles and further optimise the procedure since some high background was observed on the existing ones. Furthermore, the identification of the type of fibres i.e. using slow or fast type I, II myosin that MGF would be found to be localised in, would be important as it would suggest a possible role in the adaptive changes of the muscle in response to damage. Carrying out also in situ hybridisation experiments with a cRNA probe specific to IGF-IEa (lacking exon 5) and identifying the fibres which it would be expressed by, may signify its diverse actions compared to MGF. Recently, age-related experiments of mechanical overload looking into the two IGF-I splice variants (Owino et al., 2001) demonstrated a significant peak of MGF mRNA at 3 days after surgical ablation in young rats and when compared to mature and old rats, whereas no significant changes were observed in IGF-IEa mRNA levels during ageing or even after tenotomy.

MGF and IGF-IEa splice variants apparently yield the same mature peptide, which is derived from the highly conserved exons 3 and 4 of the IGF-I gene. A mechanism of endoproteolysis of the IGF-I pro-hormone, results in the same mature peptide, but different E peptides (Gilmour, 1994), which may function as independent growth factors (Siegfried et al., 1992). It has been suggested that IGF-I precursors could be pluripotent, in a form analogous to that of pro-hormone propiomelanocortin and proglucagon (Siegfried et al., 1992). The observation that a synthetic peptide derived from the rat Eb domain induces proliferation in epithelial cells is noteworthy (Siegfried et al., 1992). The role of the growth promoting properties of the E peptide in MGF, acting as an independent growth factor, is supported by the recent cell culture observations by Yang and Goldspink (2002). In that, muscle cell lines stably transfected with MGF was
shown to stimulate myoblast proliferation but differentiation was depressed. Addition of a synthetic MGF peptide or a medium-harvested from MGF-transfected cells into normal C2C12 cells also inhibited their differentiation. Yet, this inhibition was reversed when the peptide or the medium were withdrawn. In contrast, cells of the IGF-IEa-positive clone did form myotubes and the normal cell lines showed less cellular proliferation as well as forming myotubes. Of particular interest was the observation that when a blocking anti-IGF-I receptor was added to the cell lines that had MGF or IGF-I peptide and IGF-I receptor antibody in already, the cell proliferation induced by MGF was not inhibited whereas their stimulation by IGF-I was repressed. This result strongly suggests that MGF is involved in a different signalling pathway rather than with IGF-I receptor.

Although the Real-Time PCR method allows for a quick, accurate amplification of low-copy number of gene expression, the main purpose of this study was to assess the response of the two transcripts under conditions of damage through a quantitative analysis. Albeit the data showed significantly higher levels of the IGF-IEa transcript than MGF in the units measured, in both models of damage, the up regulation of MGF expression in response to damage in the time frames examined, was significantly more acute and potent.

As discussed in the introduction in chapter I, a combination of leader exons containing multiple transcription start sites, differential splicing and the presence of multiple polyadenylation sites results in a variety of IGF-I mRNAs, which provide numerous possible mechanisms to influence IGF-I gene expression. Alterations in nutritional status have been reported to cause differential regulation of IGF-I mRNAs by transcription initiation from two different leader exons (Adamo et al., 1991) and by alternative RNA splicing or differential polyadenylation (Zhang et al., 1997). In the latter, it was shown that fasting decreased IGF-IB but not IGF-IA expression in rat liver using both semiquantitative RT-PCR and RPA. Additionally, in vitro translation using specific antibodies for the two peptides also demonstrated that fasting caused a decrease in both peptides. It was concluded therefore, that since IGF-IA and IGF-IB are encoded by one single-copy gene and result from alternative splicing after transcription, that there was no
appreciable decrease in IGF-I gene transcription or in IGF-I mRNAs at the alternative splice site but due to other mechanisms (Zhang et al., 1997).  

The above proposal could explain the different expression patterns of the two splice variants of IGF-I in this study. The results showed that MGF expression was rapidly induced in response to damage and decreased once muscle regeneration had begun, whereas IGF-IEa splice variant induction was slower but sustained longer. Therefore, the apparent decrease in MGF transcript following damage is presumably to post-transcriptional pre-mRNA processing or that it may have a shorter half-life, being degraded quickly. Moreover, in nearly completely regenerating muscles of young rats, as described in the bupivacaine study in chapter 4, feedback regulatory mechanisms are likely to be responsible for the decreased expression of IGF-IEa mRNA by day 24 of recovery (fig. 3.3.12).

As satellite cells appear to play an important role in muscle repair and adaptation it was important to investigate the expression of a satellite cell marker under conditions of damage and regeneration and to relate this to the temporal expression of MGF and IGF-IEa. One of the most useful and suitable markers for the identification of satellite cells for this work proved to be the cell surface protein M-cadherin, since it has been shown to play a significant role in alignment and fusion of myoblasts to form and expand developing myotubes (Cifuentes-Diaz et al., 1995) and has been detected in satellite cells during regenerative responses after muscle damage (Moore and Walsh, 1993; Irintchev et al., 1994). The early and acute surge of MGF mRNA following mechanical and myotoxic damage in this study, suggests that it plays a key role in the proliferation and differentiation of satellite cells in response to stretch and damage. M-cadherin expression was at peak once damage was present i.e. at 4 days post bupivacaine injection (fig. 3.3.14) and 5 days post stretch and stimulation (fig. 5.3.7) and started to decrease once regeneration, fusion of myoblasts had begun. The mRNA results were confirmed by the presence of M-cadherin protein in the activated satellite cells of the damaged muscles, which was maximal at 4 days post bupivacaine injection (fig. 4.3.6.6) and stretch and stimulation model (fig. 6.3.6.4). The M-cadherin mRNA data demonstrated that satellite
cells appear to be activated in association with MGF rather than IGF-IEa levels of expression in response to the stimuli of damage.

The general IGF-I antibody used in the protein studies (chapter 4 and 6) showed that total IGF-I peptide was present in the damaged-regenerating muscle fibres in both bupivacaine and stretch and stimulation models. As the protein expression of total IGF-I was maximal at 14 days post bupivacaine-injection (fig. 4.3.5.9) and at 7 days post stretch and stimulation (fig. 6.3.5.6 & 6.3.5.7), it appears to correspond to the IGF-IEa peptide rather than MGF, since it seems to be in accordance with IGF-IEa mRNA expression.

In the future, the development of an antibody against rat MGF could be used to confirm the proposal that MGF has a short half-life. Western Blotting experiments can indicate whether this transcript is translated or not or that the presence of a decreased peptide product under conditions of damage may suggest that damage reduces the translatable MGF mRNA and attenuates the translational efficiency of IGF-IEa mRNA, as seen from the protein analysis using a general IGF-I antibody in this work. The use of an MGF antibody at the early phases of damage i.e. as seen at 4 days post bupivacaine injection, could pinpoint whether myoblasts (characterised by certain histochemical methods) could produce high amounts of MGF, since the expression of general IGF-I peptide was very low at that time (fig. 4.3.5.1). Additionally, using the existing M-cadherin antibody together with an antibody to MGF, the protein co-localisation of the MGF peptide in relation to the activated satellite cells of the damaged muscle fibres could be investigated. This could be performed by an immunohistochemical or immunofluorescent double labelling method. Peptide and mRNA levels of both IGF-I isoforms together, could then confirm whether MGF is concerned with the initial phase of local muscle repair via the activation of satellite cells and IGF-IEa with the further anabolic adaptation of muscle in response to a stimulus.

Our understanding of the multifunctional nature of the IGFBP family has made considerable progress, when it was believed that these proteins were simply passive carriers of IGFs. Given that the circulating IGF concentration in humans is ~100nM and the IGF-IR on most cells is typically approaching saturation at IGF concentrations of 5nM or lower, it is clear that regulation of circulating IGF bio availability is a key
function of the IGFBPs. Therefore, this could also be applied locally when there is a necessity for these growth factors to act in response to several stimuli. In this regard however, IGFBP modulation of IGF actions, the mechanisms underlying the potentiation of IGF activity by some IGFBPs are still poorly understood.

Muscle cells are known to produce and secrete IGFBPs (Funk et al., 1992; James et al, 1993). In the present work, the temporal expression pattern of two IGFBPs, IGFBP-4 and IGFBP-5 was studied in order to investigate a possible correlation with the IGF-I splice variants in response to damage. From the mRNA data shown in chapter 3 and 5, a similar expression pattern was shown for each IGF binding protein. IGFBP-5 maximal mRNA levels appeared to coincide with the phase of damage at 1 and 4 days post injury (fig. 5.3.8 & fig. 3.3.16) where myoblasts differentiate and this was confirmed by its protein expression in damaged-regenerating muscle fibres (fig.6.3.5.3 & fig. 4.3.5.3) in stretch and stimulation and bupivacaine models respectively. Subsequently, mRNA and protein levels of IGFBP-5 decreased. The results appear to coincide with previous observations of IGFBP-5 localisation and expression (Funk et al., 1992; Jennische and Hall, 2000).

On the other hand, the data presented for IGFBP-4 mRNA and protein data are slightly contradictory. While IGFBP-4 mRNA was up regulated 7 days post bupivacaine-injection (fig. 3.3.15), its protein expression was shown to decline after 4 days (fig. 4.3.5.9). In the stretch and stimulation study however, taking into consideration the protein data only, IGFBP-4 protein expression seemed to peak at 5 days post mechanical damage and then decreased (fig. 6.3.5.6. & 6.3.5.7). The accumulating data from the two IGF binding proteins demonstrated that the gene expression of IGFBP-5 was acutely regulated in response to conditions of damage and started to increase again once the muscle has fully recovered and preceded that of IGFBP-4 that was induced later. Given their action on muscle cells, these IGFBPs together with the local IGF-I expression may play a role in the adaptive changes of the damaged muscle in order for the regenerative process to continue. In the damaged muscle, the increased expression of IGFBP-5 associated with the high levels of MGF splice variant could imply a role of modulating MGF action. Still, the prospect of Western Ligand Blotting using $^{125}$I-MGF as the ligand, once the MGF peptide is available, could show a number of bands indicating whether IGFBP-5 is
binding to MGF or not, since it was shown that it acts independent of the IGF-I receptor (Yang and Goldspink, 2002). Additionally, since it was shown that during the regeneration process after muscle necrosis the expression of both types of IGF-I receptors remained essentially unchanged (Levinovitz et al., 1992), this supports the idea that the availability of the IGFs at the tissue level is determined mainly by the regulation of the amount of the IGFBPs and not by the modified expression of the IGF-receptors.

In contrast, the early increase in IGFBP-4 mRNA and its short-lived protein expression, could explain the increase in total IGF-I peptide independent of changes of its mRNA observed by other groups in muscle subjected to exercise (Eliakim et al., 1997). Therefore in the early steps, IGFBP-4 could act by quenching circulating IGF-I within the muscle. This could be supported by its significant protein expression at 4 and 5 days post bupivacaine- and stretch and stimulation-induced injury, respectively (fig. 4.3.5.3 & fig. 6.3.5.2). Although IGFBP-4 has been most distinctly expressed by connective tissue cells (Jenische and Hall, 2000) in the present study in both models of damage, its protein was expressed in regenerating muscle fibres, as also seen with IGFBP-5. At the specific tissue level thereafter, the questions on their functions include; Do IGFBPs enter the cell as part of the signalling process and if so by what mechanism? Are their functions regulated in response to different stimuli?

The results of these studies add additional insight into the complexity and implication of the IGF-I system in conditions of damage and subsequent regeneration. IGF-IEa and MGF are produced by active muscle in rodents and have been shown to be positive regulators of muscle hypertrophy (Goldspink, 1999; McKoy et al., 1999; Owino et al., 2001). However, reported here is that during damage MGF isoform is acutely induced, whereas IGF-IEa has a delayed effect that is sustained during the regenerative phase. Thus, damage differentially regulates the two IGF-I transcripts that are locally produced in skeletal muscle. Their mRNA and protein expression seem to be modulated extensively by the local productions of certain IGF binding proteins to which also play a role in local tissue repair. A decrease in MGF mRNA levels has also been implicated with cellular ageing (Owino et al., 2001) and satellite cells appear to be activated in
association with MGF expression in response to damage observed in this work. Therefore, future experiments investigating the expression of the two transcripts and activation of satellite cells in young and old muscle offer the prospect of a therapeutic approach to ameliorating muscle loss following injury, neurological conditions and to old age.
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