CELLULAR AND MOLECULAR BASIS OF PERIODONTAL REGENERATION

Thesis submitted by
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for the degree of
DOCTOR OF PHILOSOPHY

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1998
Guided tissue regeneration (GTR) is based on the insertion of a barrier membrane to physically prevent downgrowth of the gingival epithelium and to enable cells with regenerative capability to re-populate the root surface and re-build the lost periodontium. Although many clinical studies have been carried out, little is known about the cellular and molecular mechanisms underlying GTR. The aims of this study were therefore to investigate the phenotypes, functions and extracellular environment of the cells involved in periodontal regeneration. Periodontal defects of subjects were treated by GTR using expanded polytetrafluoroethylene membranes or conventional flap (CF) surgery. Clinical measurements were recorded and retrieved membranes, underlying regenerated tissue (RT), periodontal ligament (PL) and gingival tissues, gingival crevicular fluid (GCF) and serum samples obtained. The cells associated with the retrieved membranes (M cells) and those derived from the RT (RT cells) were obtained and cultured in vitro. They were found to have a fibroblast-like morphology similar to the PL and gingival cells, and flow cytometry analysis showed that they also produce higher levels of collagen type I, fibronectin and tenasin. In addition, cultures of M cells formed mineralised nodules, characteristic of osteoblasts. The RT cells expressed high levels of bone-associated proteins osteonectin, osteocalcin, bone sialoprotein, osteopontin and alkaline phosphatase activity. Moreover, expression of the receptors for transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) were also found to be up-regulated in the RT tissue, and analysis of GCF showed that TGF-β was significantly higher in GTR test samples compared with CF samples after surgery. The results of this study have demonstrated that GTR-associated cells have a fibroblast-like morphology, produce certain key connective tissue proteins, have osteoblast-like characteristics and express high levels of growth factor receptors which are likely to be involved in periodontal regeneration.
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</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>γ</td>
<td>gamma</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>M</td>
<td>mole</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<td>second</td>
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<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>ABC</td>
<td>avidin-biotin complex</td>
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<tr>
<td>aFGF</td>
<td>acidic fibroblast growth factor</td>
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<tr>
<td>AFI</td>
<td>average fluorescence intensity</td>
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<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>αMEM</td>
<td>alpha minimum essential medium</td>
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<td>BC</td>
<td>alveolar bone crest</td>
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<td>BD</td>
<td>base of the defect</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>BMP</td>
<td>bone morphogenetic protein</td>
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<td>BSP</td>
<td>bone sialoprotein</td>
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<tr>
<td>CAL</td>
<td>clinical attachment level</td>
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<tr>
<td>CEJ</td>
<td>cemento-enamel junction</td>
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<tr>
<td>CF</td>
<td>conventional flap</td>
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<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine tetrahydrochloride</td>
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<tr>
<td>DD</td>
<td>defect depth</td>
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<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<td>dexamethasone</td>
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<td>deoxyribonucleic acid</td>
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<td>dNTP</td>
<td>dioxynucleotide triphosphate</td>
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<td>DMEM</td>
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<td>extracellular matrix</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>ePTFE</td>
<td>expanded polytetrafluoroethylene</td>
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<td>gingival crevicular fluid</td>
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<td>GTR</td>
<td>guided tissue regeneration</td>
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<td>haematoxylin and eosin</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>immunoglobulin G₁</td>
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<td>monoclonal antibody</td>
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<td>membrane-associated cells</td>
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<td>messenger ribonucleic acid</td>
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<td>NGS</td>
<td>normal goat serum</td>
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<td>phosphate buffered saline</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>PPD</td>
<td>probing pocket depth</td>
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<td>REC</td>
<td>recession of gingival margin</td>
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<td>RGD</td>
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<td>Tris-buffered saline</td>
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<td>transmission electron microscopy</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<td>transport medium</td>
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ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. I. Olsen and Dr. G.S. Griffiths for their valuable advice, encouragement and guidance throughout my studies. I am also deeply grateful to Prof. H. Newman for his guidance and support in the establishment of my thesis.

Special thanks to all of my friends and colleagues in the Departments of Periodontology, Oral Medicine and Oral Pathology and the Electron Microscopy Unit at the Eastman Dental Institute, particularly to Mr. M. Parkar for his technical help and to Mr. V. Ward for providing membrane samples. I would like to thank Ms. A Petrie in the Department of Transcultural Oral Health for her advice regarding the statistical analyses in this study.

I would also like to acknowledge Marmara University (TURKEY) for providing funding to undertake this study.

Thank you very much from the bottom of my heart, my late mother and my beloved father, for your endless support and love, and teaching me to work hard in order to fulfil my dreams. And last but not least, thank you Huseyin, my husband and dearest friend. It would have not been possible to make my dreams come true without your unique friendship, precious support and immense patience, and thanks for just always being there.
DECLARATION

I declare that all the experiments in this thesis were carried out by myself, unless otherwise stated. The surgical procedures were performed by staff and postgraduate students in the Departments of Periodontology and Oral and Maxillofacial Surgery. The paraffin-embedded RT sample was prepared by Mr. P. Darkins in the Department of Oral Pathology. The membrane samples for TEM examination were prepared by Ms. N. Mordan in the Electron Microscopy Unit.
PUBLICATIONS AS A RESULT OF THIS THESIS

PAPERS


ABSTRACTS


1.1 OVERVIEW

Periodontal regeneration is a complex wound healing process which involves structural and functional rebuilding of periodontal tissues lost as a result of periodontal disease. This process requires the most coronal establishment of the gingival junctional epithelium and new formation of the periodontal ligament with collagen fibres inserting into newly-formed cementum and alveolar bone. In guided tissue regeneration (GTR), a membrane is placed surgically onto the periodontal defect between the gingival flap and root surface. The underlying principle of this procedure is the use of this membrane as a barrier, firstly to prevent the downgrowth of the gingival epithelium and, secondly to facilitate the selective re-population of the previously diseased root surface by periodontal cells capable of re-forming the cellular and extracellular components of new periodontal ligament, new cementum and new alveolar bone.

Histological studies in experimental animal models and a few human cases have demonstrated that the reconstruction of new periodontal tissues can be achieved in vivo using the principles of GTR. Several case reports and controlled clinical studies in humans have also shown significant improvements in the clinical parameters following therapy with GTR. However, the outcome of this treatment modality is still not highly efficacious and predictable. A thorough understanding of the cellular and molecular basis of periodontal regenerative healing would enable the development of more efficient, successful and predictable strategies for periodontal therapy.

1.2 PERIODONTIUM

The periodontium is a unique and topographically complex organ which is defined as the tissues supporting and investing the tooth (Ten Cate 1994). The anatomic compartments of the periodontium macroscopically include the gingiva, periodontal ligament (PL), cementum and alveolar bone, as shown schematically in Figure 1.1. The periodontium is almost entirely
derived from the ectomesenchyme, embryonic connective tissue derived from neural crest cells, apart from the gingival epithelium and cell rests of the epithelial root sheath (Ten Cate 1997) which are derived from the epithelium lining the stomatodeum (Ten Cate 1994).

![Figure 1.1. Schematic diagram showing the tissues of the periodontium.](image)

**1.2.1 Gingiva**

Clinically, the gingiva is a part of the oral mucosa that surrounds the teeth and is attached to both the tooth and the alveolar process (Figure 1.1) (Schroeder and Listgarten 1997). It is demarcated from the oral mucosa by the mucogingival line and extends to the gingival margin. The gingiva is subdivided into three topographical regions, the free, attached and interdental gingiva, but it is nevertheless an anatomical and functional unit. Histologically, the gingiva is composed of epithelium and connective tissue, including fibres, nerves, blood and lymphatic vessels.

The gingival epithelium provides a protective sheath for the underlying periodontal tissues. It is divided into three structural and anatomical regions: i) the keratinised oral gingival epithelium, covering the free and attached
gingiva; ii) the non-keratinised sulcular epithelium, forming one wall of the gingival sulcus, the other wall formed by the tooth; and iii) the junctional epithelium, providing the attachment of the epithelium to the tooth surface, extending to the cemento-enamel junction (CEJ) and also protecting the underlying PL (Hassell 1993).

Beneath the gingival epithelium resides the gingival connective tissue, also termed the lamina propria. The gingival connective tissue is highly organised and vascularised, and accounts for the major proportion of the gingiva (Schroeder and Listgarten 1997). The main compartment of the lamina propria includes a complex network of collagen fibre bundles. These supra-alveolar fibres provide support for the free and attached gingiva (Hassell 1993). They also form the coronal part of the connective tissue attachment while the apical part is formed by the fibres of the PL. The collagen fibres of the gingiva are surrounded by fibroblasts, although other cells, vascular elements and nerves are also present.

1.2.2 Periodontal ligament

The PL tissue is derived from the inner layer of the dental follicle shortly after root development starts (Ten Cate 1994), but it is also considered that cells migrating from the dental papilla to the dental follicle have the potential to form the PL during odontogenesis (Palmer and Lubbock 1995). Mature PL is a highly vascular and highly cellular soft connective tissue situated between the root of the tooth and the inner wall of the alveolar socket (Figure 1.1). It is composed mainly of fibre bundles stretching between the cementum on the root surface and the alveolar bone, inserting into both of these tissues as Sharpey's fibres. The PL fibres are located subjacent to gingival fibres and it is not possible to distinguish the border between them. The main function of the PL is tooth anchorage, although it also plays a part in fibrous tissue development and maintenance, calcified tissue development and maintenance, nutrition and innervation (Hassell 1993). Of major importance is the capacity of the PL for repair and regeneration, which is reflected in the complex and heterogeneous sub-populations of cells in this tissue (Lekic and McCulloch 1996).
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The main cell type in the PL is the fibroblast. These are scattered among the collagen fibres. Cementoblasts, osteoblasts, cementoclasts and osteoclasts, which maintain and remodel the cementum and alveolar bone on the borders of the PL, are also considered to be part of this tissue (Berkovitz et al. 1995). In addition, other mesenchymal cells, which may include progenitor cells, the epithelial rests of Malassez and inflammatory cells are among the different types of cell residing in the PL.

1.2.3 Cementum

The root surface of the tooth is covered by a calcified connective tissue, referred to as cementum (Figure 1.1) (Hassell 1993). As its principal function, cementum provides anchorage of the tooth in the alveolar socket via the collagen fibres of the PL, whose terminations, Sharpey's fibres, are embedded within the cementum. As the occlusal and incisal surfaces of teeth are worn away, new cementum deposits at the apical root area and maintains the width of the PL space. During development, as spaces appear between the epithelial cells of the Hertwig's root sheath, mesenchymal cells in the follicle come into contact with the dentine and appear to differentiate into cementoblasts which synthesise the organic matrix of cementum (Ten Cate 1994).

The cementum is classified on the basis of location, morphology and histological appearance as follows (Hassell 1993): i) the acellular afibrillar cementum, covering the enamel at and along the cemento-enamel junction (CEJ); ii) the acellular fibrillar cementum, found on the cervical and middle root portions; iii) the cellular intrinsic fibre cementum, assumed to represent repair or secondary cementum; and iv) the cellular cementum, with intrinsic cementoblast-derived and extrinsic fibroblast-derived fibres.

1.2.4 Alveolar bone

Another anatomical component of the periodontium comprises the alveolar processes, which represent the extensions of the body of the mandible/maxilla (Figure 1.1). The embryological origin of the alveolar bone stems from the initial condensation of ectomesenchyme around the early tooth germ (Ten Cate 1997). The alveolar processes are tooth-dependent,
being present as long as they house the teeth. They are comprised of three compartments: i) the alveolar bone proper, in which the Sharpey's fibres are embedded; ii) the compact bone, composed of the oral and buccal cortical plates; and iii) the cancellous bone, located between the alveolar bone proper and the cortical plates (Saffar et al. 1997).

1.2.5 Cells of the periodontium

The periodontium contains diverse populations of cells which undoubtedly have unique and specific functions. These maintain periodontal tissue homeostasis and comprise epithelial cells, mesenchymal cells (including fibroblasts, cementoblasts, osteoblasts and their progenitors), resorptive cells, and other cell types.

1.2.5.1 Epithelial cells

The main epithelial cell population of the periodontium is the gingival epithelium, which is separated from the underlying lamina propria by a basal lamina. Free and attached gingiva are covered by keratinised stratified squamous epithelium comprised of four layers: stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Schroeder and Listgarten 1997). Some of the cells in the stratum basale migrate toward the surface of the epithelium and differentiate. They first enlarge, then become flattened and elongated and eventually keratinise, whereupon they are referred to as keratinocytes.

Although the sulcular epithelium has the same four layers of cell populations, a continuous keratinised layer is absent. However, the junctional epithelium, providing the epithelial attachment to the tooth, lacks the spinosum, granulosum and corneum strata and its width is only a few cells thick, with a maximum 20 to 30-cell layer at the most coronal portion and a minimum 3 to 4-cell layer at the most apical portion (Hassell 1993).

Another epithelial cell population of the periodontium is the epithelial cell rests of Malassez, which are usually present within the PL as small clusters (Berkovitz et al. 1997). They represent the remains of the Hertwig's epithelial root sheath, which is the extension of the inner enamel epithelium and disintegrates during root development (Palmer and Lubbock 1995).
small numbers of remaining cells are surrounded by fibroblasts and the connective tissue matrix of the PL (Berkovitz et al. 1997). Although these have been readily cultured and studied (Uitto et al. 1992), their function in PL tissue homeostasis is not well understood.

1.2.5.2 Fibroblasts

Fibroblasts are found extensively in all connective tissues of the body, including the gingiva and PL. Indeed, the fibroblasts are the predominant cell type in gingival and PL connective tissues and account for about 6% of their total volume (Giannopoulou and Cimasoni 1996). Both the gingival and PL fibroblasts share a common embryonic mesenchymal lineage derived from neural crest cells (Ten Cate 1994). They not only maintain the integrity of the tissue and support the framework for other cells, but also play crucial roles in periodontal development, physiology, pathology, healing and immune reactivity (McCulloch and Bordin 1991; Fries et al. 1994; Lekic and McCulloch 1996). Fibroblasts synthesise a wide variety of extracellular matrix (ECM) components, including collagen, elastin, glycoproteins and glycosaminoglycans (Terranova and Wikesjö 1987). They also synthesise and secrete a number of enzymes that degrade the ECM, such as collagenase and matrix metalloproteinase. Their prominent nucleus and the abundance of cytoplasmic organelles, such as the rough endoplasmic reticulum, reflect their active synthetic function (Berkovitz et al. 1995). PL fibroblasts tend to be polarised with respect to their morphology and this polarisation may play an important part in their continuous migration under physiologic conditions (McCulloch and Melcher 1983a) as well as during healing after injury (Gould et al. 1980; McCulloch 1995).

Fibroblasts are characterised by their spindle or stellate morphology, presence of vimentin filaments and synthesis of collagens and fibronectin (McCulloch and Bordin 1991). However, fibroblasts are now recognised as a diverse group of cells, consisting of distinct sub-populations within the same tissue and also between different anatomical sites (Häkkinen and Larjava 1992; Fries et al. 1994; Irwin et al. 1994a; Sempowski et al. 1995; Phipps et al. 1997; Kuru et al. 1998a).
While gingival fibroblasts maintain the synthesis and integrity of gingival connective tissue, PL fibroblasts with specialised functions are considered to be responsible not only for the formation and maintenance of the PL (Berkovitz et al. 1995) but also for repair, regeneration and remodelling of the adjacent alveolar bone and cementum in vivo. They also appear to prevent ankylosis between these two mineralised tissues (Boyko et al. 1981). In addition, although the morphology of both the gingival and PL fibroblasts cultured in vitro have been reported to be similar, having a characteristic spindle-shaped, elongated appearance when examined by phase-contrast (Somerman et al. 1988; Piche et al. 1989; Ogata et al. 1995) and scanning electron microscopy (Giannopoulou and Cimasoni 1996), heterogeneous subsets of cells having different shapes (Hou and Yaeger 1993; Adams et al. 1993) and different sizes (Hassell and Stanek 1983) have also been identified.

In addition, functional differences between gingival and PL fibroblasts have been reported. Thus, in animal studies, it was found that when the roots were covered with PL cells which had been cultured in vitro and then re-implanted in vivo, they acted as progenitor cells and gave rise to new PL connective tissue (Boyko et al. 1981; Van Dijk et al. 1991; Lang et al. 1995; Lang et al. 1998). In marked contrast, gingival fibroblasts failed to produce new tissue (Boyko et al. 1981). In addition, clinical studies have indicated that new periodontal connective tissue attachment, including new cementum with inserting collagen fibres, was formed when PL cells repopulated the root surface, whereas cells derived from the gingival connective tissue lacked this ability (Gottlow et al. 1984; Gottlow et al. 1986). Moreover, certain molecular features, such as total protein and ECM production (Somerman et al. 1988; Kuru et al. 1998a) have been shown to be higher in PL compared with gingival fibroblasts. The response of these two cell types to attachment factors (Somerman et al. 1989), ECM proteins (Giannopoulou and Cimasoni 1996) and growth factors (Dennison et al. 1994) also have been found to be different. In addition, PL cells have been shown to possess osteoblast-like characteristics, including the production of osteonectin (Somerman et al. 1990; Nohutcu et al. 1996), osteocalcin (Nojima et al. 1990) and high levels of alkaline phosphatase (ALP) activity (Kawase et al. 1988; Kuru et al.
submitted). These studies provide evidence that phenotypically distinct and functionally different fibroblast sub-populations exist in the periodontium.

1.2.5.3 Osteoblasts

Osteoblasts are considered to be derived from a mesenchymal cell precursor and are the cells responsible for bone formation (Bilezikian et al. 1996). Active osteoblasts are cuboidal or columnar cells in situ, with prominent rough endoplasmic reticulum characteristic of protein-secreting cells (Arnett 1990). They are found on the surface of alveolar bone only during osteogenesis and, as bone deposition proceeds, they become trapped in the accumulating matrix and differentiate to osteocytes (Berkovitz et al. 1995). Osteoblasts synthesise and regulate the deposition of organic bone matrix, of which the main component is type I collagen (Arnett 1990). Other main constituents of bone matrix include osteonectin, osteocalcin, bone sialoprotein (BSP), osteopontin and proteoglycans (Gage et al. 1989). Expression of the enzyme ALP by osteoblasts has been found to be closely associated with new bone formation in vivo (Arnett 1990).

1.2.5.4 Cementoblasts

Cementoblasts are the other mesenchymal cell population in the periodontium. They are responsible for synthesis and secretion of the organic matrix constituents of cementum. Apart from their location adjacent to the cementum, they are not readily distinguished from the fibroblasts within the PL tissue (Berkovitz et al. 1995). As cementum formation proceeds, the cementoblasts entrapped within the cementum matrix are referred to as cementocytes. It has been suggested that there may be two populations of cementoblasts, which can be distinguished by their phenotype and developmental origin (Ten Cate 1997). Thus, while the cells associated with acellular cementum may be derived from dental follicle, the cells forming cellular cementum may originate from the progenitor cells migrating from the endosteal spaces (McCulloch et al. 1987).
1.2.5.5 Progenitor cells

It has been suggested that, in mature PL tissue, the PL fibroblast lineage comprises a renewing cell system in steady-state (McCulloch et al. 1989; McCulloch 1995). The daily rate of cell generation in normal rodent PL has been reported to be between 0.5-2% (McCulloch and Melcher 1983b). The PL is considered to contain progenitor cells which function as the precursors for the mesenchymal cell population that is continually renewed under physiologic conditions due to cell death and terminal differentiation (Aukhil 1991; Berkovitz et al. 1995).

A number of recent studies have investigated the origin and location of the progenitor cell population(s) in the PL. It has been suggested that even after the PL develops from undifferentiated mesenchymal cells embryologically, some progenitor cells remain in the mature tissue (Hassell 1993). This progenitor population within the PL has been suggested to be localised paravascularly, i.e. in close proximity to blood vessels (McCulloch et al. 1991; Lekic and McCulloch 1996). In a wound healing model system, Gould et al. (1980) found that, following partial removal of the PL, the proportion of $^3$H-thymidine labelled paravascular cells in the adjacent PL increased five-fold. The majority of such cells was localised in the central part of the PL (McCulloch and Melcher 1983b), and it was suggested that these cells may give rise to PL fibroblasts and also migrate towards the bone and cementum surfaces, where they differentiate to osteoblasts and cementoblasts, respectively (McCulloch and Melcher 1983a; Lekic et al. 1996).

The cells present in the vascular channels of alveolar bone, which migrate towards the PL, may be another source of progenitors (McCulloch et al. 1987). This suggestion is supported by a study in which root slices were cultured with cells derived from foetal rat calvaria in vitro (Melcher et al. 1987). It has been also suggested that some cells adjacent to the cementum could constitute a separate progenitor cell population, although evidence for this is very limited (McCulloch and Melcher 1983b). Alternatively, the possibility that different populations of ancestral cells may be responsible for the apparently distinct types of cells found in the mature PL cannot be excluded (McCulloch 1995). Although the precise identification and
characteristics of the progenitor cell population(s) in the PL are not yet known, such cells undoubtedly play a major role in periodontal tissue homeostasis and in the regenerative healing process, in particular.

1.2.5.6 Resorptive cells

In contrast to the highly synthetic mesenchymal cells, the resorptive cells of the periodontium are involved in removing ECM from the periodontal tissues. These cells include osteoclasts and cementoclasts or odontoclasts (Aukhil 1991). Osteoclasts are large, multinucleated and highly motile cells of haemopoietic origin which are responsible for bone degradation. They are relatively sparsely distributed in bone and do not proliferate, being terminally differentiated. Osteoclasts in the periodontium are found on the surface of the alveolar bone but do not cover the whole of the resorbing surface. Although the multinucleated cells associated with the resorption of cementum are referred to as cementoclasts, osteoclasts and cementoclasts have been suggested to be morphologically and functionally similar (Berkovitz et al. 1995).

1.2.5.7 Miscellaneous cells

Melanocytes are present in the gingival epithelium and are responsible for producing the pigment melanin. Another cell type in the gingival epithelium include the Langerhans cells, which are derived from the bone marrow and play a major role in the host immune response of the gingiva.

The cell types, other than the fibroblasts, residing in the connective tissues of the healthy periodontium include all types of blood cells, the majority being within the blood vessels (Hassell 1993). The granulocytes, including polymorphonuclear neutrophils, polymorphonuclear eosinophils and polymorphonuclear basophils, represent an important part of the host defence mechanism. The neutrophils have the capacity to migrate towards micro-organisms and destroy them by phagocytosis. Their presence in both healthy and diseased periodontium suggest that they may play important roles in normal tissue homeostasis and inflammation. The lymphocytes, mostly T lymphocytes, are also present in healthy gingival connective tissue
subjacent to the junctional epithelium. Other cells include the tissue macrophages, plasma cells and mast cells.

1.2.6 Extracellular matrix components of the periodontium

The ECM is the intercellular substance of tissues and is composed of a diverse number of secreted macromolecules (Terranova and Wikesjö 1987). It plays an active and complex role in regulating the migration, proliferation, shape and metabolic functions of the cells with which it is in contact (Hay 1981). The ECM of the soft and hard connective tissues of the periodontium is composed of collagens and many non-collagenous components, including fibronectin, tenascin, osteonectin, osteocalcin, BSP, osteopontin, proteoglycans and others, as described below.

1.2.6.1 Collagen

The collagens, the predominant ECM component of the periodontium (Mariotti 1993), are responsible for the maintenance of the framework of the tissues (Bartold 1995). In addition to their structural role, collagens have been shown to be involved either directly or indirectly in promoting cell attachment and differentiation, as a chemotactic agent for both fibroblasts and macrophages, as an antigen in immunological processes and possibly as a casual agent in some pathological conditions (Hay 1981). The collagen molecule is composed of 3 polypeptide chains called α chains and contains one or more triple helix domains (Hay 1981). As many as 19 types of structurally and functionally different collagen molecules have been identified (Bartold and Narayanan 1998).

The distribution of collagens varies considerably between different tissues. Approximately 60-65% of healthy gingival connective tissue is occupied by collagen (Hassell 1993). The total collagen content of the PL is 50% of the tissue on a dry weight basis (Embery 1990). Type I collagen, the predominant species, together with type III collagen, are uniformly distributed throughout gingival connective tissue, whereas types IV, V and VI are minor species (Becker et al. 1993; Bartold 1995). Type IV collagen is localised to the basement membrane structures (Gage 1989), such as the basement
membrane of gingival epithelium as well as blood vessels and nerves (Romanos et al. 1993). Type V collagen is distributed in the matrix of the lamina propria, in close association with cells, and type VI collagen has a microfibrillar pattern of distribution (Romanos et al. 1993). As with the gingiva, the main collagen species in the PL is type I, which forms the PL fibres and their extensions, Sharpey’s fibres (Lukinmaa and Waltimo 1992; Bartold 1995; Matsuura et al. 1995). Types V and XII are co-distributed with type III collagen which surrounds type I collagen in Sharpey’s fibres. The organic matrix of both cementum and alveolar bone is comprised of type I collagen, the main constituent, and type III collagen (Lukinmaa and Waltimo 1992; Bartold 1995). Bone collagen is virtually insoluble due to cross-links, which provide the structural and mechanical stability and normal function of the bone collagen fibrils.

1.2.6.2 Fibronectin

Fibronectin is an insoluble, high molecular weight, fibre-forming glycoprotein which is present both intra- and extra-cellularly (Yamada and Olden 1978). The structure of fibronectin consists of two identical, disulphide-linked polypeptide chains and contains a sequence, arginine-glycine-aspartate (RGD), that binds to cells, as well as other sites that bind to collagen, heparin and fibrin (Mariotti 1993; Engel 1991). In addition to its main function as an adhesive protein, fibronectin is involved in blood coagulation, wound healing and chemotaxis (Yamada and Olden 1978; Mariotti 1993). Since fibronectin has been found in high concentrations in regions of cell migration during development, this protein is also thought to be involved in cell migration in vivo (Hay 1981).

Fibronectin has been found to be widely distributed in the periodontium (Tucker et al. 1991; Steffensen et al. 1992; Romanos et al. 1993). Expression of this protein in the gingival epithelium varied from strong to weak and even to negative (Tucker et al. 1991; Steffensen et al. 1992; Romanos et al. 1993). It has been also localised immunohistochemically in the basement membrane underlying the epithelium and is most intense in the lamina propria (Tucker et al. 1991; Steffensen et al. 1992), having a fibrillar and diffuse distribution (Romanos et
al. 1993). However, fibronectin was expressed particularly strongly along the attachment sites of PL collagen fibres to the cementum but not to alveolar bone (Matsuura et al. 1995; Lukinmaa et al. 1991). It has been also observed in the endosteal spaces, periosteum and bone-lining cells at the interface between the alveolar bone (Steffensen et al. 1992). In the cementum the expression was weaker than in the PL (Zhang et al. 1993).

1.2.6.3 Tenascin

Tenascin is a large glycoprotein and has a restricted tissue distribution (Chiquet-Ehrismann et al. 1986; Mackie et al. 1987). It appears as a protein with 6 arms extending from a central core (Erickson and Inglesias 1984). Although tenascin is believed to have a prominent role in developmental processes, Saga et al. (1992) showed that transgenic mutant mice in which the tenascin gene is not present developed nevertheless normally. In contrast to most other major proteins of the ECM, the expression of tenascin is maintained only during wound healing (Mackie et al. 1988), carcinogenesis (Chiquet-Ehrismann et al. 1986) and in a few adult tissues including bone marrow (Klein et al. 1993) and periodontal tissues, in which it is unevenly distributed (Lukinmaa et al. 1991; Steffensen et al. 1992; Becker et al. 1993).

Tenascin is localised in stratified gingival epithelium close to the basement membrane (Becker et al. 1993), in the basement membrane (Steffensen et al. 1992) and less intensely in the lamina propria (Tucker et al. 1991), in which it is found in association with loosely-arranged collagen fibres in the upper connective tissue (Becker et al. 1993). Tenascin was found between less densely-packed collagen fibrils of the PL (Zhang et al. 1993) and accumulated towards the alveolar bone and cementum (Lukinmaa et al. 1991; Steffensen et al. 1992). Strong expression of this protein was seen in the periosteal and the endosteal fibrous tissues of the alveolar bone (Steffensen et al. 1992), with only weak expression throughout the alveolar bone matrix (Lukinmaa et al. 1991). Cementum also showed weak expression of this protein, which may have been deposited prior to mineralisation (Zhang et al. 1993).
1.2.6.4 Osteonectin

Osteonectin, secreted protein acidic and rich in cysteine (SPARC), is a phosphate containing glycoprotein (Gage et al. 1989). It is comprised of a single polypeptide chain and has a strong affinity for Ca^{2+} ions due to the phosphate ions and also for collagen type I (Sage and Bornstein 1991). It has been suggested that the phosphate groups may be crucial for initiating the mineralisation process (Gage et al. 1989). Osteonectin is one of the major non-collagenous proteins of bone and is also expressed in basement membranes (Bilezikian et al. 1996). It has been found in PL, particularly strongly around the Sharpey’s fibres, at the attachment sites between the PL and cementum and the PL and alveolar bone (Matsuura et al. 1995).

1.2.6.5 Osteocalcin

Osteocalcin, also called bone gla protein due to its \( \gamma \)-carboxyglutamic acid (gla) residues, is a small protein that is synthesised principally by odontoblasts and osteoblasts (Mariotti 1993). Soon after secretion, osteocalcin becomes incorporated into the mineralised matrix of bone. It has been suggested that osteocalcin is involved in the mineralisation process, as the gla residues on the protein act as calcium-binding sites (Gage et al. 1989). D’Errico et al. (1997) demonstrated the expression of osteocalcin by the cells lining the tooth root surface during development in mice.

1.2.6.6 Bone sialoprotein

BSP, also known as BSP II, is a phosphoglycoprotein containing up to 20% of sialic acid residues and also has an RGD sequence (Bilezikian et al. 1996). It exhibits a restricted pattern of expression and is found primarily in the bone (Fujisawa et al. 1995). BSP expression marks a late stage of osteoblast differentiation and an early stage of matrix mineralisation (Lekic et al. 1996). Matsuura et al. (1995) reported weak expression of BSP in the PL, especially at the attachment sites between the PL and cementum. BSP was also expressed by cells lining the root surface at early stages of cementogenesis during tooth development (MacNeil et al. 1995; MacNeil et al. 1996). These cells appeared to be responsible for the secretion of BSP.
onto the root surface which then became occupied by cementum. Although the precise functions of this protein are not yet known, it may serve as an attachment factor (Mariotti 1993) since it has an affinity for collagen fibrils and enhances the attachment of fibroblasts and osteoblasts to plastic surfaces (Fujisawa et al. 1995).

1.2.6.7 Osteopontin

Osteopontin, also termed as BSP I due to the sialic acid content, is a glycophosphoprotein found primarily in bone and also in some non-skeletal tissues such as the central nervous system, kidney and placenta (Mariotti 1993). In addition to an RGD cell-attachment sequence, it has a high affinity for Ca\(^{2+}\) ions. Although its physiological functions are also not clear, it is expressed prior to mineralisation and appears to be involved in both attachment and movement of osteoblasts and osteoclasts. MacNeil et al. (1995) suggested that osteopontin functions as an inhibitor of mineralisation during PL development, since it was distributed in a non-specific fashion throughout the PL of the developing murine molar tooth germ between 21 to 42 developmental days. In contrast, D’Errico et al. (1997) reported that osteopontin was not expressed in the PL at day 41 but was expressed by cells lining the tooth root surface. Lekic et al. (1996) demonstrated the expression of osteopontin in regenerating alveolar bone adjacent to a fenestration wound in rats, prior to the expression of BSP, and concluded that this protein was an early marker of periodontal tissue regeneration.

1.2.6.8 Proteoglycans

Proteoglycans are extracellular and cell surface-associated macromolecules composed of a protein core to which one or more glycosaminoglycan chains are attached. The glycosaminoglycans are represented by several species, including chondroitin sulphate, dermatan sulphate, heparin, heparan sulphate, keratin sulphate and hyaluronan (Mariotti 1993). Proteoglycans contribute to the overall structure of the ECM, regulate cell adhesion and growth, and have the capacity to bind and regulate the growth factor activity (Bartold and Narayanan 1998). Proteoglycans detected in gingiva and PL are decorin, biglycan, versican and
syndecan (Häkkinen et al. 1993; Bartold and Narayanan 1998). Chondroitin sulphate-rich proteoglycans, likely decorin and biglycan, were also identified in alveolar bone (Waddington and Embery 1991).

1.2.6.9 Other ECM constituents

Elastin, a very flexible and insoluble protein, is the major component of the elastic fibres of gingival and PL connective tissue (Hay 1981; Mariotti 1993; Bartold 1995). Laminin is found exclusively in basement membranes where it is believed to mediate the attachment of epithelial cells to type IV collagen (Mariotti 1993). In the periodontium, laminin was located in the basement membranes of vessels and of the oral, sulcular and junctional epithelium of gingiva in baboons (Steffensen et al. 1992). Vitronectin promotes the attachment and spreading of cells in vitro. This protein was found on the cells lining the cementum and alveolar bone (Steffensen et al. 1992) and shown also to be associated with connective tissue fibres of the gingiva and PL (Matsuura et al. 1995).

1.3 PERIODONTAL HEALING, REPAIR AND REGENERATION

The structure and integrity of the periodontium are altered by a number of inflammatory diseases, which can cause destruction to the cells and surrounding ECM of the periodontal tissues. This leads to the loss of connective tissue attachment to the tooth, exposure of the root surface to the oral cavity, resorption of the alveolar bone and ultimately tooth loss. Many attempts have therefore been made to preserve and restore periodontal tissues destroyed by these disease processes (Froum and Gomez 1993; Garrett 1996). The main goal of periodontal therapy is therefore to provide a dentition that will provide healthy, normal function throughout the life of the patient (Zander et al. 1976). The initial phase of periodontal therapy is aimed at the elimination of periodontal infection by removing root surface deposits and controlling the bacterial infection which induces pathologic and clinical changes in the periodontium (Garrett 1996). However, after this phase of therapy, the periodontal defect resulting from infection may still persist. A second phase of periodontal therapy may be attempted to alter this defect and rebuild lost periodontal tissues to their original structure and function.
using a number of surgical procedures. The clinical outcome, however, depends to some extent on the healing response of the periodontium to the surgical therapy. The success of the healing process depends on the presence of cells and regulatory factors that control the complex biological events which rebuild periodontal tissues.

1.3.1 Definitions of repair and regeneration

Healing is a response of tissues to mechanical, thermal, chemical or pathological injuries (Takata 1994). Regeneration is one type of healing in which lost cells are replaced by the same type of cells (Alison 1992) and is defined as the reproduction or reconstitution of a lost or injured part (Hurt et al. 1986). From the periodontal point of view, this definition implies new formation of the tooth's supporting tissues, including the PL, cementum and alveolar bone, in which the architecture and function are completely renewed (Garrett 1996). Healing by repair, however, involves the synthesis of connective tissue and eventually the formation of a scar tissue (Alison 1992) and is defined as the healing of a wound by a tissue in which architecture and/or function is not fully restored to the original (Hurt et al. 1986). Regeneration and repair in periodontal healing can be mediated by 'new attachment' or 'reattachment'. New attachment is considered to be the reunion of connective tissue with root surfaces that have lost their PL (Hurt et al. 1986). This association occurs by the formation of new cementum with inserting collagen fibres. In contrast, reattachment refers to the reunion of connective tissue with a root surface on which viable periodontal tissue is present (Hurt et al. 1986).

1.3.2 General principles of wound healing

Depending on the wound type, wound healing is divided into three categories: i) healing by primary intention with apposed edges and minimal tissue loss; ii) healing by secondary intention with separated edges and extensive tissue loss; and iii) healing by third intention with more extensive tissue loss treated by either graft or flap rotation (Messadi and Bertolami 1991). Healing of a wound requires complex interactions involving multiple cell types, various growth factors, cytokines and the ECM. Although the
healing process is continuous, it has been sub-divided into 3 phases: i) the inflammatory phase, ii) the proliferative phase, and iii) the remodelling phase (Sempowski et al. 1995).

1.3.2.1 The inflammatory phase

The initial response to injury is inflammation which involves vascular and cellular components and includes a directed and sequential migration of neutrophils, monocytes and lymphocytes into the wound area over the first several days (Deuel et al. 1991). The injury to the soft tissue causes the disruption of blood vessels. This results in leakage of plasma proteins from damaged and undamaged blood vessels adjacent to the wound (Messadi and Bertolami 1991) and consequently in the formation of clot which is rich in fibrin. The fibrin clot is an important early component of healing (Wikesjö et al. 1992) and contains many proteins, including fibronectin, which can be cross-linked to fibrin, collagen and other ECM proteins to stabilise the wound mechanically (Alison 1992). The injury also leads to the activation and aggregation of platelets. Activated platelets release several growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-β1 (TGF-β1) (Assoian et al. 1984). A number of specific substances including bradykinin, histamine, serotonin, heparin and prostaglandin cause an increase in the permeability of undamaged vessels adjacent to the injured area (Messadi and Bertolami, 1991). The action of inflammatory mediators, such as interleukin-1 and tumour necrosis factors, and the increase in vascular permeability activates endothelial cells. This leads to the adherence of circulating polymorphonuclear leukocytes (neutrophils) to endothelial cells. The neutrophils marginate, cross the vessel wall and migrate to the site of injury. They appear within the first 6 to 12 hours of injury and help to prevent infection by phagocytosis of micro-organisms. They also eliminate injured, dead tissue by releasing proteases and lysosomal enzymes (Messadi and Bertolami 1991). Fibronectin and enzymes released from neutrophils act as chemoattractants for macrophages which develop from circulating monocytes (Alison 1992). These cells are found in wounds after the initial stages of the healing process.
and help to phagocytose micro-organisms and tissue debris. They also have a role in initiation of tissue repair via secretion of many biologically active substances, such as chemotactic agents, growth factors and cytokines (Wikesjö et al. 1992). During the later stages, lymphocytes are the main inflammatory cells which appear at the wound site. They secrete many factors which influence the functions of not only macrophages but also fibroblasts, endothelial and epithelial cells.

1.3.2.2 The proliferative phase

The proliferative phase of healing begins approximately 2 to 4 days after injury and continues up to 2 to 3 weeks (Deuel et al. 1991). During this period, the wound is occupied by the granulation tissue which consists of fibroblasts, macrophages and newly-formed blood vessels within the matrix of fibrin, fibronectin, collagens, glycoproteins and glycosaminoglycans (Messadi and Bertolami 1991). Fibroblasts, activated by growth factors produced by inflammatory cells, proliferate and migrate into the wound, and synthesise a wide variety of ECM proteins, including collagen in abundance, fibronectin, proteoglycans and other proteins. While fibronectin acts as an adhesive substratum on which fibroblasts migrate, collagens and glycosaminoglycans contribute to tissue structure. Formation of new blood vessels begins at the wound edge. This process has a key role in delivering nutrients and oxygen and removing metabolic by-products. Activated endothelial cells also produce collagenase and other ECM-degrading enzymes, thus facilitating the growth of new capillaries through the ECM (Alison 1992).

1.3.2.3 The remodelling phase

Remodelling is the final phase of wound healing, beginning simultaneously with granulation tissue formation and continuing for months after re-epithelialization has occurred (Messadi and Bertolami 1991). The newly-formed, cell-rich granulation tissue undergoes maturation and subsequent remodelling to meet functional demands (Wikesjö et al. 1992). The main process of this phase is active collagen turnover.
1.3.3 Healing of periodontal tissues

Healing of a periodontal defect following surgery is a complex process which involves different structures and cell types. While the gingival epithelium and connective tissue form one of the margins of a periodontal wound, the other wound margin is formed by an avascular, calcified and previously diseased root surface (Wikesjö et al. 1992). It has been proposed that fibrin is the essential precursor to enhance the adhesion of connective tissue cells to the root surface during the early phases of healing following periodontal surgery (Polson and Proye 1983). Thus, new connective tissue attachment to the root surface and the outcome of periodontal surgery appears to be critically dependent on the attachment between the fibrin clot and the root (Wikesjö et al. 1992). The gingival epithelial and connective tissue cells, PL cells and osteoblasts are the major, crucial cell types which participate in the healing of the periodontal tissues following surgery. The role of these cells during periodontal healing will be discussed in detail below (section 1.3.5).

1.3.4 Clinical procedures for periodontal regeneration

Many attempts have been made to achieve periodontal regeneration using a variety of natural and synthetic materials and surgical procedures (Froum and Gomez 1993; Garrett 1996).

1.3.4.1 Open flap debridement

The open flap debridement procedure is a surgical procedure in which the gingival flap is raised, the granulation tissue removed and the root surfaces are instrumented (Ramfjord and Nissle 1974). The flap is then replaced to its original position and sutured. Clinical studies have shown that this procedure leads to an arrest of the progression of the periodontal lesion, resulting in some improvements in clinical parameters (Rosling et al. 1976; Froum and Gomez 1993). However, histological studies have demonstrated that the healing response is neither structurally nor functionally identical to a healthy periodontium in certain features. For example, soft tissue adhesion to the root surface frequently occurs with the formation of a long junctional epithelium (Stahl et al. 1982). Nonetheless, the open flap debridement
procedure, also referred to as conventional flap surgery, has been widely used as the basic periodontal surgical therapy and has served as a control in many clinical trials of other procedures (Cortellini et al. 1993a; Cortellini et al. 1993b; Kiliç et al. 1997).

1.3.4.2 Root surface treatment

One of the important components of the periodontal healing process is the root surface which becomes exposed to the oral environment as a result of periodontal disease. This surface functions as one of the wound margins, serving as a source for the renewal of structural components and a surface for new cell attachment and fibre development. Selective alterations of the root surface have been carried out so that it does not contain cytotoxic contaminants, provides chemotactic stimulus for connective tissue cells and supports the migration and attachment of the cells involved in healing (Lowenguth and Blieden 1993).

Citric acid-induced demineralisation of the root surface is aimed at exposing collagen fibres present in cementum or dentine in order to form a barrier against epithelial migration and also allow interdigitation of collagen fibres from the healing connective tissues (Froum and Gomez 1993). Early animal studies demonstrated that topical application of citric acid on previously denuded root surfaces improved the rate of connective tissue healing compared to open flap debridement and resulted in the formation of new attachment and new cementum (Ririe et al. 1980; Polson and Proye 1983). However, in another animal study citric acid provided little additional benefit over root planing (Nyman et al. 1980) and the procedure did not demonstrate significant clinical improvement in humans (Marks and Mehta 1986). Another agent used for the same purpose has been tetracycline HCl, which has produced similar equivocal results (Lowenguth and Blieden 1993).

Terranova and Wikesjö (1987) proposed a possible therapeutic role for ECM proteins. However, topical application of fibronectin onto root surfaces did not result in additional bone fill (Terranova et al. 1987), and the addition of laminin did not improve the response (Smith et al. 1987).
1.3.4.3 Coronally positioned flap

In order to exclude the gingival epithelium from the healing periodontal wound or to delay the migration of the epithelial cells, mucoperiosteal flaps were displaced in a more coronal position. Such coronally positioned flaps have been used mainly in conjunction with other approaches, such as citric acid conditioning (Froum and Gomez 1993), and histological studies in animal models have demonstrated effective results in achieving periodontal regeneration (Crigger et al. 1978). However, in humans this procedure has had some technical difficulties as placement of sutures over occlusal surfaces may result in rupture of the sutures with subsequent release of the flaps. Therefore, the interdental sutures were placed to secure the coronally positioned flaps in humans.

1.3.4.4 Grafting

Bone grafts, of natural or synthetic materials, have been widely used to fill the defects in alveolar bone destroyed by periodontal disease. These procedures have been carried out as adjuncts to open flap debridement surgery, to accelerate periodontal regeneration and obtain improvement in clinical parameters (Brunsvold and Mellonig 1993). The 3 types of grafts which are used most frequently are: i) autogenous bone grafts taken from one part of a patient’s body and transferred to another part in the same individual; ii) allografts taken from donors and transferred to a recipient host of the same species; and iii) alloplasts, synthetic and inert materials such as hydroxyapatite (Brunsvold and Mellonig 1993).

Froum et al. (1975) reported that over 70% of intraosseous defects were filled when the autogenous bone graft was used. This procedure resulted in significant bone fill compared to the open flap debridement procedure (Froum et al. 1976). Although several reports have supported the success of autogenous bone grafts, the problems concerning the need of a donor site, time and limited amount of donor material has led to the development of allografts. Urist (1965) showed that demineralisation and freeze-drying of cortical bone allograft material enhances its osteogenic potential. Pearson et al. (1981) noted substantial bone fill using decalcified freeze-dried bone compared to surgical debridement. Although bone grafts
were considered to be inductive to the formation of alveolar bone, it has been noted that they act as space-fillers and the procedure results in new connective tissue attachment with long junctional attachment and very limited regeneration (Wikesjö et al. 1992). Thus, more effective and more predictable procedures are required to completely restore form and function of the periodontal ligament, cementum and alveolar bone.

1.3.5 Guided tissue regeneration

In 1976, Melcher suggested that the outcome of healing is determined to a large extent by the types of cell that repopulated the wound site. This was supported by the results of a number of experimental studies in animals. In these, teeth which were periodontally healthy or affected by periodontal disease were extracted. The roots were separated from the crowns, subjected to mechanical treatment and then transplanted back into the same animal (Karring et al. 1980; Nyman et al. 1980; Karring et al. 1985). The root surfaces came into contact with different types of cell of the periodontium during healing period. The long junctional epithelium was established as a result of the proliferation and migration of gingival epithelial cells along the root surface. However, when the cells of the lamina propria populated the previously diseased root surfaces, soft connective tissue adhesion occurred which may consequently result in root resorption, but a new connective tissue attachment failed to form (Nyman et al. 1980). The extensive osteogenesis resulting in ankylosis and root resorption was the outcome of healing, when osteoblasts from the adjacent healthy alveolar bone migrated to the wound site and came into contact with the root surface (Karring et al. 1980; Nyman et al. 1980). New connective tissue attachment failed to form on the root surfaces deprived of their PL tissue (Nyman et al. 1985), whereas new cementum with inserting fibres was observed when viable PL tissue was preserved prior to transplantation (Karring et al. 1980) or when PL cells from the remaining healthy PL proliferated and repopulated the root surface (Nyman et al. 1980; Nyman et al. 1982a; Karring et al. 1985). Isidor et al. (1986) suggested that the repopulation of a root surface by cells from the PL is a prerequisite for new attachment formation.
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These observations have led to the development of a new treatment modality known as guided tissue regeneration (GTR). GTR is based on the directed repopulation of the previously diseased root surface by selected PL cells. In the clinical situation, a barrier membrane is surgically placed over the periodontal defect to cover it completely. The membrane is adapted to the tooth and covered by the gingival flap. In this technique, gingival epithelium and connective tissue are physically excluded from the wound area and at the same time a space is created between the membrane and the root surface of the tooth to allow the PL cells to migrate, proliferate and eventually repopulate the previously exposed root surface (Wikesjö et al. 1992; Gottlow 1993).

1.3.5.1 Early studies

Early experimental animal studies involved the use of barrier membranes to facilitate the proliferation of the various periodontal tissue components and thereby to alter the healing response following periodontal surgery (Nyman et al. 1982a; Gottlow et al. 1984; Caffesse et al. 1988; Aukhil et al. 1987). Nyman et al. (1982a) prepared a periodontal wound where the buccal alveolar bone at the mid-root level was removed to create a fenestration. Following removal of the PL and cementum, a barrier was placed onto the fenestration wound so that the gingival epithelium and connective tissue were prevented from coming into contact with the root surface during healing. The results showed that new attachment with fibres inserting into new cementum was re-established by the PL cells. Similar histological results were observed on the previously diseased root surfaces following membrane placement (Gottlow et al. 1984; Caffesse et al. 1988; Sander and Karring 1995). These findings suggested that the PL cells posses the ability to reform new attachment and new cementum and barrier membranes are effective in promoting this regenerative healing process and preventing epithelial downgrowth.

In humans, the formation of new attachment on previously diseased root surfaces using barrier membranes was first described in 1982 by Nyman et al. (1982b). Further, a clinical trial by Gottlow et al. (1986) provided evidence that the use of barrier membranes based on the principles of GTR
was a predictable procedure for treating advanced periodontal disease in humans.

1.3.5.2 Membrane materials in GTR

There are 5 criteria which are considered to be important in the design of barrier membranes used for GTR (Scantlebury 1993; Hardwick et al. 1995). These include i) biocompatibility; ii) cell-occlusiveness; iii) spacemaking; iv) tissue integration; and v) clinical manageability. In order to achieve the mechanical tissue separation and support, various types of materials have been developed which can be grouped together as either non-resorbable or resorbable membranes.

1.3.5.2.1 Non-resorbable membranes

Among the first non-resorbable membranes to be used was cellulose acetate filter which was not produced for medical use (Nyman et al. 1982a; Nyman et al. 1982b). Although early experiments indicated new attachment formation at the most apical part of the defect, the gingival epithelium migrated around the membranes into the wound area and interfered with healing, leading to secondary pocketing, recession and membrane exposure. Subsequently, a membrane that was made of expanded polytetrafluoroethylene (ePTFE) material was designed specifically for GTR. This membrane consisted of 2 parts: i) a collar portion, having open pores to allow ingrowth of connective tissue and to prevent epithelial migration, and ii) an occlusive portion, preventing the flap tissues from coming into contact with the root surface (Scantlebury 1993). Because the space which was defined and protected by the membrane determined the volume of tissue that could be regenerated, the material was redesigned with a stiff central portion to treat osseous defects (Scantlebury 1993; Hardwick et al. 1995) and reinforced with titanium for both osseous and periodontal defects (Hardwick et al. 1995; Cortellini et al. 1995; Sigurdsson et al. 1995b).

Since these membranes are made of a non-resorbable material, a second surgical procedure is necessary to remove them (Scantlebury 1993). This procedure therefore has the disadvantage of the additional trauma to the patient as well as the healing periodontal tissues (Gottlow 1993).
Moreover, the use of these non-resorbable membranes has been associated with membrane contamination and/or infection when the membrane is exposed to the oral cavity (Selvig et al. 1990; Tempro and Nalbandian 1993; Grevstad and Leknes 1993; Nowzari et al. 1996; Nowzari and Slots 1994). Attempts to overcome these problems and limitations have led to the development of resorbable barrier membranes.

1.3.5.2.2 Resorbable membranes

The resorbable biomaterials most commonly used as barrier membranes to facilitate GTR are collagen, synthetic poly-lactide and poly-glycolide polymers and co-polymer membranes (Gottlow 1993). Other resorbable materials include oxidised cellulose, freeze-dried dura mater and skin (O'Neal et al. 1994). The rationale for using collagen is that it is the main constituent of periodontal connective tissue, chemotactic for fibroblasts, a cross-linked bioabsorbable material and has the capability to enhance clot formation (O'Neal et al. 1994). Although resorbable membranes eliminated the need of surgical removal, they also have been found to have certain limitations. A major difficulty is that the device must be biologically stable and remain intact sufficiently long to enable tissue regeneration to occur. Moreover, the material should not generate deleterious immune responses. Most importantly, the resorption process itself must not interfere with the regeneration of periodontal tissues (Hardwick et al. 1995).

1.3.5.3 Clinical applications of GTR

The principles of GTR have been used for treatment of both the interproximal intraosseous defects and furcation defects in humans. Histological evaluation of the outcome of this therapy in humans has presented some difficulties due to ethical considerations. Clinical parameters are therefore used to assess the healing response. They include clinical attachment level (CAL), probing pocket depth (PPD), gingival recession and bone fill as well as bone density and height using radiographs (Garrett 1996). The results are assessed by comparing pre- and post-treatment data.
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Human clinical studies using non-resorbable membranes have demonstrated that GTR therapy significantly improves the clinical outcome compared to conventional flap surgery. Thus, several short-term clinical studies reported significant reductions in PPD (ranging from 3.5 to 5.9 mm) and significant gains in CAL (ranging from 3 to 6 mm) and bone levels (ranging from 2.7 to 4.7 mm) following GTR treatment (Gottlow et al. 1986; Pontoriero et al. 1988; Gottlow 1993; Tonetti et al. 1993; Cortellini et al. 1993a; Cortellini et al. 1993b; Cortellini et al. 1995; Cortellini et al. 1996a; Kiliç et al. 1997; Eickholz et al. 1998). Cortellini et al. (1993a; 1993b) treated interproximal intraosseous defects using ePTFE membranes and observed a significant gain in CAL, reduction in PPD and new formation of alveolar bone 1 year post-operatively, while Pontoriero et al. (1988) demonstrated complete resolution of more than 90% furcation defects 6 months following GTR therapy. When the clinical efficacy of ePTFE membranes and titanium-reinforced ePTFE membranes were compared, significant clinical improvements were obtained in both of the membrane groups, but the gain in CAL in the titanium-reinforced group was greater than that in the ePTFE group (Cortellini et al. 1995).

Clinical studies using various types of resorbable barrier materials also produced favourable clinical outcomes (Becker et al. 1996; Cortellini et al. 1996b; Eickholz et al. 1998). Polson et al. (1995) demonstrated significant reduction in PPD and gain in vertical and horizontal attachment levels in furcation defects following the use of resorbable barrier membranes. However, Cortellini et al. (1996b) obtained similar clinical outcomes in patients treated by GTR using either resorbable or non-resorbable membranes, whereas Hugoson et al. (1995) found a significant improvement in gingival recession at the sites treated with resorbable membranes compared with non-resorbable membranes at 1 year post-operatively.

The significant improvements in clinical parameters, which were obtained in short-term GTR studies, have been shown to be maintained in long-term studies over periods of 1 to 5 years (Gottlow et al. 1992; Weigel et al. 1995; Machtei et al. 1996; Cortellini et al. 1996a). However, long-term stability of clinical attachment following GTR could be influenced by factors, such as oral hygiene, compliance with a supportive periodontal care program.
and cigarette smoking (Machtei et al. 1996; Cortellini et al. 1996b). The biological principles of GTR have also been applied recently for bone regeneration (Nowzari and Slots 1995).

1.3.5.4 GTR combined with other clinical procedures

Histological and clinical studies on the use of barrier membranes have provided extensive evidence that periodontal regeneration is practically feasible. However, the clinical results remain variable and unpredictable. Studies have shown that treatment of Class III furcation defects at mandibular molars using GTR results in partial healing where complete closure of the defect is obtained infrequently (Pontoriero et al. 1989; Eickholz et al. 1998). The biologic principles and materials of the different regenerative procedures have therefore been combined in order to achieve a greater degree of clinical success. For example, GTR procedures used in combination with root conditioning and/or grafts have resulted in improved outcomes (McClain and Schallhorn 1993; Kiliç et al. 1997). Moreover, antimicrobial agents have either been applied topically before membrane placement (Sander et al. 1994) or incorporated in the resorbable membranes (Dowell et al. 1995), in order to reduce the possibility of bacterial contamination during healing. However, the addition of the antibiotic metronidazole did not appear to improve periodontal regeneration beyond that of the membrane alone (Sander et al. 1994; Dowell et al. 1995). Several recent experimental studies in animals have used growth factors alone or in conjunction with either GTR or with GTR and root conditioning. The results have shown that growth factors had significantly increased potential for inducing regenerative healing of periodontal tissues (Sigurdsson et al. 1995a; Sigurdsson et al. 1995b; Cho et al. 1995; Park et al. 1995). However, the therapies using the growth factors were considered to be in the experimental stage, and therefore no growth factor therapy has received approval by the Food and Drug Administration to treat periodontitis in humans (The American Academy of Periodontology 1996). Nevertheless, Howell et al. (1997) recently used recombinant human PDGF and insulin-like growth factor (IGF) to treat periodontal osseous defects in humans and
reported that the application of these factors significantly increased the formation of alveolar bone compared to conventional flap surgery.

### 1.3.5.5 Cells and ECM proteins in periodontal regeneration

Recognition that the cells involved in GTR are key factors which ultimately determine successful regeneration has prompted a number of investigations aimed at understanding this process at the cellular and molecular levels. Morphological analysis of furcation defects treated by GTR in dogs showed that the wound was occupied primarily by granulation tissue containing numerous infiltrated inflammatory cells and blood vessels, at 2 weeks (Matsuura et al. 1995). At 4 weeks, however, the defect was nearly filled by new connective tissue containing fibroblast-like cells. The cells colonising the periodontal wound area were found to be derived from within 200 μm of the adjacent unwounded PL (Gould et al. 1980; Iglhaut et al. 1988) as well as from alveolar bone (Iglhaut et al. 1988). At 8 weeks, new PL associated with newly-formed bone was evident (Matsuura et al. 1995).

Pritlove-Carson et al. (1992) observed variable amounts of tissue adherent to ePTFE membranes removed from periodontal surgical sites of patients. Immunohistochemically, vimentin-positive mesenchymal cells and keratin-positive epithelial cells were identified in the tissues adherent to the membrane as well as in regenerated soft tissues taken from the healing periodontal defect (Pritlove-Carson et al. 1992; Pritlove-Carson et al. 1994).

Recently, the cells associated with ePTFE membranes were cultured and studied in vitro (Wakabayashi et al. 1996; Wakabayashi et al. 1997; Grosso et al. 1997; Kuru et al. 1997a; Kuru et al. 1997b). These cells appeared to be fibroblast-like in morphology, formed mineralised nodules in vitro (Wakabayashi et al. 1996; Wakabayashi et al. 1997; Grosso et al. 1997) and produced ECM proteins associated with both soft (Kuru et al. 1997a) and hard connective tissues (Kuru et al. 1997b), certain proteases (Wakabayashi et al. 1996; Grosso et al. 1997) and cytokines (Wakabayashi et al. 1997). Furthermore, culture media in which these cells had been incubated in vitro inhibited osteoclast differentiation (Rowe et al. 1996).
The identification and localisation of ECM proteins, which are expressed during periodontal tissue regeneration, have been studied in animals and only in a few human cases. Type I collagen, along with type III, was found to be sparsely distributed and not well organised (Matsuura et al. 1995; Pritlove-Carson et al. 1994). Collagen type IV was only found at basement membranes associated with blood vessels and epithelium (Pritlove-Carson et al. 1994). Fibronectin was localised between the inflammatory cells in the newly-formed connective tissue and at the attachment sites of the PL to the root surface (Matsuura et al. 1995). The expression of bone-associated proteins, including osteocalcin, osteonectin and BSP, were observed in the newly-formed cementum and newly-formed bone as well as in the connective tissue in close proximity to the hard tissues (Amar et al. 1995; Matsuura et al. 1995; Ho et al. 1995; Amar et al. 1997).

1.3.6 Growth factors in periodontal healing

Growth factors are substances capable of initiating the proliferation of cells that are in a quiescent state (Hefti 1993) by stimulating DNA synthesis and progression of the cell cycle (O'Neal et al. 1994). They have primarily a paracrine or autocrine action and exert their effect via binding to specific transmembrane receptors on target cells (Alexander and Damoulis 1994) which generate a cascade of intracellular molecular signals (Sporn and Roberts 1991). These polypeptide growth factors thus regulate a number of processes in vitro in addition to activation and proliferation, including cell migration and the synthesis of ECM proteins, all of which are essential events in wound healing (Deuel et al. 1991; Kiritsy et al. 1993). Moreover, Terranova and Wikesjö (1987) suggested that growth factors may also have the potential to biochemically mediate periodontal wound healing and regeneration when applied topically. The nature and role of some of these factors [e.g. PDGF, TGF, fibroblast growth factor (FGF), epidermal growth factor (EGF), IGF and bone morphogenetic proteins (BMPs)] in wound healing and their effect on periodontal regeneration are discussed below.
1.3.6.1 Platelet-derived growth factor

PDGF plays an important role not only in wound healing but also in embryogenesis, neoplasia and fibrotic responses associated with inflammatory disease (Sporn and Roberts 1991; Hu et al. 1995; Horner et al. 1996; Ataliotis and Mercola 1997). It is released mainly from platelets and is also synthesised by macrophages, fibroblasts, endothelial cells, skeletal myoblasts and kidney epithelial cells (Sporn and Roberts 1991; Plemons et al. 1996). PDGF consists of a dimer of two glycoprotein subunits, A and B (Sporn and Roberts 1991). There are therefore three combinations of the two chains, PDGF-AA, PDGF-BB and PDGF-AB. PDGF acts by binding to two distinct cell surface receptors, termed PDGFR-α and PDGFR-β, on target cells.

A number of in vitro studies have demonstrated that PDGF can stimulate proliferation (Piche and Graves 1989; Boyan et al. 1994; Dennison et al. 1994; Anderson et al. 1998), DNA synthesis (Matsuda et al. 1992; Oates et al. 1993; Blom et al. 1994) and collagen production by PL cells (Matsuda et al. 1992). It is also chemotactic for PL cells (Matsuda et al. 1992; Boyan et al. 1994; Nishimura and Terranova 1996) as well as for osteoblasts (Hughes et al. 1992). PDGF-BB was more effective than the other isoforms in promoting mitogenesis and chemotaxis of PL cells in vitro (Boyan et al. 1994) and also acted synergistically with other growth factors both in vitro and in vivo (Lynch et al. 1987; Rutherford et al. 1992; Matsuda et al. 1992; Hefti 1993).

Experimental studies in animals examined the efficacy of PDGF-modulated GTR on furcation defects (Cho et al. 1995; Park et al. 1995) and fenestration wounds (Wang et al. 1994b) and concluded that this therapy effectively promoted periodontal regeneration. When PDGF was applied in combination with IGF to periodontal defects in dogs, enhanced formation of new PL, cementum and alveolar bone was observed during the early phases of healing (Lynch et al. 1991). This combination has recently been applied locally to periodontal defects in humans and appears to significantly promote bone regeneration (Howell et al. 1997).
1.3.6.2 Transforming growth factor

The TGFs are a family of structurally and functionally different proteins that have been isolated from normal and neoplastic tissues (Massague, 1987; Barnard et al. 1990; Sporn and Roberts, 1991). The two best characterised are TGF-α, primarily a growth stimulator and TGF-β, primarily a growth inhibitor (Massague 1987; Sporn and Roberts 1991). TGF-α stimulates epithelial and endothelial cells and acts through the receptor of a different growth factor, EGF (Hefti 1993). TGF-β is encoded by 5 different genes yielding the 5 isoforms TGF-β1 to TGF-β5 which display different spatial and temporal patterns of expression during healing (Levine et al. 1993; Frank et al. 1996). TGF-β is present in high concentration in platelets (Hefti 1993) and is also produced by both activated macrophages and neutrophils, which are present during the initial phases of wound healing (Igarashi et al. 1993). Three distinct receptors, type I, type II and type III, have been identified on almost all normal cells and most neoplastic cells (Barnard et al. 1990).

The biological effects of TGF-β in vitro are highly diverse. It has been found to be chemotactic for macrophages (Barnard et al. 1990) and gingival and PL cells (Postlethwaite et al. 1987; Nishimura and Terranova 1996), stimulated the proliferation of gingival and PL cells (Postlethwaite et al. 1987; Oates et al. 1993; Anderson et al. 1998), inhibited the growth of epithelial, endothelial and certain mesenchymal cells (Lynch et al. 1989; Barnard et al. 1990; Matsuda et al. 1992; Lu et al. 1997) and selectively stimulated the synthesis of ECM components such as collagen, fibronectin, tenascin and proteoglycans (Irwin et al. 1994b; Matsuda et al. 1992; Lynch et al. 1989; Barnard et al. 1990). In addition, TGF-β1 alone and also in combination with PDGF increased the proliferation of PL cells significantly more than gingival cells (Dennison et al. 1994). However, when collagen sponges impregnated with TGF-β1, IGF and FGF were placed into fenestration defects in dogs, this combination was found to have no effect on bone regeneration (Selvig et al. 1994).
1.3.6.3 Fibroblast growth factor

The FGFs are a family of polypeptides which are potent mitogens and chemoattractants for endothelial and mesenchymal cells (Caffesse and Quinones 1993). Two of the most widely studied forms of this family are acidic FGF (aFGF) and basic FGF (bFGF). aFGF stimulates endothelial cell proliferation (Hefti 1993). bFGF is widely distributed and found in nearly all tissues, including brain, retina, kidney, placenta, gingiva, PL and bone (Hefti 1993; Bilezikian et al. 1996; Gao et al. 1996; Murata et al. 1997).

FGF has been reported to stimulate PL and endothelial cell migration (Terranova et al. 1989b), increase DNA synthesis and enhance proliferation (Blom et al. 1994; Takayama et al. 1997) and inhibit the induction of ALP activity and mineralised nodule formation by PL cells in vitro (Takayama et al. 1997). The in vivo use of bFGF in conjunction with autografts has had a beneficial effect on mandibular bone healing in rabbits (Eppley et al. 1991). However, in combination with other growth factors it did not show any effect on periodontal healing (Selvig et al. 1994).

1.3.6.4 Epidermal growth factor

EGF is a small polypeptide which stimulates the proliferation of epithelial, endothelial and mesodermal cells (Caffesse and Quinones 1993; Bilezikian et al. 1996). It is present in most human extracellular fluids and secretions including plasma, saliva, milk, amniotic fluid and urine (Sporn and Roberts 1991). EGF is mitogenic for PL cells (Matsuda et al. 1992; Blom et al. 1994) and was found to stimulate the growth of gingival cells in vitro (Irwin et al. 1994b). It also showed a slightly increased chemotactic effect on PL cells but suppressed their collagen synthesis (Matsuda et al. 1992). The effects of EGF on periodontal regeneration remain to be investigated.

1.3.6.5 Insulin-like growth factor

The IGFs are a family of single-chain proteins (Sporn and Roberts 1991). IGF-I and IGF-II are anabolic peptides structurally and functionally related to insulin. They are synthesised by liver, smooth muscle and placenta and transported via the plasma (Caffesse and Quinones 1993). They are also present in skeletal tissues, via de novo synthesis by bone cells
and release of the stored peptides from the bone matrix (Bilezikian et al. 1996). Both gingival and PL cells were found to exhibit dose-dependent migratory responses when incubated with IGF-I (Matsuda et al. 1992; Nishimura and Terranova 1996) and IGF-II (Nishimura and Terranova 1996). In addition, IGF-I increased DNA and protein synthesis in PL cells (Blom et al. 1992; Matsuda et al. 1992). IGF-I has been reported to act synergistically with other growth factors to enhance epidermal and connective tissue wound healing (Lynch et al. 1987). Moreover, the short term application of IGF-I in combination with PDGF was found to increase the healing response following periodontal surgery in animals (Lynch et al. 1991; Giannobile et al. 1996) and in humans (Howell et al. 1997).

1.3.6.6 Bone morphogenetic proteins

The BMPs are part of the large TGF-β superfamily (Wozney 1995; Bilezikian et al. 1996). Localisation of members of the BMP family in embryological development of the skeleton has provided strong evidence of an important role in mediating skeletal patterning as well as skeletal cell differentiation (Bilezikian et al. 1996). Furthermore, the BMPs are considered to be responsible for the inductive and regenerative ability of demineralised bone allografts used in periodontal therapy (Urist 1965). In addition, BMP-2 was found to stimulate osteocalcin and ALP expression by cultured PL cells and was chemotactic for connective tissue stem cells in vitro (Hughes 1995). The BMPs also induced the formation of cartilage and bone tissues when implanted with a carrier into a soft tissue site (Wozney 1995). Moreover, the topical application of BMPs onto periodontal defects resulted in the establishment of new attachment, regeneration of alveolar bone (Sigurdsson et al. 1995a) and induction of cementum formation in animals (Ripamonti et al. 1996).

1.4 GINGIVAL CREVICULAR FLUID

1.4.1 Definition and production of GCF

Gingival crevicular fluid (GCF) is an extracellular fluid that accumulates in the gingival crevice. It has been considered as an
osmotically mediated transudate as the result of increased rate of capillary transudation or an inflammatory exudate in the presence of inflammation (Løe and Holm-Pedersen 1965; Pashley 1976). Early studies have demonstrated that the epithelium lining the gingival sulcus was permeable to the molecules of serum regardless of the health of the gingival tissues (Brill and Krasse 1958; Brill and Björn 1959). It was suggested that GCF occurs naturally as well as from inflammation in the surrounding tissues. Further, the presence of fluid in the crevice appeared to be related to the changes in the gingival vasculature induced by the inflammatory process (Brill and Krasse 1958; Egelberg 1966).

1.4.2 Collection and quantification of GCF

Collection of GCF is site-specific, non-invasive and relatively easy to perform (Embery and Waddington 1994). Among the methods of GCF collection, the most common method is the use of pre-cut absorbent filter paper strips (Griffiths et al. 1988). In the extracrevicular method of sampling, the strips are fitted closely to the tooth surface, the gingival margin and attached gingiva (Brill and Krasse 1958; Løe and Holm-Pedersen 1965). In the intracrevicular method, however, the strips are placed at the entrance of the gingival crevice parallel to the tooth crown and then either inserted just into the crevice (Løe and Holm-Pedersen 1965) or inserted in an apical direction until mild resistance was felt (Brill and Björn 1959). The strips are left for various periods of time ranging from 3 sec to until the paper is saturated (Lamster et al. 1985; Offenbacher et al. 1993). The volume of GCF collected on paper strips can then be quantified using a disclosing dye (Brill and Björn 1959), weighing (Weinstein et al. 1967) or using an electronic device called Periotron (Alexander et al. 1996). Other collection methods include the use of capillary tubes and gingival washing, which is preferred for collecting the cells present in the crevice (Lamster and Grbic 1995; Salonen and Paunio 1991).

1.4.3 Constituents of GCF

In recent years, attention has focused on the constituents of GCF as potential diagnostic or prognostic markers of periodontal disease
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(Offenbacher et al. 1993; Embery and Waddington 1994; Lamster and Grbic 1995). GCF is now known to contain a wide range of biochemical components arising from i) microbial plaque, e.g., endotoxins, enzymes and metabolic end-products; ii) host inflammatory cells, e.g., enzymes degrading proteins and glycoproteins; iii) host factors, e.g., immunoglobulins, cytokines; and iv) host tissue breakdown products, e.g., collagen and collagen cross-links, proteoglycans and glycoproteins such as osteocalcin and osteonectin (Embery and Waddington 1994).

1.4.4 GCF as the periodontal wound fluid

The components present in GCF may, however, represent not only the products of tissue inflammation and breakdown but also tissue formation. Thus, GCF may also be considered as the fluid of healing periodontal wounds, and in recent years several investigators have attempted to find potential ‘healing’ markers in GCF. The levels of procollagen III aminoterminal propeptide (Talonpoika and Hämäläinen 1992), procollagen I carboxyterminal propeptide (Talonpoika and Hämäläinen 1993), fibrin and fibronectin (Talonpoika et al. 1993) have therefore been measured in GCF samples of patients with periodontitis before and after non-surgical periodontal therapy consisting of scaling and root planing. Ten days after periodontal treatment, all of these substances were found to be elevated, suggesting increased synthesis of types I and III collagen and degradation of the subgingival fibrin clot. Furthermore, non-surgical therapy reduced the levels of proteins (Mäkelä et al. 1991), interleukin-6 (Guillot et al. 1995) and bacterial proteases (Eley and Cox 1995) in GCF. Thus far however, growth factors have never been investigated in GCF following periodontal therapy, surgical or non-surgical.

1.5 SUMMARY AND AIMS

The periodontium is a highly specialised organ composed of soft connective tissues, the gingiva and periodontal ligament, and hard connective tissues, the cementum and alveolar bone. Periodontal diseases are chronic inflammatory diseases resulting in the breakdown of the periodontium and ultimately tooth loss. A number of clinical procedures have
been developed to restore damaged and lost periodontal tissues, the GTR technique thus far appeared to be the most promising. This treatment modality is based on the surgical insertion of a barrier membrane to physically exclude gingival epithelium and connective tissue from the root surface and to enable PL cells to re-populate the root surface and re-build the lost periodontal tissues. Histological studies showed that GTR therapy using both non-resorbable and resorbable membranes resulted in the formation of new PL with fibres inserting into the new cementum and new alveolar bone. Clinical studies in humans have also revealed significant clinical improvements such as reduction in PPD, gain in CAL and some degree of defect fill.

The histological events and clinical outcomes associated with the GTR procedure have been relatively well documented. However, clinically successful results depend on the presence of the appropriate tissue elements in the adjacent undamaged tissue. Since the type of healing is determined by the cells repopulating the wound area, the precise knowledge of the cells involved in periodontal regeneration are necessary to understand and modulate GTR processes. Although a few recent studies reported certain characteristics of the cells involved in GTR, their precise functional properties remain uncertain. For example, although these cells can form mineralised nodules in vitro, it is not yet known whether they directly produce major components associated with the soft and hard connective tissues. Since gingival and PL connective tissues appear to comprise sub-populations of cells with distinct phenotypes and functions, different sets of GTR cells may also have distinct roles during periodontal healing.

Periodontal wound healing requires the migration of cells with regenerative capability to the wound site, followed by the activation and proliferation of these cells and the synthesis of specific components of the ECM. However, very little is known about the basic biologic mechanisms which underlie the GTR process. While in vivo studies have demonstrated that growth factors either alone or in combination are beneficial for periodontal regeneration, the role of these factors in the healing wounds is still not clear. For example, these growth factors exert their function via binding to their respective receptors on the surface of target cells, but the
expression of these receptors have not yet been identified in regenerating periodontal tissues. Moreover, although the growth factors promote cell proliferation, migration and matrix synthesis in vitro and thus are likely to play a major role during various phases of the healing process in vivo, their temporal distribution and physiologically active levels in the periodontal wound are also not yet known. Such information is essential if they are to be valuable tools as potential markers for evaluating the healing process and for improving the efficacy of clinical treatment.

Thus, basic information about the phenotypic features of the cell populations and the underlying mechanisms that control cellular events is required to develop more successful regenerative techniques. The aims of the present study are therefore:

i) to examine and identify the phenotypes of the cells associated with periodontal regeneration,

ii) to isolate and investigate their functional activities in comparison with normal gingival and PL cells,

iii) to study the expression of certain growth factor receptors in normal and regenerated tissues of the periodontium, and

iv) to measure the levels of the growth factors TGF and PDGF in GCF collected from patients treated by either GTR or conventional surgical procedures.
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MATERIALS AND METHODS

2.1 CLINICAL METHODS

2.1.1 Subject population and ethical requirements

The patient population included subjects who had been referred to the clinics of the Eastman Dental Hospital by their dental practitioners for treatment of dental and/or periodontal problems. All were medically fit and in good general health. The protocol was approved by the Eastman Joint Research and Ethics Committee. Subjects were given written and verbal information about the nature and purpose of this project and their right to withdraw from the study at any time without it affecting their future treatment. Informed consent forms (Appendix 1) were signed by the participants prior to treatment.

2.1.2 Surgical procedures

The surgical procedures were performed by postgraduate students and staff of the Departments of Periodontology and Oral and Maxillofacial Surgery at the Eastman Dental Hospital. Periodontal surgical procedures were performed in the dental chair using local anaesthesia whereas tooth extraction procedures were performed under general anaesthesia.

2.1.2.1 GTR surgery

The patient population comprised 94 subjects (34 males and 60 females; 22 to 65 years of age; mean age 44.2 ± 10.2) with moderate to advanced periodontal disease who were referred to the Periodontology Clinic at the Eastman Dental Hospital. All subjects received initial phase periodontal treatment comprising oral hygiene instruction, scaling and root instrumentation. Subject inclusion was based on the following criteria at reassessment after completion of initial therapy: presence of at least one tooth with a residual probing pocket depth (PPD) of ≥5 mm associated with CAL of ≥5 mm and radiographic evidence of bone loss. Intraosseous 1-, 2- and 3-
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wall defects and combined defects as well as furcation defects were included.

Following intracrevicular incisions, full thickness mucoperiosteal flaps were raised on both buccal and lingual/palatal surfaces of the teeth and alveolar process. Vertical releasing incisions were placed as required for proper access to the defect area. Granulation tissue associated with the osseous defect and adherent to the alveolar bone was removed and the exposed root surfaces were instrumented. An ePTFE membrane (Gore-Tex periodontal material, W.L. Gore and Associates; Flagstaff, AZ, USA) with appropriate size and shape (interproximal, single tooth or wraparound) was placed so that it fully covered the defect area and extended 2-3 mm beyond the margin of the defect. It was then tightly adapted to the tooth by Teflon sutures. The flaps were repositioned and sutured to completely cover the membrane. Following surgery, the subjects were instructed to rinse with 0.2% chlorhexidine twice daily for a period of 2 weeks. The sutures were removed 2 weeks after surgery and professional tooth cleaning was performed at 2 week intervals for the first 6 weeks. The membranes were removed approximately 6 weeks after placement and the sutures were removed 1 week after membrane removal. The subjects were recalled for professional tooth cleaning 5 weeks after the last visit and then at 1 month intervals up to 6 months after the surgery.

2.1.2.2 Conventional flap surgery

Twelve patients (4 males and 8 females; 23-58 years of age; mean age 39.8 ± 9.4) volunteered to participate in this part of the study following completion of the initial phase of periodontal therapy, comprising oral hygiene instruction, scaling and root instrumentation. All subjects had moderate to advanced periodontal disease and required conventional flap (CF) surgery for at least one quadrant of either their maxilla or mandible.

Following reverse bevel incisions, mucoperiosteal flaps were raised on the buccal and lingual/palatal aspects of the teeth. Granulation tissue was removed and the exposed root surfaces were instrumented.
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Recontouring or resection of alveolar bone was not performed. The flaps were replaced to their original position and secured with sutures. For 2 weeks following surgery, subjects were advised to rinse their mouth with 0.2% chlorhexidine twice a day. The sutures were removed 1 week after the periodontal operation. The subjects were recalled for professional tooth cleaning routinely for up to 6 months.

2.1.2.3 Crown lengthening surgery

Crown lengthening surgery was performed on 9 quadrants of 7 patients (1 male and 6 females; 16 to 51 years of age; mean age of 36.4 ± 13.6) who had no evidence of periodontal disease. The gingival tissue was resected using reverse bevel incisions. Vertical releasing incisions were used when necessary to allow adequate elevation and mobilisation of buccal and lingual/palatal mucoperiosteal flaps. Approximately 2-3 mm of crestal alveolar bone was resected to lengthen clinical crowns by using a dental bur for gross reduction, leaving a thin plate of bone for removal with a bone chisel. The flap was apically repositioned and sutured. A periodontal dressing was placed. The dressing and sutures were removed after 1 week of periodontal operation and then the subjects were recalled for professional tooth cleaning routinely for up to 6 months.

2.1.2.4 Tooth extraction

Tooth extraction procedures were performed under general anaesthesia by staff of the Victor Goldman Unit, Department of Oral and Maxillofacial Surgery of the Eastman Dental Hospital. The subject population included 38 subjects (12 males and 26 females; 19 to 31 years of age; mean age 25.1 ± 4.9) undergoing removal of at least one tooth other than for periodontal reasons. Other criteria used in selection of teeth included no caries or endodontic involvement. A total number of 119 periodontally-healthy, fully-erupted mandibular/maxillary third molars (2 to 4 teeth per subject) were extracted according to the procedure described by Peterson et al. (1997). Briefly, an elevator was used first to help mobilise
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the tooth. Then forceps were adapted to the tooth, seated apically as far as possible, and the tooth was removed by applying the basic extraction movements.

2.1.3 Clinical measurements

An assessment of all teeth scheduled for periodontal surgical treatment or extraction was carried out initially in order to define their periodontal status. Tooth extraction and crown lengthening procedures were performed on periodontally healthy teeth which had PPD of \( \leq 3 \) mm with no bleeding on probing, and showed no evidence of radiographic bone loss. Only those teeth where GCF samples were to be collected were subjected to the more detailed periodontal examination described below.

2.1.3.1 Pre- and post-surgical clinical measurements

Clinical measurements were made to the nearest millimetre using an EN15 periodontal probe, by a single investigator immediately before the surgery and 6 months post-operatively. The measurements were recorded at 6 sites of each tooth, which were mesiobuccal, mid buccal, distobuccal, distolingual/palatal, mid lingual/palatal and mesiolingual/palatal. The clinical measurements recorded included the following (Cortellini et al. 1993a): 1) PPD, the distance from the gingival margin to the base of the periodontal pocket; 2) CAL, the distance from the cemento-enamel junction (CEJ) to the base of the pocket; and 3) recession of marginal gingiva (REC), the distance from the CEJ to the gingival margin (Figure 2.1).

2.1.3.2 Intra-surgical measurements

Intra-surgical measurements were recorded only for periodontal defects treated by GTR, immediately after debridement of the defects, as described by Cortellini et al. (1993b). These measurements, as shown in Figure 2.1, are i) the distance from the CEJ to the most coronal extension of the alveolar bone crest (CEJ-BC); ii) the distance from the CEJ to the base
of the defect (CEJ-BD); and iii) the defect depth (DD), defined as (CEJ-BD)-(CEJ-BC).

Figure 2.1. Illustration of the pre-surgical (1, 2 and 3) and intra-surgical measurements (4, 5 and 6) recorded for the GTR subjects. (1) Probing pocket depth, (2) clinical attachment level, (3) recession, (4) the distance from the CEJ to the most coronal extension of the alveolar bone crest, (5) the distance from the CEJ to the base of the defect, and (6) defect depth.

In addition, the distance from the CEJ to the most coronal extension of the newly-regenerated tissue (CEJ-RT) was recorded after membrane removal. The design of clinical measurements of the GTR group subjects is outlined in Figure 2.2.
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2.1 Procedure

Oral hygiene instruction, Scaling, Root instrumentation

Membrane placement

Membrane removal

Recall

<table>
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<th>Procedure</th>
<th>Time (Week)</th>
<th>Clinical measurement</th>
</tr>
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<tbody>
<tr>
<td>Oral hygiene instruction, Scaling, Root instrumentation</td>
<td></td>
<td>PPD, CAL, REC</td>
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<td>Membrane placement</td>
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<td>Membrane removal</td>
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<td>CEJ-RT</td>
</tr>
<tr>
<td>Recall</td>
<td>26</td>
<td>PPD, CAL, REC</td>
</tr>
</tbody>
</table>

Figure 2.2. The study design of clinical measurements recorded for the GTR subjects. The measurements are probing pocket depth (PPD), clinical attachment level (CAL), gingival recession (REC), the distance from the CEJ to the base of the defect (CEJ-BD), the distance from the CEJ to the alveolar bone crest (CEJ-BC), defect depth (DD) and the distance from the CEJ to the newly-regenerated soft tissue (CEJ-RT).

2.1.4 Sample collection

2.1.4.1 Tissue samples and retrieved membranes

A total of 34 clinically healthy gingival tissue samples were obtained from two separate sources. The first source was the gingival tissue (n = 7) resected from the subjects who underwent crown lengthening surgery, as described above in 2.1.2.3. The resected gingival tissue was placed into a tube containing a 'transport medium' (TM) consisting of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies Ltd.; Paisley, UK), 10% foetal calf serum (FCS) (PAA; Linz, Austria), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 0.25 μg/ml amphotericin B (Gibco).
The second source was the gingival tissues (n = 27) which remained firmly attached to the collar part of extracted, healthy third molars. Immediately after extraction as described in 2.1.2.4 above, the tooth was placed into TM. The attached gingiva was then removed from the tooth under sterile conditions using a scalpel.

PL tissue samples were obtained from 119 extracted, healthy third molars which had no apparent pathology (Piche et al. 1989; Hou and Yaeger 1993). After extraction the tooth was placed into TM. Under sterile conditions, the tooth was secured using forceps and the PL tissue scraped from the middle third of the root surface using a scalpel. Care was taken to avoid any gingival or apical connective tissue contamination.

A total of 98 ePTFE barrier membranes removed approximately 6 weeks after placement were collected from 94 subjects who were treated based on the principle of GTR, as described in 2.1.2.1. The membranes were immediately placed into TM and then treated in several different ways as described for each of the experiments. In some instances, membranes were divided into half and membrane pieces were then used for different types of experiments.

Samples of regenerated periodontal tissues (RT) underlying the ePTFE barrier membranes were obtained from 7 of the subjects treated by GTR. In these cases, a small portion of the RT was removed from the most coronal part of the newly-regenerated soft periodontal tissue after removal of the membrane, because excessive formation of this tissue was apparent. These RT samples were also placed immediately into TM.

### 2.1.4.2 Subject population and site selection for GCF collection

GCF samples were collected from subjects treated either with GTR or OF surgery. The GTR group included 24 subjects (7 males and 17 females; 24 to 59 years of age; mean age 40 ± 10) who received GTR treatment for at least one interproximal intraosseous defect, as described in 2.1.2.1. GCF samples were collected from sites designated as GTR test and GTR control sites. The GTR test sites, 2-4 per subject, were the sites adjacent to the
periodontal defect treated with ePTFE membrane and thus expected to show regeneration, as shown schematically in Figure 2.3. The GTR control sites were defined as sites within the same surgical field but at least one complete unit away from the defect and were therefore not associated with GTR treatment (Figure 2.3). These samples would thus represent a periodontal wound, but at a site not expected to show regeneration.

![Figure 2.3. Illustration of the GTR test sites (T) associated with the membrane (M) and the GTR control sites (C) where the GCF samples are collected.]

The CF group consisting of 12 subjects (4 males and 8 females; 23 to 58 years of age; mean age 39.8 ± 9.4) scheduled to receive periodontal surgical therapy, as described in 2.1.2.2. GCF samples were collected from 2-6 sites within the surgical field which had periodontal pockets of ≥ 5 mm.

2.1.4.3 GCF collection

GCF samples were collected from the GTR group immediately before surgery and at 2, 4, 6, 7, 12 and 26 weeks post-surgery, as shown in Figure 2.4. For CF group, the GCF was collected immediately before surgery and at 1, 6, 12 and 26 weeks post-operatively (Figure 2.4).
Figure 2.4. The design of GCF collection. GCF samples were collected at a number of time points from subjects of the GTR group and the conventional flap (CF) group.

The volume of GCF collected was determined by weighing the sample, using modifications of the method of Cimasoni and Giannopoulou (1988). Whatman 3 MM chromatography paper (Whatman Lab. Sales Ltd.; Maidstone, UK) was cut to 2x10 mm strips (Griffiths et al. 1988). Each strip was placed individually into a 250 μl microcentrifuge tube (Central Lab. Supplies; Basingstoke, UK) containing a plastic cup positioned half way down the tube, as shown in Figure 2.5. The inserted plastic cup had a hole in the middle to allow eluant to be collected in the bottom half of the tube (Figure 2.5). Prior to sampling, each tube including inserted cup and paper strip was weighed using a Cahn 25 microbalance (Cahn Instruments Inc.; Cerritos, CA, USA) accurate to within 0.01 mg. Repeat weighing of pre-sample variations showed no variation (< 0.02 mg) (Griffiths et al. 1998).
Figure 2.5. A microcentrifuge tube specially prepared for the storage of GCF absorbed paper strip. The tube has an inserted plastic cup with a hole in the middle. The paper strip is stored within this tube until the elution step.

Precautions were taken to prevent contamination of the paper strips with saliva and/or plaque (Griffiths et al. 1992). The area from which GCF was to be collected was isolated with cotton rolls and saliva was removed from the tooth surfaces and gingival margin using a fine bore, high power suction tip. Supragingival plaque, if present, was gently removed using a periodontal probe. Care was taken to avoid any mechanical injury to the gingival tissues. The paper strip was then placed at the entrance of the crevice (Löe and Holm-Pedersen 1965) and remained in position for 2 min in order to maximise the amount of fluid obtained. The strip was placed back into the same tube and reweighed. The amount of GCF taken onto the strips was calculated by subtracting the pre-sampling weight from the post-sampling weight. The weight of the fluid was converted to volume by assuming that the density of GCF was 1.0 (Cimasoni and Giannopoulou 1988). For each subject in each group, the strips were pooled at each time of sampling. For the GTR group, the pooled samples of the control sites were kept separate from the test site samples at each time point. All samples were stored in tubes at -70°C prior to use.
2.1.4.4 Blood

10 ml of venous blood was collected from the GTR and CF subjects who had provided GCF samples. These were taken prior to surgery and also on the day of membrane removal for the GTR group, and treated according to the method of Hudson and Hay (1989). Briefly, the samples were allowed to clot at room temperature for 1 h. Once the clot formed, it was loosened from the walls of the container using an orange wood stick to aid clot retraction. The serum was collected, transferred to a 15 ml conical tube (Falcon, Becton and Dickinson; Oxford, UK) and centrifuged at 750 rpm for 5 min. The serum was again transferred to another tube, followed by centrifugation at 1250 rpm for 15 min. Serum samples were transferred to a new tube and heated in a water bath at 56°C for 30 min to destroy the heat-labile components of complement. The samples were then stored at -70°C prior to use.

2.2 LABORATORY METHODS

2.2.1 Preparation and histological examination of tissues

2.2.1.1 Preparation of fresh-frozen tissue samples

The samples of gingiva, PL, RT and retrieved ePTFE membrane were washed in phosphate-buffered saline (PBS) (Gibco), oriented onto cork discs and embedded in Tissue-Tek OCT (Miles Inc.; Elkhart, IN, USA). The gingival samples were oriented so that each section to be cut contained both epithelium and connective tissue. The PL tissue samples scraped from the root surfaces of extracted teeth of the same individual were placed together in embedding material (Berkovitz et al. 1997). The membrane samples were placed in a longitudinal orientation into embedding material so that any tissue attached to both sides of membrane could be examined. The samples were frozen immediately by immersing into liquid N₂ for 1-2 min and then stored at -70°C prior to use.

The 7 µm thick sections were cut using a cryostat (5030 Microtome, Bright Instrument Co. Ltd.; Huntingdon, UK) and transferred to glass slides
(BDH Lab. Supplies; Poole, UK), left at room temperature overnight to dry and then either used the day after or stored at -70°C prior to use.

2.2.1.2 Preparation of a paraffin-embedded RT sample

One of the 7 RT samples was prepared for histological examination by the Department of Oral Pathology at the Eastman Dental Institute. This tissue sample was washed in PBS and immediately placed into 4% paraformaldehyde for 24 h. The sample was then dehydrated in an ascending series of alcohol (70, 90, 100 and 100% of alcohol; 45 min for each step) and immersed in xylene (Solmedia; Romford, UK) 3 times, for 2 h each. The paraffin waxing process was carried out at 65°C for 3 h followed by for a further 2 h under vacuum at 65°C. The sample was embedded in fresh paraffin (Solmedia), 5 μm thick sections were cut using a microtome (Anglia AS200, Life Science International; Runcorn, UK), collected on glass slides and stored at room temperature prior to use.

The sections were de-waxed and dehydrated immediately prior to staining, as follows. The sections were warmed at 60°C for 10-15 min and, while still hot, immersed in xylene at room temperature for 30 min. The sections were then dehydrated in ascending concentrations of alcohol, as described above.

2.2.1.3 Haematoxylin and eosin (H&E) staining

The sections of both fresh-frozen and paraffin-embedded specimens were stained with H&E according to the protocol of Drury et al. (1967). They were first stained with Mayers’ haematoxylin for 1 min, followed by washing in running tap water for 2-3 min. Excess stain was removed by differentiating in 1% HCl in 70% alcohol for 10 sec, which changed the haematoxylin from blue to red. After washing in running tap water for 5 min, the blue colour was regained and the sections were then counter-stained with 1% aqueous eosin for 2 min. Excess stain was washed off with water and the sections were dehydrated in ascending concentrations of alcohol (from 70 to 100%), dried for 2-3 min and mounted in DePeX (Merck Ltd.;
Poole, UK). The sections were examined under the light microscope (Olympus BX 50; Japan) and photographs were taken on Kodak 64T film using an Olympus PM-C35DX camera fitted with an Olympus PM-30 Exposure Control Unit.

2.2.1.4 Immunohistochemical staining

An indirect immunostaining technique was used for the sections of the fresh-frozen tissue samples. The sections were fixed with ice cold acetone-methanol (1:1 v/v) for 10 min and washed three times with PBS, for 5 min each. Circular wax lines were made around each individual section on the slide using a Dakopen (Dako; Glostrup, Denmark), in order to prevent overflow of the antibody and to minimise the amount of antibody required. The sections were then treated with 0.3% H₂O₂ in methanol for 10 min to inhibit endogenous peroxidase and washed with PBS.

The following incubations were carried out, all in a humidified chamber at room temperature. The sections were treated with 20% normal goat serum (NGS) (Gibco) in PBS for 30 min to block non-specific binding of antibody. After washing three times in PBS, the sections were incubated with the primary antibodies diluted in PBS containing 20% NGS for 1 h. Non-specific mouse immunoglobulin G₁ (IgG₁) antibody (Dako) was used as negative control. They were washed and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:100 in PBS containing 20% NGS) for 1 h. After washing, they were incubated with 3,3’-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co.; St. Louis, MO, USA) for 10 min and the reaction terminated by adding PBS. The sections were counter-stained with Mayers’ haematoxylin for 45 sec and left under running tap water for a further 10 min. They were dehydrated in ascending concentrations of alcohol, dried for 2-3 min at room temperature and mounted in DePeX. The sections were examined and photographs taken as described above.
2.2.2 RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

The retrieved membrane samples were washed extensively with PBS and RNA extracted from them according to the method of Chomczynski and Sacchi (1987). Briefly, the samples were placed individually into 1 ml disposable polypropylene tubes and 200 µl of a denaturing solution was added which consisted of 4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl and 0.1 M β-mercaptoethanol. Then 20 µl of 2 M sodium acetate (pH 4), 200 µl of water-saturated phenol and 40 µl of chloroform-isooamyl alcohol (49:1, v/v) were added in sequence. The mixtures were shaken vigorously for 15 sec, cooled on ice for 10 min and the tubes centrifuged at 13 000 rpm for 10 min. After centrifugation, the RNA remained in the aqueous phase, while DNA and proteins were in the interphase. 200 µl of the aqueous phase was removed and transferred to a polypropylene tube, mixed with 200 µl of isopropanol and left at room temperature for 10 min to precipitate the RNA. After centrifugation at 13 000 rpm for 20 min, the RNA pellet was resuspended in 50 µl of diethyl pyrocarbonate (DEPC)-treated water.

The reverse transcriptase reaction was then performed, using all the RNA obtained, in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol (DTT) (Sigma), 3 mM MgCl₂, 500 µM of each dioxynucleotide triphosphate (dNTP) (Boehringer Mannheim UK Ltd.; Lewes, UK), 1µg of oligo deoxythymidine (dT₁₈) (Boehringer) and 50 U of reverse transcriptase (Stratagene Ltd.; Cambridge, UK) in a total volume of 50 µl. The reaction was incubated at 37°C for 1 h. PCR amplification to detect the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Ercolani et al. 1988), was performed using 5 µl of the cDNA products, 1 U of Taq polymerase (Stratagene) in 20 mM Tris-HCl (pH 8.7), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 1 mg/ml bovine serum albumin and 50 pM of each of the dNTPs, in a final volume of 50 µl. The sequence of the sense primer was 5'-CACCCATGGCAAATTCCATGGCA and that of the antisense primer 5'-TCTAGACGGCAGGTGCAGTCCACC. The PCR amplification
programme consisted of initial denaturation at 94°C for 3 min, followed by 35 cycles each consisting of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, extension at 72°C for 1.5 min and a final extension at 72°C for 10 min. The amplified products were detected, following electrophoresis in a 2% agarose gel, by staining with ethidium bromide (Sigma). A 1 Kb DNA ladder (BRL; Paisley, Scotland) was used as a DNA size marker.

2.2.3 Transmission electron microscopy (TEM)

The preparation of membrane samples for TEM examination was carried out by the Electron Microscopy Unit at the Eastman Dental Institute.

Twelve retrieved membrane samples were washed extensively with PBS. They were cut into 3 pieces corresponding to the coronal, middle and apical regions, then fixed in 2.5% glutaraldehyde (v/v) in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 3 h. Specimens were subsequently post-fixed in similarly buffered 1% osmium tetroxide at 4°C for 2 h. They were then dehydrated in ascending concentrations of alcohol (20, 50, 70, 90 and 100%), infiltrated with propylene oxide (3 x 10 min changes) and embedded in Araldite (Agar Scientific Ltd.; Stansted, UK). In order to check the orientation of the specimen, semi-thin sections (1 μm) were cut with a glass knife and stained with 1% toluidine blue. Then ultra-thin sections (90-100 nm) were cut on a Reichert-Jung Ultramicrotome (Cambridge Instruments; Cambridge, UK) and collected on 200 mesh formvar carbon-coated copper grids (Agar). The sections were stained with uranyl acetate and Reynold’s lead citrate (Reynolds 1963). Both sides of the membrane, i.e. the external side facing the gingival flap and the internal side facing the root and regenerating periodontal tissue, were examined by TEM in a JEOL 100CX II (JEOL; Hertfordshire, UK). The images were recorded on Kodak EM film 4489 (Kodak; New York, USA).
2.2.4 Cell culture

2.2.4.1 Preparation of explants

Gingival cells were obtained from healthy gingiva of subjects undergoing crown lengthening surgery and from the collar part of extracted teeth (Somerman et al. 1988). The PL cells were obtained from the PL tissue scrapped from the middle third of root surfaces (Rose et al. 1987) and RT cells from the RT of GTR subjects. The membrane-associated cells (M cells) were obtained from retrieved ePTFE membranes and also from the cells which had dissociated from the membrane into the TM during transportation. The TM was therefore centrifuged in order to collect these dissociated cells, as described below.

2.2.4.2 Culture and maintenance of cells

All cell culture procedures were performed under sterile conditions. The explant samples were removed from TM and washed with PBS supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. The gingival samples were cut into 1-2 mm³ pieces, whereas both the PL and RT samples were cut into pieces less than 0.5 mm³ using a sterile scalpel. The cut tissues were washed twice as described above. They were collected and placed into separate sterile 25 cm² tissue culture flasks (Nunc; Denmark) and incubated in 2 ml of culture medium consisting of alpha Minimum Essential Medium (α-MEM) (Gibco) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

The collar part of the membrane was dissected and discarded and any Teflon sutures which remained attached were removed. The remaining part of the membrane was cut into approximately 4x4 mm pieces and washed as described above. Several different approaches were developed and used in order to obtain the M cells. In one, each of the membrane pieces was placed into a well of a 6-well tissue culture plate (Falcon, Becton Dickinson Labware; Oxford, UK) and overlaid with a glass cover slip No. 1 (BDH). This prevented the membrane pieces from floating and also enabled
the cells attached to both the internal and external sides of the membrane to be obtained on the surfaces of the dish and cover slip. In a second procedure, the surface of the retrieved membrane was scrapped with a scalpel and dislodged cells collected and cultured. In another approach, 2 to 3 membrane pieces obtained from the same individual were placed into 25 cm² tissue culture flask, 5 ml of culture medium added and the flask gently shaken to help dissociate the membrane-attached cells. The other source of M cells was the cells which became dissociated during transportation and remained in the TM. These cells were obtained from the TM by centrifugation at 1100 rpm for 10 min, the pellet washed, centrifuged and resuspended in the culture medium. The medium used for the initial culture of the M cells contained additional amounts of amphotericin B (0.25 μg/ml) and ampicillin (100 μg/ml) (Sigma) to minimise fungal and bacterial contamination.

Incubation of explants was carried out at 37°C in a humidified atmosphere of 5% CO₂. After 7 days of incubation, 3 ml of additional culture medium was gently placed into each flask. The cultures were examined using a phase-contrast microscope (Telaval 31, Carl Zeiss; Germany) and the culture medium was changed every 2 or 3 days until the cells around the explant fragments were observed to be confluent. The cultures were then passaged as follows. The culture medium was removed and the cells washed twice with PBS and incubated with 2 ml of trypsin-ethylenediamine tetra acetic acid (EDTA) (0.25% trypsin, 1 mM EDTA) (Gibco) at 37°C for 5 to 10 min. The flasks were shaken vigorously in order to detach the cells from the surface. When the cells became round and were observed, under the phase-contrast microscope, to be floating in the media, 8 ml of fresh culture medium was added to neutralise the trypsin. Cells still remaining attached to the surface of the flask were obtained by vigorous pipetting of the neutralised medium. The cell suspension was then centrifuged at 1100 rpm for 7 min. The supernatant was removed and the pellet resuspended in the culture medium and re-cultured in 80 cm² tissue culture flasks (Falcon) until confluent monolayers were again obtained. These cells were defined
as the first passage. The cells from all samples were expanded by repeated sub-culture after diluting 1:4 and re-incubating until confluence was again achieved.

An aliquot of each sample at early passage was stored to use the cells for future experiments as follows. After trypsinization of the cells, as described above, the pellet was resuspended in the culture medium containing 0.5% (v/v) dimethyl sulfoxide (DMSO) (Sigma). Approximately 1 ml of this suspension containing a high density of cells (approximately 5x10^5-10^6) was placed into cryotube vials (Nunc), which were stored in a pre-freezing container (Marathon; London, UK) at -70°C for 24 h. The vials were then transferred to liquid N\textsubscript{2}. The cells were used between the third and eighth passages in the experiments described below.

2.2.4 Cell viability

The cultured cells were examined daily by phase-contrast light microscopy and the viability determined by trypan blue exclusion, as follows. After the cells were trypsinized, centrifuged and resuspended, 10 µl of the cell suspension was mixed with 10 µl of trypan blue (38%) (Sigma). Then 10 µl of this mixture was placed in the calibrated chamber under the cover slip of a Neubauer haemocytometer (Weber, UK) and examined under the light microscope. The viable cells, which were seen as bright and colourless, and the dead cells, which were seen as dark blue, were counted in the haemocytometer.

2.2.4.4 Cell growth

The growth of the cell cultures was evaluated at 0, 2, 4, 6, 8 and 10 days. Day 0 was designated as the day after the cells were seeded into culture flasks. The cells were placed in duplicate into 25 cm\textsuperscript{2} flasks containing 5 ml of culture medium at an initial density of 10^5 cells per flask. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}, with the media being changed every second day. For determination of
cell numbers, the cells were washed twice with PBS, harvested using 0.25% trypsin and 1 mM EDTA and counted as described above (section 2.2.4.3).

2.2.5 Immunocytochemical staining of antigens in cultured cells

The qualitative examination of cellular antigens in the cultured cells obtained from the M, RT, PL and gingival explants was carried out using an indirect avidin-biotin complex (ABC) immunostaining technique. Approximately 500 cells were seeded onto glass cover slips (22x22 mm) previously placed into the wells of 6 well-plates. The cultures were grown to log phase, washed with PBS and fixed in ice-cold acetone-methanol (1:1) for 10 min. They were treated with 10% NGS for 30 min at room temperature to block non-specific binding and with 0.1% saponin (Sigma) in Tris-buffered saline (TBS) to permeabilise the cell membranes. After each incubation step, the cells were washed 3 times for 5 min each with TBS. Primary monoclonal antibodies (mAbs) were diluted in TBS containing 10% NGS and 0.1% saponin. Non-specific mouse IgG1 antibody (Dako) was used as negative control. Incubation with primary antibodies was carried out for 1 h at room temperature. Following incubation with biotin-conjugated secondary antibody (anti-mouse antibody raised in rabbit) diluted in TBS, 10% NGS and 0.1% saponin for 1 h, the HRP-conjugated avidin (Dako) was applied and incubated for 30 min. Approximately 100 µl of DAB substrate solution was added and the reaction terminated after 5 min by adding TBS. The development of a brown colour indicated the expression of the antigen. The cells were counter-stained with Mayers’ haematoxylin, the cover slips removed from the dishes and mounted in DePeX and examined by light microscopy.

2.2.6 Alkaline phosphatase (ALP) activity

The ALP activity of the cultured cells was measured colourimetrically using para-nitrophenyl phosphate as the substrate (ALP kit 104-LL; Sigma). This substrate is hydrolysed by the enzyme ALP to para-nitrophenol and inorganic phosphate. Under alkaline conditions, para-nitrophenol is
converted to a yellow product and its absorbance is subsequently measured at 405 nm ($A_{405}$).

The cells were seeded into 96-well plates at a density of $10^4$ cells per well and incubated in culture medium in the absence and presence of $10^{-6}$ M dexamethasone (DEX) (Sigma). Wells containing culture medium only were used as controls. For each of the cell lines, 6 replicate wells each of the untreated and DEX-treated cells were used in these experiments. After 4 and 7 days of incubation, the cells were washed twice with PBS and solubilised by the addition 50 µl/well of 1% Triton-X (BDH) for 20 min. Then 50 µl of the assay mixture (pH 10.3), consisting of 1.5 mM 2-amino-2-methyl-1-propanol (Sigma) and 4 mM para-nitrophenyl phosphate disodium (Sigma) was added to each well. The mixture was incubated for 30 min at 37°C and the reaction was terminated by adding 150 µl of 1 M NaOH. The release of para-nitrophenol from para-nitrophenyl phosphate, corresponding to the relative amount of ALP activity was determined by measuring the $A_{405}$ using a spectrophotometer (Titertek Multiskan Plus). The unit of ALP activity was determined using a standard curve of the $A_{405}$ of the reaction product, as described below.

The standard curve was prepared individually for each experiment. Dilutions were made of a standard by adding 50 µl of para-nitrophenol (10 µM/ml) to 10 ml of 0.02 M NaOH and then making serial dilutions of this solution, according to the manufacturer’s (Sigma) instruction. The standard dilutions, prepared fresh for each experiment, were placed into duplicate wells and the $A_{405}$ was measured, as described above. The standard curve was constructed by plotting the $A_{405}$ versus Sigma Units. The International Units (U/L) of the experimental samples were calculated from this standard curve by multiplying by 16.7.

2.2.7 von Kossa staining

In order to detect calcium phosphate nodules in cultures, von Kossa staining was used (Wakabayashi et al. 1996). This method is based on the principle of substituting silver for calcium ions, followed by exposure to light
until the silver salts are reduced to metallic silver when they can be visualised as a dark brown colour. The cells were seeded into 6-well plates at a density of $3 \times 10^4$ cells per well and incubated in culture medium until they reached confluence. Then the wells were divided into 3 groups and the media were replaced with either i) nodule growth medium consisting of culture medium supplemented with 50 μg/ml ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma); or ii) nodule growth medium containing also $10^{-8}$ M DEX; or ii) culture medium only which was used as control. The media were replaced every other day for a further period of 2 and 4 weeks at which time the cells in duplicate wells were examined for the formation of mineralised nodules by von Kossa staining (Wakabayashi et al. 1996). The medium was removed and the cells were fixed with acetone-methanol (1:1) for 10 min. After washing with distilled water twice, for 5 min each, the cells were exposed to 5% aqueous silver nitrate (Sigma) for 30 min in the dark. They were then exposed to light for 60 min. The cell cultures were examined using phase-contrast light microscopy for the presence of dark brown nodules and micrographs were taken on a black and white film (TMAX 100, Kodak; Japan) using Yashica 108 camera (Japan).

The von Kossa staining was also performed on tissue sections after fixing with acetone-methanol (1:1) for 10 min and following the protocol described above.

2.2.8 Examination of cells using flow cytometry (FCM)

2.2.8.1. The principles of the FCM

FCM is a technique in which certain physical and chemical features of individual cells are measured simultaneously as they move in a fluid stream past a fixed laser beam (Shapiro 1988). The excitation source of the instrument used in this study (the Becton Dickinson FACScan flow cytometer; Oxford, UK) was an argon-ion laser. The physical characteristics of the individual cells are measured by the scattering of the incident laser light (Figure 2.6). The light reflected at low angles by the particles (i.e., the cells) is detected along the axis of the incident light in the forward direction.
and referred to as forward scatter (FSC) (Figure 2.6). This is considered to be proportional to the relative size of the cells (Ormerod 1994). The light reflected at greater angles is detected at 90° to the light axis and is referred to as orthogonal or side scatter (SSC) (Figure 2.6). This is proportional to the relative cytoplasmic granularity or internal complexity of the cells (Ormerod 1994).

![Figure 2.6](image)

Figure 2.6. The laser light applied to a cell in the flow cytometer is reflected at various angles. Forward scatter is detected along the axis of the incident light whereas side scatter detected at 90° to the light axis.

The cells can also be treated with fluorochrome reagents which react with specific cellular components such as DNA and cell-associated proteins (Bou-Gharios et al. 1994; Kuru et al. 1998a; Lopes et al. 1998). When the laser light is applied to the cell, the fluorochrome absorbs the energy from the laser and subsequently releases this energy by emitting fluorescence (Shapiro 1988), which is measured by a different optical detector. The intensity of the fluorescence is considered to be proportional to the quantity of the bound fluorochrome. The development of fluorochrome-conjugated antibodies has thereby enabled the relative level of many specific cell antigens to be measured quantitatively.

### 2.2.8.2 Preparation of the cells for FCM

Confluent cultures were prepared for FCM according to the method of Bou-Gharios et al. (1994). Briefly, the cells were washed twice with PBS,
detached using 20 mM EDTA (pH 7.2) (Sigma) in PBS at 37°C for 10 min and scraping with a rubber policeman. It was necessary to use EDTA instead of trypsin to detach the cells because trypsin is likely to remove the extracellular domain of many cell surface-associated proteins. The cells were centrifuged at 1100 rpm for 7 min and fixed with 1% paraformaldehyde in PBS for 30 min, followed by permeabilisation with 0.1% saponin for 10 min. This procedure enables antibodies, which are of high molecular weight, to enter the cells and react with intracellular antigens, thus enabling both these and cell surface antigens to be measured (Sumner et al. 1991; Bou-Gharios et al. 1994).

The cells were washed in buffer containing PBS, 2% FCS and 0.05% sodium azide (ICN Medicals; Ohio, USA) (PBS/FCS) and centrifuged at 1100 rpm for 10 min. Aliquots of 10^5 cells were placed into separate round bottom polystyrene tubes (Becton Dickinson Labware; Oxford, UK). The primary antibodies (i.e., mouse mAb or rabbit polyclonal antibody), diluted in PBS/FCS containing 0.1% saponin, were added and the suspension incubated at room temperature for 60 min. Mouse IgG1 served as negative control for mouse mAbs while PBS/FCS only served as negative control for rabbit polyclonal antibodies. The cells were washed and centrifuged as described above. Fluorescein isothiocyanate (FITC)-labelled secondary antibody (Dako), diluted in washing buffer containing 0.1% saponin, was then added at room temperature for 30 min. Following the washing and centrifugation steps described above, the cells were re-suspended in 400 μl of PBS/FCS. The size, granularity and the fluorescence intensity of 10 000 individual cells were measured by FCM using the FACScan flow cytometer.

### 2.2.8.3 Settings and acquisition

Before the measurements were made, the electronic system of the instrument was optimised as follows. One of the negative control samples was introduced to the flow cytometer. As the properties of the cells in this sample were being detected by the instrument, the electronic system was adjusted for collecting the data. The Voltage of each detector was set
Chapter 2

individually and subsequent fine adjustments were made by setting the Amplifier Gain, as shown in Table 2.1. A linear Mode of data collection was used for the FSC and SSC parameters, while a logarithmic mode was used for the fluorescence parameter, as suggested by the manufacturer of the FACScan in order to span the range of values obtained (Table 2.1). The Threshold, which is the lower level of electronic detection, was adjusted to 52 for the FSC parameter. These settings were kept constant throughout all experiments performed in this study.

Table 2.1. The electronic settings of the flow cytometer.

<table>
<thead>
<tr>
<th>Detector</th>
<th>Voltage</th>
<th>Amplifier Gain</th>
<th>Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSC</td>
<td>E-1*</td>
<td>4.26</td>
<td>Linear</td>
</tr>
<tr>
<td>SSC</td>
<td>326</td>
<td>1.00</td>
<td>Linear</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>417</td>
<td>-</td>
<td>Logarithmic</td>
</tr>
</tbody>
</table>

* signal is multiplied by 0.1

Acquisition is the function of collecting and storing the data. During the acquisition, the samples as a single cell suspension are introduced into the instrument through an injection port which delivers 60 μl/min of sample through the sample chamber. Each cell is subjected to the laser beam as it passes through the sample chamber. The optical data representing the parameters of the FSC, SSC and fluorescence intensity are collected by the detectors and the electronic signals transmitted to a computer (Quadra 650, Apple Macintosh) and stored.

2.2.8.4 Analysis of the FCM data

Analysis of stored data was performed using the CELLQuest Software programme (Becton Dickinson). For the first stage of analysis, a FSC versus SSC dot plot was produced. The cells serving as negative control were displayed on a dot plot diagram, each cell being represented by a single dot. A 'gate' was then created by manually selecting the majority of
cells in the population, while at the same time eliminating cell aggregates
and cell debris by leaving them outside the gate. The software programme
subsequently generated the arbitrary units of FSC and SSC parameters for
the cells within the selected gate.

The second stage of analysis measured the fluorescence intensity of
the sample corresponding to the relative level of the cell-associated antigen.
A histogram, which is a frequency distribution of arbitrary fluorescence
levels on a logarithmic axis, was displayed for the negative (control) cells
which had been treated with non-specific primary antibody or PBS/FCS
without primary antibody. The negative and positive areas were then
designated manually. These areas were also applied to the histograms of
the cells treated with the specific primary antibody. The arbitrary units of the
average fluorescence intensity (AFI) and the percentage of negative and
positive cells were then generated by the software programme.

2.2.9 Analysis of GCF and serum samples

2.2.9.1 Elution of GCF samples

The GCF blotted paper strips within the tubes, which had been stored
at -70°C, were allowed to thaw at room temperature for at least 30 min.
Elution of the GCF samples was carried out by a centrifugal method
(Griffiths et al. 1988). Briefly, 50 µl of PBS was added to each tube followed
by centrifugation at 11 000 rpm for 15 min. This elution step was repeated,
so a total volume of 100 µl of eluant was collected at the bottom of each
tube after centrifugation. The strips were discarded and the GCF eluants
transferred to 500 µl polypropylene tubes and stored at 4°C for not more
than 24 h prior to use in the experiments described below.

2.2.9.2 Detection of PDGF-AB by sandwich (‘capture’) ELISA

The levels of PDGF-AB in the GCF samples were determined also
using a commercially available capture enzyme-linked immunosorbent assay
(ELISA) kit (Quantikine DHD00, R&D Systems; Minneapolis, MN, USA). As
mentioned previously in section 1.3.6.1, PDGF consists of two chains, A and
B, which form three compositions of isoforms as AA, AB and BB. The principle of this assay was based on sandwiching the samples and standards between a mAb for PDGF-AA, which had been pre-coated onto a microtiter plate, and a polyclonal antibody for PDGF-BB. In the first step of the assay, both AA and AB isoforms of PDGF molecule bind to the mAb on the plate. In the second step, the polyclonal antibody used in this assay binds to the AB but not AA isoform because it recognises only the B chain. The only form which can be detected by this assay is, therefore, the PDGF-AB.

The eluted GCF samples (40 µl of each) were diluted a further 5-fold with PBS, bringing the final volume to 200 µl. The standards of recombinant human PDGF-AB were prepared, according to the manufacturer's instructions, at concentrations of 0, 31.2, 62.5, 125, 250, 500, 1000 and 2000 pg/ml. An aliquot of 50 µl of assay diluent was added into each well of 96-well plate which was pre-coated with mAb against PDGF-AA. 200 µl of sample and of standards were then added, covered with an adhesive strip and incubated for 2 h at room temperature. Each well was aspirated and washed by filling with 400 µl of wash buffer and then removing it completely. This washing procedure was repeated for a total of 3 times. Then 200 µl of HRP-conjugated rabbit polyclonal antibody against PDGF-BB was added to each well and the plate covered with a new adhesive strip and incubated for 2 h at room temperature. The plate was washed 3 times, as described above. The substrate solution (tetramethylbenzidine/H₂O₂) (200 µl per well) was added, incubated for 20 min at room temperature and the reaction was terminated by adding 50 µl of 2 M H₂SO₄. A₄₅₀ was determined by a microtiter plate reader (Titertek). The reading was repeated at the wavelength of 570 nm and subtracted from the reading at 450 to correct for optical imperfections in the plate.

2.2.9.3 Detection of TGF-β1 by sandwich (capture) ELISA

The levels of the growth factor TGF-β1 in the GCF and serum samples were determined using a commercially available sandwich ELISA
kit (Quantikine DB100, R&D Systems; Minneapolis, MN, USA). In this assay, the soluble TGF-β1 receptor which binds to active TGF-β1 had been pre-coated onto a 96-well polystyrene microplate. In order to activate latent TGF-β1 present in the GCF and serum samples so that it becomes immunoreactive and eventually detectable by this assay, the samples were treated as follows. An aliquot of 40 μl of each sample was transferred to a polypropylene tube and activated by adding 40 μl of 2.5 M acetic acid/10 M urea. After mixing and incubation at room temperature for 10 min, the acidified sample was neutralised by adding 40 μl of 2.7 M NaOH/1M HEPES and mixed. The activated GCF and serum samples were further diluted 4- and 10-fold, respectively, with a diluent provided by the manufacturer of the assay kit.

Standards of recombinant human TGF-β1 were prepared, according to the manufacturer's instructions, at concentrations of 0, 31.2, 62.5, 125, 250, 500, 1000 and 2000 pg/ml. An aliquot of 200 μl of standard and of the activated samples were added to each well of the pre-coated microplate, the plate was then covered with an adhesive strip and incubated at room temperature for 3 h. Each well was aspirated and washed with 400 μl of wash buffer provided by the kit, repeating the process twice for a total of 3 washes. After adding 200 μl of HRP-conjugated polyclonal antibody against TGF-β1, the plate was covered with a new adhesive strip and incubated at room temperature for 1.5 h. The aspiration and washing steps were repeated, as described above. Following addition of 200 μl of a substrate solution (tetramethylbenzidine/H₂O₂) into each well and incubation at room temperature for 20 min, 50 μl of 2 M H₂SO₄ was added to stop the reaction. A₄₅₀ was measured using a Titertek spectrophotometer reader. The plate was also read at 570 nm and this reading was subtracted from the reading at 450 nm in order to correct for optical imperfections in the plate.
CHAPTER 3
PHENOTYPIC EXAMINATION OF CELLS INVOLVED IN GTR

3.1 INTRODUCTION

It has been suggested that the nature and structure of a healing wound is determined to a large extent by the cells that repopulate the wound area (Melcher 1976). Thus, the phenotypes of the repopulating cells play a crucial role in determining the successful outcome of periodontal wound healing after surgery, including GTR surgery. The external surface of a barrier membrane, when inserted surgically to promote periodontal regeneration, comes into contact with the gingival epithelium and connective tissue. The internal surface, however, faces the root surface of the tooth, the periodontal defect and alveolar bone, and subsequently the newly-regenerated periodontal tissue when the membrane has been left in situ for 6 weeks (Pritlove-Carson et al. 1994; O'Neal et al. 1994; Amar et al. 1997; Lekovic et al. 1998). These surrounding host tissues play a part in the GTR process and some of these may remain adherent or attached to the barrier membrane when it is surgically removed. However, fibroblast-like and epithelial-like cells were found to be associated with both surfaces of the retrieved membranes when examined by immunohistochemistry (Pritlove-Carson et al. 1992), scanning electron microscopy (SEM) (Selvig et al. 1990; Selvig et al. 1992) and TEM (Grevstad and Leknes 1992).

Recent light microscopic, electron microscopic and microbiological cultivation studies have also shown that oral bacteria colonised the membranes which may become partially exposed during the 6-week retention period (Selvig et al. 1990; Selvig et al. 1992; Grevstad and Leknes 1993; Tempro and Nalbandian 1993; Nowzari and Slots 1994; Nowzari et al. 1995; Novaes Jr. et al. 1995; Nowzari et al. 1996). The distribution and composition of microbial species on barrier membranes have been studied in detail, although little data are available on the specific host cells associated with the membranes.
Chapter 3

Periodontal regeneration is a complex wound healing process which involves specific cells and factors at different phases of healing. As mentioned in section 1.3, early wound healing comprises a fibrin clot and platelets, neutrophils, macrophages and lymphocytes (Polson and Proye 1983; Messadi and Bertolami 1991). Secretion of biologically active substances by these cells at this phase of healing results in the stimulation of mesenchymal, endothelial and epithelial cells around the wound margin, which then migrate into the wound and proliferate (Ighlaut et al. 1988; O'Neal et al. 1994).

After the placement of an ePTFE membrane over the periodontal defect, the wound area underlying the membrane is filled by a soft, jelly-like tissue as observed at the time of membrane removal (Tonetti et al. 1993; Cortellini et al. 1993a). This newly-formed tissue was found to be composed of epithelial and mesenchymal cells (Pritlove-Carson et al. 1994) and ECM proteins which comprise the soft (Pritlove-Carson et al. 1994; Matsuura et al. 1995) and hard connective tissues (Amar et al. 1995; Matsuura et al. 1995; Amar et al. 1997). Despite a number of clinical studies of GTR, the biological events taking place during periodontal regenerative wound healing and the nature and structure of the soft tissue that forms underneath the barrier membranes are still not completely known, thus limiting the development of successful regenerative techniques. The aims of this part of the study were therefore:

i) to determine whether any host cells remained adherent to the membranes after they were removed from the patients;
ii) to determine the morphological features of the cells associated with the retrieved membranes by TEM; and
iii) to examine and identify the phenotypes of the cells present in the newly-regenerated tissue (RT) in comparison with normal gingival and PL tissues.
3.2 MATERIALS AND METHODS

3.2.1 Preparation and examination of membranes and tissue samples

Five retrieved ePTFE membranes (single tooth), 7 RT, 4 healthy gingiva and 4 PL tissues were prepared for histological examination as described in 2.2.1.1 and 2.2.1.2. Tissue sections of all these samples were stained with H&E, as described in 2.2.1.3.

Fifteen retrieved membranes (8 interproximal, 1 single tooth and 6 wraparound) were used to extract mRNA, as described in 2.2.2. After the reverse transcriptase reaction was performed using all the RNA obtained, PCR amplification was carried out to detect the GAPDH gene, as described in 2.2.2, in order to determine the presence of intact membrane-associated cells.

Twelve retrieved membranes (8 interproximal, 3 single tooth, 1 wraparound) were used to examine the cells which remained attached to the membrane at the ultrastructural level. The interproximal membranes were cut through the interproximal area, obtaining two symmetrical pieces. All membranes were then cut into 3 pieces corresponding approximately to the coronal, middle and apical regions, as described in 2.2.3. The longitudinal alignment of the membrane pieces allowed to examine both internal and external surfaces of them by TEM.

Upon removal, a cut was made to identify the mesial side of the membrane in subsequent histologic and electron microscopic examinations. This marker and the notes recording the location of the membrane in the mouth enabled the membrane to be orientated during the embedding procedures. The internal and external surfaces of the membrane, and thus the in situ orientation, were therefore readily identified.

3.2.2 Immunohistochemical staining

Immunohistochemical staining method was carried out, as described in section 2.2.1.4, to identify the cell phenotypes in sections of tissue specimens (6 RT, 4 PL and 4 gingival samples). The following mouse mAbs were used as the primary antibody: vimentin (Sigma), cytokeratin
(Boehringer), CD31 (Dako), CD3 (Dako), CD20 (Dako), CD68 (Dako), HLA-DR (Dako). The primary antibodies, their antigen specificity and working dilutions are detailed in Table 3.1. Mouse non-specific IgG, served as negative control. HRP-labelled anti-mouse IgG raised in rabbit (diluted 1:100) was used as the secondary antibody.

In addition, the presence of calcium salts, indicative of mineralisation, were visualised in tissue sections by von Kossa staining, as described in 2.2.7.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin</td>
<td>Mesenchymal cells</td>
<td>1:100</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>Epithelial cells</td>
<td>1:100</td>
</tr>
<tr>
<td>CD31</td>
<td>Endothelial cells</td>
<td>1:100</td>
</tr>
<tr>
<td>CD3</td>
<td>T lymphocytes</td>
<td>1:200</td>
</tr>
<tr>
<td>CD20</td>
<td>B lymphocytes</td>
<td>1:100</td>
</tr>
<tr>
<td>CD68</td>
<td>Macrophages</td>
<td>1:100</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Antigen-presenting cells</td>
<td>1:100</td>
</tr>
</tbody>
</table>

### 3.3 RESULTS

#### 3.3.1 Light microscopic examination

The examination of longitudinal sections of retrieved ePTFE membranes stained with H&E revealed that little material remained attached to them. The adherent material was much thicker at the coronal open pore structure portion of the membranes and decreased towards the apical part. In addition, some areas devoid of any adherent material were observed, especially at the middle third and apical third. Figure 3.1 shows the middle part of the retrieved membrane along with pink stained adherent material on both surfaces of the membrane as well as between the fibrillar structures of the membrane. The thickness and extent of the adherent material varied
widely between membrane samples, but the amounts on the internal and external surfaces of the same membrane were similar. No host cells could be identified.

In addition, the samples of fresh-frozen, OCT-embedded ePTFE membranes were difficult to cut with the cryostat, possibly due to their fibrillar structure, and some sections of the membrane were therefore often deformed or fragmented, making the light microscopic examination equivocal. However, light microscopy of the RT sections stained with H&E clearly revealed granulation tissue infiltrated by numerous inflammatory cells, including lymphocytes and polymorphonuclear cells, as shown in Figure 3.2. Disorganisation of the RT tissue was a common observation in all samples. In the areas where the tissue was highly vascularised, inflammatory infiltration was denser and polymorphonuclear cells could be observed both within and around the blood vessels. Intact connective tissue and epithelium-like tissue were also identified in the samples. Erythrocytes were present both intra- and extra-capillary, possibly due to ruptured blood vessels.

As in many other studies, healthy gingiva was found to be well-organised under light microscope (Figure 3.3). Multi-layered cuboidal epithelial cells were clearly evident accompanied by highly structured rete pegs. The underlying connective tissue contained numerous elongated fibroblast-like cells among the fibres, with only a few inflammatory cells. Similarly, the elongated fibroblast-like cells were the only cellular structure embedded among the fibres, with no sign of inflammatory cells in sections of normal PL tissue (not shown).
Figure 3.1. Longitudinal section of a retrieved ePTFE membrane (arrows) and adherent material stained with H&E. The pink-stained adherent material is visible on both the internal (I) and external (E) surfaces as well as between the fibrillar structures of the membrane (original magnification $\times 100$).

Figure 3.2. Light photomicrograph of an RT sample stained with H&E. Note the fibrous connective tissue (CT) and numerous small, intensely-stained inflammatory cells (original magnification $\times 100$).
Figure 3.3. A section of normal gingiva stained with H&E. Note the well-organised tissue with epithelium (EP), underlying connective tissue (CT) and the structure of rete pegs (original magnification x 100).

Figure 3.4. Analysis of membrane-associated GAPDH mRNA by RT-PCR. Lane 1 is the 1 Kb DNA ladder and lanes 2-4 are the DNA fragments corresponding the GAPDH gene of 3 different retrieved ePTFE membranes and adherent cells. The arrow indicates a size of 600 bp.
3.3.2 Detection of GAPDH gene in cells associated with membranes

As noted above, little adherent material was found to be attached to the membranes after retrieval. Therefore, the membranes were further examined using molecular biology techniques to assess the presence of intact human mRNA, and thus intact cells. The RNA was extracted as described in 2.2.2. Four of 15 samples were found to contain detectable levels of RNA. PCR amplification was then carried out using the mRNA of only those 4 samples. The predicted 600 basepair (bp) DNA fragment of GAPDH was obtained from 3 out of 4 samples, as shown in Figure 3.4. This finding provides evidence that some intact cells remained attached to the membranes after surgical removal.

3.3.3 TEM observations

The external and internal surfaces of the retrieved membrane samples were examined by TEM to identify the cellular structures which remained adherent to the membranes, as detected above (section 3.3.2). Ultrastructural inspection revealed that both surfaces were covered with variable amounts of adherent material comprising bacteria and host cells, with some surface areas which did not contain any cells. In addition, it was noted that a space or vacant area was present between the adherent material and the membrane surface. The extent of bacterial colonisation and the density of cellular structures varied from membrane to membrane, the most commonly occurring features are described below.

External coronal region. In all samples, this region was covered by dense bacterial aggregates. The bacterial colonisation directly adjacent to the membrane surface appeared to be aligned at a perpendicular angle to the membrane surface (Figure 3.5). A layer with a striated appearance was observed at the interface between the bacteria aggregation and the membrane surface, as shown in Figure 3.5. The bacteria remote from the membrane showed no apparent alignment and distributed more randomly (not shown). In addition, a few bacterial colonies were infiltrated into the fibrillar open pore microstructure of the membrane. Gram-positive and
Gram-negative bacteria could be identified on the basis of the differences in morphology of their cell wall by TEM at high magnification. Abundant aggregates of micro-organisms were found to consist mainly of Gram-positive cocci and rods, and filaments within a fibrillar inter-bacterial matrix. Gram-negative micro-organisms with characteristic trilaminar cell wall were also observed, as shown in Figure 3.5, although at lower frequency. Spirochaetes were rarely encountered.

**Internal coronal region.** All membrane samples had some degree of bacterial colonisation on the internal surface, but far less compared to the external surface. The morphotypes of bacteria seen on the internal surface were similar to those seen on the external surface. Erythrocytes among the bacteria were also apparent.

**External middle region.** Although micro-organisms extended into this region, their intensity decreased towards the apical portion. Host cells, mainly neutrophils containing phagocytosed bacteria, individual epithelial-like cells and numerous erythrocytes were apparent (Figure 3.6). In a few membrane samples, some areas were found to be free of any bacterial and/or host cells.

**Internal middle region.** The majority of host cells close to the coronal region were large numbers of polymorphonuclear cells, which appeared to be engaged in phagocytosis and ingestion of bacteria (Figure 3.7). However, towards the apical region these cells were replaced by sparsely distributed fibroblast-like cells having a well-developed rough endoplasmic reticulum, suggestive of biosynthetic activity. They were occasionally associated with collagen fibres (Figure 3.8).

**External apical region.** Individual, or 3 to 4 closely-packed, fibroblast-like cells were a common feature in this region (Figure 3.9). No micro-organisms were evident. In addition, areas free of any cellular structures were frequently observed.

**Internal apical region.** Fibroblast-like cells, similar to those present on the external surface of the same region, were present in the internal surface of apical region. These cells were associated with thin and/or stratified collagen fibres oriented in several directions (Figure 3.10).
Figure 3.5. TEM examination of the external coronal region of a retrieved membrane. Note the striated structure (arrowhead) between the membrane (M) and bacteria, which align perpendicularly. A few Gram-negative (G-) bacteria with typical trilaminar cell wall are seen among the majority of Gram-positive (G+) bacteria (bar = 2 μm).

Figure 3.6. Ultrastructural examination of cells in the external middle region of the membrane. The host cells include an epithelial-like cell (EP) with surface projections (arrows), a neutrophil (N) with large phagocytic vacuoles and erythrocytes (ER). A small colony of coccoid micro-organisms (C) is also present (bar = 5 μm).
Figure 3.7. Cells associated with the internal middle region of the membrane. Note neutrophils with typical multi-lobed nuclei and numerous vacuoles, particularly present in areas close to the coronal portion (bar = 3 μm).

Figure 3.8. An individual fibroblast-like cell (FC) on the internal middle region of the membrane. Note the randomly oriented thin collagen fibres (arrows) surrounding the cell. M represents the area previously occupied by the membrane (bar = 3 μm).
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Figure 3.9. TEM examination of the external apical region of the membrane. Note the fibroblast-like cells (FC) and a phagocytic cell containing large vacuoles (arrows) in this region. M represents the area previously occupied by the membrane (bar = 5 μm).

Figure 3.10. Extracellular structures in the internal apical region of the membrane (M). Note the characteristic stratified appearance of collagen fibres (arrows) (bar = 50 nm).
3.3.4 Phenotypes of the cells in tissue samples

An immunohistochemical staining technique was used to identify the cell phenotypes in the tissue samples of RT, PL and gingiva. In the present study, the brown colour which is produced by the enzyme HRP at the last step of this staining technique, indicates the presence of the antigen.

**Mesenchymal cells.** Immunohistochemical staining of the RT sections for vimentin, a protein of intermediate filaments present only in mesenchymal cells, showed that the majority of the cells in this tissue stained positive for this protein, suggesting their mesenchymal origin (Figure 3.11a). Strong staining was also observed for vimentin in cells in the connective tissues of both the gingiva and PL, but the gingival epithelium was negative (Figures 3.11b and c).

**Epithelial cells.** When the tissue sections were stained for cytokeratin, a cytoskeletal protein present only in epithelial cells, the positive-stained cells in the RT samples formed either a continuous band, as shown in Figure 3.12a, or an extensive network or well-organised rete pegs resembling those present in normal gingival tissues. The gingival epithelium also stained strongly positive for cytokeratin, as shown in Figure 3.12b, whereas no staining was observed in the PL tissue sections (not shown).

**Endothelial cells.** Many thin-walled blood vessels which stained positive for the CD31 antigen were observed throughout the RT samples (Figure 3.13), suggesting that active angiogenesis was taking place in these samples. The formation of new blood vessels is one of the characteristic and important features of a wound at the early phase of healing. Although some blood vessels were also observed in the gingival and PL samples (not shown), their distribution was far less frequent compared with those in the RT samples.

**Inflammatory cells.** The inflammatory infiltrate was clearly evident when sections were stained with specific mAbs against T and B lymphocytes and macrophages. The small T lymphocytes were distributed heterogeneously in all the RT sections (Figure 3.14). In contrast, B lymphocytes were not detected in 4 out of 6 RT samples and the remaining
2 samples showed only a few cells which stained positively for CD68 (not shown). However, the relatively large macrophages were widely distributed throughout all the RT samples. The gingival tissue samples showed a minimal level of inflammation, comprising a few T lymphocytes and macrophages, whereas inflammatory cells were absent in the PL tissue samples examined (not shown). Table 3.2 summarises the results of immunohistochemical staining for the cell phenotypes.

**HLA-DR.** Immunostaining for the HLA-DR antigen, molecules which are normally expressed by antigen-presenting cells such as B cells, macrophages and Langerhans cells, revealed very strongly positive staining throughout the RT (not shown), again indicating the ongoing inflammatory phase of the healing process. In contrast, HLA-DR staining in the normal gingival and PL samples was far less intense (not shown).

None of the sections of tissue samples treated with non-specific mouse IgG1 exhibited staining. Figure 3.15 shows no brown colour in the RT tissue section, only blue counter-staining of haematoxylin. Similar observations were made for the negative control sections of other tissue samples (not shown).

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<th>Tissue</th>
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++ large number of cells  
+ cells present  
-/+ small or variable number of cells  
- cells absent
Figure 3.11. Vimentin-positive mesenchymal cells (brown staining) in the RT (a), PL (b) and gingival (c) tissue samples (original magnification x 400).
Figure 3.12. Cytokeratin-positive epithelial cells (arrows) in RT (a) and gingival (b) tissue samples. The epithelial cells in the RT section are distributed in a relatively disorganised fashion (a). The epithelial cells in the samples of normal gingiva are visualised only in the clearly distinguishable and well-organised epithelium (b) (original magnification $\times 400$ in a, $\times 100$ in b).
Figure 3.13. An RT sample showing randomly orientated blood vessels (arrows) as identified by CD31. Note the very thin, mostly one cell layer thick walls of the blood vessels. Erythrocytes and leukocytes are clearly visible within some blood vessels (original magnification x 200).

Figure 3.14. T lymphocytes in the RT sample. Note heterogeneous distribution of these cells as identified by CD3 (original magnification x 200).
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Figure 3.15. A control section of an RT sample treated with non-specific mouse IgG1, showing no brown staining (original magnification x 200).

Figure 3.16. Calcified areas (arrows) in an RT sample visualised by von Kossa staining as dark brown colour (original magnification x 200).
3.3.5 Signs of calcification in the RT samples

von Kossa staining was utilised to visualise possible calcified areas in the tissue samples. A few small nodules were observed in all 7 of the RT samples, with one sample having a very large nodule surrounded by fibroblast-like cells (Figure 3.16). None of the PL and gingival tissue sections was found to have mineralised areas (not shown).

3.4 DISCUSSION

The cells are a key factor in achieving successful periodontal regeneration by GTR using barrier membranes to guide the cellular elements during early healing and to promote regeneration. Histological observation of the RT samples showed that they resembled the periodontal soft tissues obtained from periodontal osseous defects in both humans and animals treated with GTR membranes (Pritlove-Carson et al. 1994; Matsuura et al. 1995; Amar et al. 1995; Amar et al. 1997). As demonstrated by other investigators (Pritlove-Carson et al. 1992; Tempro and Nalbandian 1993), light microscopic observation indicated that only very small amounts of material remained adherent to the membranes upon removal. However, because the data obtained by histological examination was equivocal, a number of other methods were used in order to determine whether any host cells remained attached to the ePTFE membranes after removal.

Firstly, RT-PCR was used to examine the presence of the mRNA corresponding to the GAPDH, a general, housekeeping enzyme present in many cells. The finding that GAPDH transcripts were present in the retrieved membrane samples demonstrated that the membrane-associated material contained cells which were intact, since the mRNA had not been degraded. This result is consistent with the TEM findings here and other studies which also showed the presence of adherent intact cells (Selvig et al. 1990; Grevstad and Leknes 1992; Selvig et al. 1992; Tempro and Nalbandian 1993). The GAPDH transcripts detected in this study indicated the presence of such host cells since this gene is present only in human cells and not in bacteria (Ercolani et al. 1988). The absence of mRNA in
some membrane samples may have been due to the very small number of adherent host cells on some membrane surfaces, as shown using TEM.

The results of TEM observations also revealed the presence of intact host cells which had remained attached mainly to the apical and middle portions of the membranes. The coronal portion comprised a very large accumulation of micro-organisms, as previously shown (Selvig et al. 1990; Selvig et al. 1992; Tempro and Nalbandian 1993; Grevstad and Leknes 1993). PL fibroblasts examined under TEM have previously been described as containing a prominent nucleus and large numbers of organelles, including Golgi complex and rough endoplasmic reticulum (Berkovitz et al. 1995). In the present study, some of the cells associated with the membranes also had a fibroblast-like appearance, including an extensive rough endoplasmic reticulum suggestive of active biosynthetic activity. These fibroblast-like cells were observed mostly in the internal middle region and both the internal and external surfaces of the apical region of the membranes, consistent with the study of Selvig et al. (1990). The finding that intact and highly structured collagen fibres were also localised in close proximity to these cells (Selvig et al. 1990; Selvig et al. 1992) strongly suggests that the cells were functionally active and likely to play an important part in the formation of the connective tissue components that mediate new attachment. These structural elements, adherent to the middle portion of the internal surface, are particularly critical since this region of the membrane faces the periodontal defect where the regenerative healing process takes place. It is therefore likely that cells in this portion could have a major role in periodontal regeneration.

Epithelial-like cells were rarely observed (Selvig et al. 1992), and only at the external surface of the membrane. These cells might be desquamated gingival epithelial cells since they were individual and not seen as a cluster of cells, in contrast with the findings of Grevstad and Leknes (1992). Inflammatory cells, particularly neutrophils which appear to be involved in early healing events, were actively phagocytic and found to be associated with both micro-organisms and fibroblast-like cells, as previously observed (Selvig et al. 1990; Grevstad and Leknes 1992; Tempro
and Nalbandian 1993). In addition to the low frequency of fibroblast-like and epithelial-like cells, there were some areas which were free of any cells (Grevstad and Leknes 1992; Selvig et al. 1992), probably because of the surface roughness of the ePTFE created by the overlapping fibrils (Salonen and Persson 1990; Payne et al. 1996). In addition, cell-free areas could have resulted from the membrane having been retrieved without also removing some closely-associated tissue. Thus the finding of cell-free areas on retrieved membranes does not necessarily mean that these areas had previously not been in close contact with cellular structures in situ.

Human gingival fibroblasts cultured directly on the ePTFE membrane have previously been shown to have an abnormally rounded morphology (Payne et al. 1996). In another in vitro study, cultured gingival fibroblasts seeded onto the membrane showed decreased synthesis of DNA, collagen and glycosaminoglycans compared to the cells grown directly on plastic surface of the culture dish (Locci et al. 1997). Furthermore, the capability of these cells to migrate over ePTFE in response to a chemoattractant was less than that over plastic surface (Payne et al. 1996). This may be caused by the composition of the ePTFE, which is in the form of a solid sheet of Teflon surrounded by a coarsely textured fibres, a microstructure which appears to be less favourable for migration of gingival epithelial cells compared to a milipore filter (Salonen and Persson 1990). In addition, epithelial cells cultured on the ePTFE have been found to have decreased proliferation and to exhibit some signs of degeneration, possibly because of the low protein-binding capacity of the Teflon material (Salonen and Persson 1990).

Micro-organisms were consistently present on all retrieved membranes examined in this study. This is not unexpected, since they have been previously observed by SEM and TEM as well as detected by microbial cultivation and DNA probe analysis (Selvig et al. 1990; Selvig et al. 1992; Grevstad and Leknes 1993; Tempro and Nalbandian, 1993; Nowzari and Slots 1994; Novaes Jr. et al. 1995; Nowzari and Slots 1995; Nowzari et al. 1995; Nowzari et al. 1996). The majority of bacterial colonies, surrounded by an inter-microbial matrix, were found to accumulate on the coronal part of
the retrieved membranes, both on the external surface and with less density on the internal surface. This observation is consistent with the findings of Tempro and Nalbandian (1993) and Grevstad and Leknes (1993), who observed that the coronal portion of the barrier material was heavily colonised by oral bacteria, some of which invaded the open microstructure, with decreasing density towards the middle portion (Selvig et al. 1990). The finding that the external surface tended to contain more bacteria than the internal surface was also observed by Nowzari and Slots (1995) and Nowzari et al. (1996). Although Novaes et al. (1995) found that bacterial contamination extended to the apical portion, even on the internal surface, the apical portions examined in this study appeared to be free of any bacterial contamination. However, similar findings were reported by Selvig et al. (1990), who showed by SEM that the localisation of bacterial colonies extended only to the middle portion of the membrane.

The micro-organisms associated with the retrieved ePTFE membranes appeared to be cocci, rods and filaments, although the particular bacteria species were not identified further. However, the ultrastructural morphology of the micro-organisms was similar to the findings of the previous studies (Selvig et al. 1990; Selvig et al. 1992; Tempro and Nalbandian 1993; Grevstad and Leknes 1993; Novaes Jr. et al. 1995) and to the subgingival plaque formed in periodontal pockets (Listgarten 1976), the majority of bacteria being cocci and rods with Gram-positive cell membranes (Tempro and Nalbandian 1993; Grevstad and Leknes 1993).

The presence of bacterial colonies in situ may have resulted from contamination during handling and insertion, inadequate marginal sealing by gingival flap coverage after implantation or premature exposure due to gingival recession. Bacteria found on the middle region may also be the result of smearing over the membrane during removal (Selvig et al. 1992). Moreover, it was recently shown that the periodontal pathogen P. gingivalis is able to migrate through ePTFE membranes in vitro (Ricci et al. 1996), and it is therefore possible that some bacterial cells could also have reached the internal surface of the membrane by passing through the membrane in vivo, while others could have become trapped within the fibrillar structure.
Putative periodontal pathogens have been shown to colonise ePTFE membrane surfaces within 4 h of incubation in vitro, with the strongest adherence affinity of Streptococcus mutans (Wang et al. 1994a). This occurs apparently within 3 min of intraoral manipulation in vivo (Nowzari et al. 1996), indicating that in patients with high subgingival levels of bacterial pathogens barrier membranes are at high risk of becoming contaminated.

Selvig et al. (1992) suggested that the extent of membrane exposure and bacterial colonisation at the time of membrane removal could be an indicator of the long-term success or failure of the regenerative procedure. A negative correlation has been found between the clinical attachment gain and the extent of membrane contamination (Selvig et al. 1992), particularly on the internal surface (Nowzari et al. 1995), and the quantity of microorganisms (Nowzari and Slots 1994; Nowzari et al. 1996). All the membranes examined by TEM in the present study showed some degree of exposure at the coronal margins at the time of removal, although the membrane exposure was not measured and recorded here.

The space between the adherent cellular elements and the membrane surface which was frequently observed during TEM examination could have occurred during the embedding and preparation procedures (Grevstad and Leknes 1992) or as a result of the embedding material, which did not adhere to the PTFE material (Tempro and Nalbandian 1993).

In this study, it was assumed that the external surface of the membrane was apposed to the inner side of the gingival flap, whereas the internal surface of the membrane faced the root surface, newly-regenerated tissue and alveolar bone in a coronal-apical direction. However, 3-, 2- and 1-wall combined intraosseous periodontal defects included in this study may have variable shapes. Therefore, the newly-regenerated tissue may not always face the same region of the membrane. Thus, the location and composition of cells derived from each of the membranes may not be identical. This was assessed by using immunohistochemistry to identify the cell phenotypes in the RT removed from the periodontal defects treated using GTR, and also in the soft connective tissues of the gingiva and PL.
Vimentin is a protein of filaments of the cytoskeleton of mesenchymal cells. Vimentin filaments are also called intermediate filaments because their size is intermediate between the thin and thick filaments of muscle (Berkovitz et al. 1995). Immunostaining with anti-vimentin antibody revealed that the majority of the cells in the RT sections were positive for this protein, as also observed by Pritlove-Carson et al. (1994). Some cells of mesenchymal origin include fibroblasts, osteoblasts, cementoblasts and their precursors, it was not possible to identify the specific cell types by these experiments alone. However, it was notable that all the RT samples were found to also contain some epithelial cells, identified as cytokeratin-positive cells. Although the origin of these epithelial cells are unknown, membrane exposure might have allowed some epithelial downgrowth into the newly-formed periodontal tissue. Pritlove-Carson et al. (1994) also reported an epithelial cell phenotype, staining positive for keratin 19, in the regenerated periodontal tissue obtained from periodontal defects treated by GTR. These observations suggest that some epithelial cells are present in the regenerated periodontium, either as a result of contamination or membrane exposure. Alternatively, it is also possible that these cells may be derived from the rests of Malassez.

Amar et al. (1995) analysed the morphology of the cell population of human newly-formed periodontal soft tissues removed at 6 weeks following ePTFE placement. They used a calibrated grid and reported that 94% of the cells were fibroblasts and endothelial cells. The inflammatory cells, including polymorphonuclear leukocytes, monocytes, lymphocytes, macrophages accounted for approximately 5% of the cell population. Although in the present study the numbers of cells were not counted, the inflammatory cells appeared to constitute a much greater proportion than reported in the study of Amar et al. (1995), perhaps because the healing process was still ongoing, as suggested by the extensive vascularisation.

The RT samples in this study were removed from the most coronal part of the healing periodontal defect and the findings therefore reflect only the structure and cellular phenotypes of the most coronal part of the newly-regenerated tissue and may not reflect the characteristics of the tissue in
general. However, in a study performed by Matsuura et al. (1995), beagle dogs were sacrificed at 2, 4 and 8 weeks after GTR surgery, thus allowing the regenerating periodontium at the furcation areas to be studied at these 3 different time points. Two weeks after GTR surgery, the furcation was occupied by granulation tissue composed of numerous infiltrated inflammatory cells and blood vessels. This tissue was partially replaced with a newly-formed fibrous connective tissue, which almost filled the defect at 4 weeks. At 8 weeks, the fibrous connective tissue was further replaced and periodontal regeneration was nearly complete, but inflammatory cells were still evident at the most coronal part of the healing tissue. In the present study, the RT samples obtained at approximately 6 weeks had the similar appearance of a healing connective tissue with numerous inflammatory cells and vimentin-positive mesenchymal cells, and closely resembled the most coronal part of a healing furcation defect rather than the deeper or apical parts (Matsuura et al. 1995).

In the present study, healthy gingival and PL tissues were used to characterise the cell phenotypes in the normal soft connective tissues of the periodontium. These 'control' tissues were obtained from subjects with normal healthy periodontium and not from the same patients who had provided the RT samples. Although an ideal control tissue would be the healing tissue of a periodontal defect that had received the same surgical treatment as the test defects, without a membrane, this approach can only be carried out in animal studies (Amar et al. 1997).

Several histological studies have shown that GTR results not only in the formation of new PL, but also the hard connective tissues of the periodontium—the cementum and alveolar bone. The possible presence of such hard tissue formation was visualised using von Kossa staining. The procedures used for preparing cryostat sections for immunohistochemistry also permitted the use of this staining technique, since the sections were not decalcified. Mineralised nodules have previously been detected in tissue samples obtained under GTR membranes (Amar et al. 1995; Piattelli et al. 1996; Amar et al. 1997). During the ongoing process of healing and subsequent remodelling, these nodules may fuse together and form the
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regenerated alveolar bone. The results of the von Kossa staining demonstrated the presence of hard connective tissue forming cells, i.e., osteoblasts, cementoblasts or their precursors, in the newly-formed periodontal tissue.

3.5 CONCLUSIONS

In conclusion,

i) intact fibroblast-like cells remain adherent to the retrieved membranes along with inflammatory cells;

ii) the coronal portion of membranes are also colonised by bacteria;

iii) RT samples resemble a granulation tissue comprising fibroblast-like cells, newly-formed blood vessels and numerous inflammatory cells;

iv) the presence of epithelial-like cells in RT suggests that exclusion of gingival epithelium might not be fully achieved.

Thus, the membranes and RT samples, along with the PL and gingiva, could be used to isolate, culture and study further the precise phenotypes and functions of the cells, as described in detail below.
CHAPTER 4
PHENOTYPES AND FUNCTIONS OF GTR-ASSOCIATED CELLS IN VITRO

4.1 INTRODUCTION

The main cell types of the periodontium are epithelial cells, fibroblasts, cementoblasts and osteoblasts. Among these cells, the fibroblast is the predominant cell type of the gingiva and PL, and play fundamental roles in normal function and pathology (McCulloch and Bordin 1991). They synthesise a variety of extracellular matrix (ECM) proteins including collagens and glycoproteins, and regulate tissue homeostasis via the synthesis of enzymes which degrade the ECM (Ohshima et al. 1995; Alvares et al. 1995; Lekic and McCulloch 1996). Studies have provided evidence that the cells derived from the gingiva and PL exhibit differences in total protein content (Mariotti and Cochran 1990), growth rate (Mariotti and Cochran 1990; Ogata et al. 1995), synthesis and expression of ECM molecules (Hou and Yaeger 1993; Kuru et al. 1996, Kuru et al. 1997c) and response to growth factors (Mailhot et al. 1995), chemical mediators (Ogata et al. 1995) and attachment proteins (Somerman et al. 1989).

Being a major component of a healing wound, the fibroblast plays a fundamental role during periodontal repair and regeneration processes (Lekic and McCulloch 1996). A number of studies, including this (see Chapter 3), have demonstrated that fibroblast-like cells remained adherent to non-resorbable membranes following retrieval (Selvig et al. 1990; Selvig et al. 1992). In addition, the present immunohistochemical results (see Chapter 3) showed that the newly-regenerated periodontal tissue beneath the barrier membranes also contained fibroblast-like cells. Cell culture was therefore used to study the features of these GTR-associated cells. Although these cells have been examined in vitro (Wakabayashi et al. 1996; Rowe et al. 1996; Wakabayashi et al. 1997; Grosso et al. 1997; Kuru et al. 1997a), their phenotypic and functional properties have not been fully characterised. Therefore, the aims of this part of the study were:
i) to isolate and culture the GTR-associated cells from retrieved ePTFE membranes (M cells) and regenerated tissues (RT cells) in vitro;  
ii) to investigate their morphological characteristics;  
iii) to identify their phenotypes; and  
iv) to examine their functional properties in comparison with the fibroblast-like cells derived from normal PL and gingiva.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture and growth

The retrieved ePTFE membranes and RT, PL and gingival tissue samples were used as explants to obtain cell cultures, as described in section 2.2.4.1. The cultures of these cells were examined under the light microscope. The cell numbers of each cell type were determined at the first day after seeding (day 0) and at 2, 4, 6, 8 and 10 days by direct counting using a haemocytometer, as described in section 2.2.4.4, in order to evaluate the proliferation rate of the cells. In addition, the viability of the cells were checked by trypan blue exclusion.

4.2.2 Assays

The cultured cells were grown to early confluence on glass cover slips and an indirect immunocytochemical staining method was employed to identify their phenotypes, as described in section 2.2.5. The primary antibodies (1:100 dilution) used were mouse mAbs against human vimentin, 5' prolyl-4-hydroxylase (Dako) and cytokeratin. The former two identified the fibroblast-like cells while the latter identified the epithelial-like cells. The negative control cells were treated with non-specific mouse IgG1 as the primary antibody. After immunostaining, the cells were counter-stained with haematoxylin and examined by light microscopy.

In order to study functional properties, the activity of alkaline phosphatase (ALP), an enzyme considered to be involved in bone formation, was measured by a colourimetric assay, at 4 and 7 days, as described in section 2.2.6. Furthermore, the effect of dexamethasone (DEX) on the
activity of this enzyme was also investigated because this hormone has been considered to induce differentiation of osteoprogenitor cells. The formation of mineralised nodules by cultured cells, suggestive of an osteoblast-like property, was visualised during 2 and 4 weeks of culture using von Kossa staining, as described in section 2.2.7.

4.2.3 Statistical analysis

The data of the cell growth were found to be not normally distributed and therefore non-parametric statistical methods were used. The data were first analysed using the Kruskal-Wallis test for days 0, 2, 4, 6, 8 and 10 separately. When statistical significance was found (p < 0.05), the data for that particular day were further analysed, using the Mann-Whitney U test, to assess the differences in cell numbers between specific cell cultures.

The distribution of the data of ALP activity assay was also not normal. Therefore non-parametric statistical methods were used and the data are expressed as the median value instead of the mean and the distributions of the data are presented as the 25 and 75 percentile (25 and 75% of the observations) instead of the standard deviation. The results were analysed separately for days 4 and 7 of incubation using the Kruskal-Wallis test. When statistically significant differences were found (p < 0.05), further analysis was performed using the Mann-Whitney U test to determine where the differences lay. Statistical significance between days 4 and 7 as well as between basal and DEX-induced ALP activity of each cell type was determined by the Wilcoxon Signed Ranks test.

4.3 RESULTS

4.3.1 Culture of cells

4.3.1.1 M cells

The retrieved ePTFE membranes were incubated in order to obtain viable cultures of M cells which had remained attached to the membranes, as indicated by the results of the RT-PCR and TEM experiments (Chapter 3). Since extensive bacterial colonisation was observed on the coronal
portion of the membranes by TEM (see Figures 3.5), this portion was discarded, only the middle and apical portions of the membranes being used for tissue culture. Additional anti-bacterial and anti-fungal agents were also added to the primary M cultures to prevent possible contamination.

Several approaches described in section 2.2.4.2 were used to obtain the M cells, as no standardised protocol was available to isolate these cells. When the membrane pieces were immobilised under glass cover slips or the surfaces of the membrane were scraped and incubated in vitro, no outgrowths of cells were observed. However, the culture of M cells was successfully achieved when the membrane pieces were placed into a culture flask containing culture medium and then shaken gently to detach the cells from the membranes. M cells were also readily obtained from the cells which had initially dissociated from the membrane and remained in the TM used for transportation of the retrieved membranes.

Elongated spindle-shaped fibroblast-like M cells were first seen between 7 and 14 days of culture, as shown in Figure 4.1a. Despite the opaque nature of the membrane, some M cells were observed to be growing directly on membranes (Figure 4.1b).

4.3.1.2 RT cells

The spindle-shaped RT cells were first observed between 3 and 7 days after the RT samples were explanted and incubated in vitro (Figure 4.2a). The RT cells migrated from the explants and rapidly reached confluence, as shown in Figure 4.2b. In addition to fibroblast-like cells, viable cuboidal epithelial-like cells (Figure 4.2c) were also observed in all except one primary RT culture. These epithelial-like cells formed a highly condensed sheet-like layer and outnumbered the fibroblast-like cells during the initial culture period (between 5 and 10 days). The proliferation of epithelial-like cells appeared to decline after approximately 10-15 days, whereas the fibroblast-like cells continued to grow rapidly. When the primary RT cultures were trypsinized for sub-culture, the epithelial-like cells were not detected after passage 1.
Figure 4.1a. Phase-contrast photomicrograph of spindle-shaped M cells (arrows) associated with a retrieved membrane (M) cultured in vitro for 10 days. The small, circular cells scattered throughout are erythrocytes (original magnification x 100).

Figure 4.1b. The M cells (arrows) growing on the retrieved membrane after 14 days in culture. The horizontally-orientated structures at the background are the fibrils of the ePTFE membrane (original magnification x 200).
Initially randomly orientated cells (a) formed a highly condensed and parallel orientated culture (b) around a small fragment of the explant (RT) (original magnifications x 200).

Spindle-shaped fibroblast-like cells and cobblestone-like epithelial cells (EP) derived from an RT sample. While the epithelial cells grow as a continuous sheet, the fibroblast-like cells are individually scattered and randomly orientated. Note the small isolated islands of the fibroblast-like cell population (arrows) remained entrapped among the epithelial cells (original magnification x 100).
4.3.1.3 PL cells

Outgrowths of PL cells were first observed between 4 and 10 days after initiating the incubation of the PL explants, as shown in Figure 4.3. It was notable that only spindle-shaped fibroblast-like cells but no epithelial-like cells were observed in these primary cultures of PL tissue.

4.3.1.4 Gingival cells

Spindle-shaped fibroblast-like gingival cells were first observed to be individually scattered around the gingival tissue fragments after 3 to 6 days of explantation, as shown in Figure 4.4. Some of these were in contact with the tissue while others appeared to have first migrated and then proliferated. The cuboidal epithelial-like cells were also observed in almost all primary gingival cultures (not shown). Between 10 and 15 days of incubation, such cells appeared to contain large vacuoles, suggestive of deterioration, and partially detached as a sheet from the surface of the culture dish. These areas of the culture dish then became overgrown by proliferating fibroblast-like cells in the following 2 to 3 days.

After the primary cultures of gingival explants were trypsinized, the fibroblast-like cells were readily detached whereas the epithelial cells showed a relatively higher resistance to trypsin treatment. Following collection of the detached fibroblasts, the epithelial cell sheets were observed by phase-contrast microscopy to remain attached to the surface of the culture dishes. Thus, the spindle-shaped fibroblast-like cells were the only cell morphology encountered in subsequent sub-cultures.
Figure 4.3. Phase-contrast photomicrograph of fibroblast-like PL cells migrating from the tissue (PL) explanted for 12 days. Note mostly parallel orientation of these cells (original magnification x 200).

Figure 4.4. Phase-contrast photomicrograph of fibroblast-like cells growing out of the gingival tissue explant (G), after 3 days in culture (original magnification x 200).
4.3.2 Morphology of cultured fibroblast-like cells

The primary cultures of all fibroblast-like cells derived from the membrane, RT, PL and gingival explants appeared to be spindle-shaped in morphology (see Figures 4.1a, 4.2a and b, 4.3 and 4.4). These cells possessed an oval nucleus with more than one nucleolus in most cases. The M cells appeared to be well-spread and larger than the others, whilst the RT cells were observed to be less spindle-shaped, with fewer elongated cytoplasmic processes.

When primary cultures of M, RT, PL and gingival cells were subcultured and examined at sub-confluence by phase-contrast microscopy, the cultures appeared to be composed of a mixture of cells with variations in shape and size, ranging from well-spread, large, trapezoidal cells to thin, long, spindle-shaped cells. All the fibroblast-like cultures formed continuous palisades of tightly packed cells when confluent (data not shown). While most cells orientated parallel to their long axis, some others also orientated perpendicularly. The morphological appearances of cultures derived from different types of explants were very similar at this stage.

4.3.3 Establishment of cell lines

The data of establishment of cell lines are summarised in Table 4.1. Out of total 81 retrieved ePTFE membranes used as explants to obtain the M cells, 22 yielded outgrowth of viable cells, 44 resulted in no cell attachment or growth, the remaining 15 became contaminated by bacteria (12 of 15) or fungi (3 of 15), within the first week of incubation. During the subsequent culture period, 17 cell lines (21% of the total M cultures) were successfully maintained.

Out of 7 RT samples used for cell culture, emigrating cells were seen at 5 RT cultures, 2 RT explants did not yield any outgrowth of cells, and 4 RT cell lines were established on prolonged culture, which was the highest success rate (57%). Five PL primary cell cultures were grown from the PL tissue samples of 38 patients (13%) and 9 gingival cell lines were successfully obtained from 37 corresponding tissues (24%).
Table 4.1. The explants and cell culture.

<table>
<thead>
<tr>
<th>Explant</th>
<th>No of explants</th>
<th>No of primary cell cultures</th>
<th>Cell lines maintained</th>
<th>Success of cell culture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>81</td>
<td>22</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td>RT</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>57</td>
</tr>
<tr>
<td>PL</td>
<td>38</td>
<td>5</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>Gingiva</td>
<td>37</td>
<td>15</td>
<td>9</td>
<td>24</td>
</tr>
</tbody>
</table>

RT = regenerated tissue
PL = periodontal ligament

4.3.4 Growth characteristics and viability

The growth properties of the cultured cells were determined by direct counting of the cell numbers at specific time points over a culture period of 10 days (Figure 4.5). All cell types showed an initial lag phase followed by a log phase. While the RT and PL seemed to reach the plateau by 8 days of culture, the M and gingival cells appeared to be still in log phase at day 10. The most rapidly proliferating cells were found to be derived from gingival tissue, and these also grew to the highest density, followed by the RT and PL cells.

When cell numbers of each cell type were tested at each specific day, no statistical significance was observed on days 0, 2, 8 and 10 (p > 0.05). However, at day 4 the numbers of M and PL cells were found to be significantly lower than that of the gingival cells (p < 0.05). The number of M cells was also significantly lower at day 6 compared with that of the gingival cells (p < 0.05). Trypan blue exclusion showed that over 90% of the cells in all cultures were viable.
4.3.5 Expression of cell-specific antigens

The expression of cell-specific antigens in the cultured cells was investigated using the indirect ABC immunocytochemical staining technique. Figures 4.6a and b show that the M and RT cells stained intensely positive for 5' prolyl-4-hydroxylase, an enzyme involved in collagen synthesis. The brown staining, which indicates the presence of this enzyme, were especially prominent in regions close to the cell nuclei. The intensity of brown colour seemed to gradually decrease from the cell nuclei towards the cell membrane; no staining was visible in regions adjacent to cell membrane and in the cytoplasmic processes. Similar staining patterns were also observed in the PL and gingival cells (not shown).

Vimentin, a cytoskeletal protein of intermediate filaments of mesenchymal cells, is considered to identify the fibroblastic phenotype. This protein was visualised as numerous thin filaments distributed uniformly
throughout the cytoplasm of all the cell cultures examined. Figures 4.7a and b show very strong expression of this protein in the M and RT cells, respectively, as a complex and prominent network of these filaments. The PL and gingival cells also expressed high levels of this antigen, with similar distribution (not shown). It was notable, however, that cytokeratin, an antigen that forms keratin filaments characteristic of epithelial cells, was not detected in any of the cells in the M, RT, PL and gingival cultures (not shown). Light microscopic observation of the control cultures, which were treated with non-specific mouse IgG1 as the primary antibody, showed that there was no reaction (i.e., no brown colour) in the M and RT cells (Figures 4.8a and b) as well as in the PL and gingival cells (not shown).

**Figure 4.6.** Expression of 5’ prolyl-4-hydroxylase in the M (a) and RT (b) cultures. All cells express this enzyme particularly in regions close to the cell nuclei. Note that the majority of the RT cells are smaller than the M cells. The cell nuclei are seen blue due to haematoxylin counter-staining (original magnification x 200).
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Figure 4.7. Expression of vimentin in the M (a) and RT (b) cultures. All cells demonstrate positive staining for this protein throughout their cytoplasm. Note the fibrillar appearance of vimentin positive filaments especially in areas where cell cytoplasm is well-spread (original magnification x 200).

Figure 4.8. Control cells in the M (a) and RT (b) cultures. Note the absence of brown colour. Only blue colour of haematoxylin is visible (original magnification x 200).
4.3.6 Alkaline phosphatase activity

ALP activities of M, RT, PL and gingival cell cultures were measured after 4 and 7 days of incubation in the absence and presence of DEX, using a colourimetric assay as described in section 2.2.6. A standard curve was prepared for each individual experiment and the International Units (U/L) of the experimental samples were then calculated from this curve of Sigma Units. A representative standard curve is shown in Figure 4.9.

![Standard Curve of ALP Activity](image)

**Figure 4.9.** A standard curve of ALP activity.

4.3.6.1 Basal levels of ALP activity

Basal ALP activity in the RT cells were found to be higher than in any of the other cells after 4 and 7 days of incubation, as shown in Table 4.2. Thus, on day 4, enzyme activity in the RT cells (122 U/L) was significantly elevated compared with that in the M cells (33 U/L), the PL cells (34 U/L) and the gingival cells (47 U/L) \( (p < 0.01) \). However, ALP levels in the M, PL and gingival cells were not significantly different from each other \( (p > 0.05) \). By 7 day, ALP activities in the M, RT and PL cells were found to increase by 1.6-, 1.1- and 1.7-fold, respectively, whereas the level in the gingival cells
declined. The RT cells (134 U/L) still had the highest activity, which differed significantly from the M cells (52 U/L), the PL cells and gingival cells (59 and 42 U/L, respectively; p ≤ 0.01). Although the levels of ALP activity in the M and PL cells were higher than the gingival cells, the differences were not statistically significant (p > 0.05).

4.3.6.2 DEX-induced levels of ALP activity

DEX is a synthetic glucocorticoid which has been reported to selectively stimulate the proliferation and differentiation of osteoprogenitor cells in vitro (Bellows et al. 1990). This hormone has also been shown to up-regulate ALP activity in PL cells (Kuru et al. 1998a) and osteoprogenitor cells derived from bone marrow (Chen et al. 1997). Table 4.2 also shows the ALP activities in the M, RT, PL and gingival cells which had been treated with DEX for 4 and 7 days. As with the basal levels, the RT cells exhibited the highest activity compared to the other cells after 4 and 7 days of incubation in the presence of DEX. Thus, on day 4, DEX-induced activity in the RT cells (216 U/L) was found to be significantly greater than in the M cells (36 U/L), the PL cells (45 U/L) and the gingival cells (46 U/L) (p ≤ 0.01). Although the levels of ALP activity in both the PL and gingival cells were similar and slightly elevated compared with the M cells, these differences were found to be not statistically significant (p > 0.05). By 7 days, the DEX-treated RT cells had the overall highest ALP activity (349 U/L) under all conditions in this experiment, significantly elevated compared with the M cells (64 U/L) (p ≤ 0.01) and the PL and gingival cells (78 and 50 U/L, respectively; p ≤ 0.001). Although the M cells had slightly lower ALP activity than the PL cells, the difference was found to be not significant (p > 0.05). However, both the M and PL cultures showed higher DEX-induced activity than the gingival cells, and these differences were statistically significant (p < 0.05). The DEX-treated cultures of M, RT and PL cells showed 1.7-, 1.6- and 1.7-fold increases in ALP activity from day 4 to day 7, whereas the level in gingival cells remained almost unchanged.
Table 4.2. Basal and dexamethasone (DEX)-induced ALP activity (U/L) in membrane-associated (M), regenerated tissue (RT), periodontal ligament (PL) and gingival cells.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Basal activity</th>
<th>DEX-induced activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 4</td>
<td>Day 7</td>
</tr>
<tr>
<td>M</td>
<td>33</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>(28/150)</td>
<td>(30/232)</td>
</tr>
<tr>
<td>RT</td>
<td>122</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>(37/155)</td>
<td>(50/517)</td>
</tr>
<tr>
<td>PL</td>
<td>34</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>(23/64)</td>
<td>(22/105)</td>
</tr>
<tr>
<td>Gingival</td>
<td>47</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>(33/65)</td>
<td>(25/55)</td>
</tr>
</tbody>
</table>

Data are presented as the median and the numbers in brackets are the 25/75 percentiles of the distribution.

A summary of the effects of DEX on ALP activity in the M, RT and PL cells at days 4 and 7 of incubation is shown in Figure 4.10. The level of ALP activity in the RT cells exhibited a marked increase (176%) in response to DEX following 4 days of incubation and this increase was highly significantly different from the level of the corresponding non-treated RT cells (p < 0.001). While both the M (110%) and PL cells (131%) also showed a highly significant response to DEX (p < 0.001), ALP activity in the gingival cells showed only a very slight increase (104%) which was not statistically significant (p > 0.05). As at day 4, ALP activity in the RT cultures incubated with DEX was found to be significantly enhanced compared with the corresponding basal level at day 7 (256%; p ≤ 0.001). Similar highly significant responses to DEX were observed in cultures of the M (122%) and PL (131%) cells at day 7 (p ≤ 0.001). Unlike at day 4, DEX was found to significantly induce ALP activity in gingival cells (119%) by day 7 (p < 0.05).
300 Day 4 Day 7

Cell type

Figure 4.10. Effects of dexamethasone on the ALP activities of membrane-associated (M), regenerated tissue (RT), periodontal ligament (PL) and gingival (G) cells after 4 and 7 days of culture.

*p < 0.05, 
**p ≤ 0.001 compared to the basal ALP level of corresponding cells, using the Wilcoxon Signed Ranks test.

4.3.7 Mineralised nodule formation

Cultures of the M, PL and gingival cells grown under normal and special culture conditions were examined for their ability to form mineralised nodules after 2 and 4 weeks of culture. Nodule formation by the RT cells was not determined. The cells were incubated in a special nodule growth medium consisting of culture medium supplemented with ascorbic acid and \( \beta \)-glycerophosphate, as described in section 2.2.7. These were designated as test group 1. Duplicate cultures, which were incubated in nodule growth medium also containing DEX, were designated as test group 2. The cells in normal culture medium served as a negative control group. The morphological appearance of the M, PL and gingival cells were routinely examined under phase-contrast microscope, throughout the culture period, and the formation of mineralised nodules visualised by von Kossa staining were recorded.
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The M cells grown in control medium were very tightly packed and orientated in parallel arrays, as shown in Figure 4.11a. In contrast, the M cells grown in both test culture media crossed over each other and formed multilayers (Figure 4.11b). Confluent cultures of the PL cells grown in control and both of the test culture media showed patterns similar to those of the M cells (not shown). The gingival cells, however, appeared to form only parallel-aligned monolayers in all cultures, similar to M cells in control medium.

After 2 weeks of culture, von Kossa staining revealed individual nodules with irregular shapes in both test groups of the M cultures, an example of which is shown in Figure 4.12. These nodules varied in size and shape and were distributed homogeneously throughout the culture (Figure 4.13). There were no differences in either the size or the quantity of nodules between the test group 1 and test group 2 of the M cultures. In contrast, M cells grown in control medium had only a few mineralised nodules (data not shown). The PL cells incubated in both special and normal culture medium also formed nodules, but these were smaller and far more sparse compared to those in the M cells. The results of von Kossa staining are summarised in Table 4.3.

By 4 weeks, the numbers of individual nodules seen in both M test cultures increased (Table 4.3). Some appeared to fuse together and form large aggregates which covered most of the surface of the culture dish (Figure 4.14). As at 2 weeks, the control M cultures also formed nodules at 4 weeks, but these were smaller and less dense compared to those in both test M cultures. Although the PL cells exhibited some nodule formation (not shown), their size and amount did not substantially increase at 4 weeks (Table 4.3). The gingival cultures did not form any mineralised nodules under any culture conditions (not shown).
Figure 4.11. Confluent cultures of M cells incubated in normal (a) and special (b) medium for 4 weeks. Note the monolayer of tightly-packed cells in (a) compared with multilayers formed by overcrossing cells in (b) (original magnifications x 100).

Figure 4.12. High magnification of a mineralised nodule (MN) formed by the M cells. The structures seen at the background are closely packed M cells (test group 1) (original magnification x 200).
Figure 4.13. Distribution of the nodules (arrows) in the M cell cultures (test group 1) following 2 weeks of incubation in special nodule growth medium (original magnification x 100).

Figure 4.14. Distribution of the nodules in the M cell cultures (test group 1) following 4 weeks of incubation. The aggregates of nodules (dark areas) cover the majority of the culture surface as the mineralisation process proceeds with time (see Figure 4.13, same culture at 2 weeks) (original magnification x 100).
Table 4.3. Formation of mineralised nodules by cultured cells.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Week 2</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test 1</td>
<td>Test 2</td>
</tr>
<tr>
<td>M</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>PL</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gingiva</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

M = membrane-associated  
PL = periodontal ligament  
+++ = aggregates of nodules  
++ = large numbers of individual nodules  
+ = small numbers of individual small nodules  
- = absence of nodules

4.4 DISCUSSION

In this part of the study, certain morphological, phenotypic and functional properties of the cultured cells associated with periodontal regeneration were compared with cells derived from normal PL and gingival tissues in vitro. Initially, no method was available for obtaining cell cultures associated with periodontal regeneration. Cultures of M cells associated with the retrieved ePTFE membranes were obtained using a number of different methods. The most successful were those using the membrane pieces directly or collecting the dissociated cells from the TM. Although membrane pieces placed between the surface of the culture dish and cover slip did not yield outgrowths except for one instance in the present study, Wakabayashi et al. (1996; 1997) reported the establishment of M cultures using a similar method. In those studies, the cover slips overlying the membrane pieces were held in place by sterile vacuum grease, whereas it was not used in the present study.

Cultures of spindle-shaped fibroblast-like cells, as previously observed on the retrieved membranes by TEM (section 3.3.3), were readily cultured in vitro (Kuru et al. 1997a). These M cells were morphologically similar to those identified by Wakabayashi et al. (1996). They were first seen after 7 days of incubation, similar to the reports of Grosso et al. (1997)
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and Wakabayashi et al. (1996; 1997) who observed cells migrating from the membranes between 10 and 14 days. In addition to the use of the membranes, the present study showed, for the first time, that the cells which became detached from the membranes and remained in the TM during transportation could also be successfully cultured. This confirms the observation, again for the first time, that some cells remain associated and grow on the membranes in vitro. Since these fibroblast-like cells grow by adhering to a suitable substratum in vitro (e.g., the surface of a plastic culture dish), this function was apparently provided by the membrane explant itself. This suggests that the membrane explants can usefully be incubated to provide an additional source of proliferating cells.

The presence of bacterial colonies on retrieved membranes, as observed by TEM (see section 3.3.3), made it difficult to obtain primary cell cultures free of any contamination. Therefore, the most coronal part of membrane, the portion having the highest bacterial accumulation, was discarded, and primary incubation of membranes was carried out in the presence of both anti-fungal and anti-microbial agents. Despite these efforts, 15 out of 81 membrane cultures became contaminated at an early stage of incubation. Nevertheless, approximately 20% of membrane explants successfully yielded M cell cultures in vitro. Since the success of the establishment of cells isolated from retrieved ePTFE membranes has not been reported by researchers studying M cells (Wakabayashi et al. 1996; 1997; Grosso et al. 1997), it was not possible to compare those results with the present study.

The ePTFE membranes retrieved from GTR patients were readily obtained since these are normally removed approximately 6 weeks after placement as a part of the routine procedure for GTR therapy. However, the newly-regenerated tissue underlying the membranes at the time of removal was available only very infrequently. Therefore, a small portion of excessive tissue was removed from only 7 out of 98 periodontal defects treated with GTR.

The RT samples were treated similarly to the gingival explants, by cutting them in small pieces and incubating with a minimum amount of
culture medium for about one week to allow the tissue fragments to adhere to the surface of culture dishes. Using this technique, spindle-shaped RT cell cultures were established from approximately 50% of explant samples. The present study, thus far the first to describe the culture of cells from human periodontal regenerative tissue (Kuru et al. 1997a; 1997b), showed that the RT cells were smaller and had far less pronounced cytoplasmic processes compared with the M cells. Although both the M and RT cells are thought to be involved in periodontal regeneration, the reason why they exhibit different morphological appearances is unclear and need to be further analysed, since they may reflect functionally distinct populations.

The present study (see Chapter 3) and a previous study by Pritlove-Carson et al. (1994) have both shown that epithelial cells are present in the regenerated periodontal tissue. Hence it was likely that some epithelial-like cells would grow out of the RT explants initially. These results indicate that the exclusion of gingival epithelial cells by barrier membranes in GTR is not fully achieved, although the precise origin of the epithelial cells in the RT samples is still not certain.

In the present study, the PL tissue was scraped only from the middle third of the root surface with a scalpel (Somerman et al. 1988; Pontoriero et al. 1988; Hou and Yaeger 1993; Ogata et al. 1995; Kuru et al. 1996; Kuru et al. 1998a) in order to avoid any contamination of gingival or apical tissues. The PL explants were incubated immediately with culture medium unlike the study of Mariotti and Cochran (1990) who first treated the samples with trypsin and collagenase for 1 hour. Approximately 13% of PL explants gave rise to monolayer outgrowth, similar to the study of Piche et al. (1989) who obtained viable cell populations from 10% of the PL samples. In contrast, Somerman et al. (1988) and Adams et al. (1993) reported more than 90% and 76% successful cell outgrowth, respectively.

The gingival tissue was obtained from the patients undergoing crown lengthening surgery or from gingival tissue which remained attached to extracted teeth (Kuru et al. 1996; Bent et al. 1997; Kuru et al. 1998a). The gingival cell cultures were established from 24% of gingival explants, at
lower incidence compared to the study of Somerman et al. (1988) who reported emigrating cells from 48% of gingival explants.

The morphological characteristics of the PL and gingival cells closely resembled those described previously in vitro. In addition to the spindle-shaped, fibroblast-like cells (Piche et al. 1989; Somerman et al. 1990; Adams et al. 1993; Hou and Yaeger 1993; Giannopoulou and Cimasoni 1996; Kuru et al. 1998a), epithelial-like cells have been also observed in explant cultures of gingival tissues (Adams et al. 1993; Ohshima et al. 1993; Kuru et al. 1998a) and, at lower incidence, of PL tissues (Uitto et al. 1992; Adams et al. 1993). In the present study, although epithelial-like cells were present in almost all of the primary gingival cultures, there was no evidence of such cells in the primary PL cultures. These cells were also absent from gingival sub-cultures, as seen both microscopically and as evidenced immunocytochemically by the absence of cytokeratin staining.

Wakabayashi et al. (1996) reported that the cells cultured from ePTFE membrane formed palisades of interdigitating cells at confluence, as also observed in the present study. Ogata et al. (1995) demonstrated that when the gingival and PL cells were sparse there was no significant difference in morphology, but at confluence gingival cells grew in monolayer, whereas PL cells formed multilayers. However, the cell cultures examined in this study formed monolayers with parallel orientation and/or multilayers with random orientation, in agreement with the study of Piche et al. (1989). However, when the cells were grown in the special nodule growth medium, only the M and PL cell cultures formed multilayers whereas the gingival cells did not.

Adams et al. (1993) reported changes in the fine structure of fibroblast-like PL cells after the ninth sub-culture suggesting that they were undergoing differentiation or senescence. Based on this observation, only the cells between the third and eighth passages were used in the present study.

In a recent study, Wakabayashi et al. (1998) compared the proliferation rate of cells isolated from the membrane with that of gingival cells of the same patient and found that gingival cells proliferated
significantly faster than the membrane-associated cells. Similarly, the M cells showed far slower proliferation than the gingival cells as well as the RT and PL cells in the present study. This is probably due to the close association of M cells with the ePTFE membrane, since gingival fibroblasts grown directly on such membranes in vitro have been shown to proliferate more slowly compared with their counterparts grown on plastic (Locci et al. 1997). Although cell numbers were not determined in the study of Grosso et al. (1997), they observed that the cells isolated from GTR membranes tended to grow more slowly than the cells isolated from membranes used for guided bone regeneration. The fibroblast-like cells obtained from healing periodontal granulation tissues have been also reported to proliferate more slowly than normal PL fibroblasts (Häkkinen and Larjava 1992). In the present study, the proliferation rate of RT cells was found to be slower than the gingival fibroblasts but higher than the M and PL cells. Since there is no published data available, thus far, on the growth characteristics of the RT cells, no comparison could be made regarding the proliferation rate of these cells. Although both the M and RT cells could be considered to be associated with periodontal regeneration, why the RT cells proliferated far more rapidly than the M cells remained unclear.

In this study, gingival cells were found to proliferate faster than the PL cells. A similar finding was also reported by Giannopoulou and Cimasoni (1996), whereas Somerman et al. (1988) did not find any differences in proliferation rates between the PL and gingival cells. In contrast, in the study of Ogata et al. (1995) the PL cells have been reported to exhibit a higher growth rate than the gingival cells.

The M, RT, PL and gingival cells examined immunocytochemically between passages 3 to 8 were found to express vimentin, suggesting their mesenchymal origin. Pritlove-Carson et al. (1992; 1994) have also identified the cells attached to the retrieved membranes as vimentin-positive. The enzyme 5' prolyl-4-hydroxylase, which plays a key part in the synthesis of collagen (Hay 1981), catalyses the post-translational modification of collagen by hydroxylation of proline into hydroxyproline (Gage et al. 1989). It is characteristically associated with the fibroblastic phenotype (Wilkinson
et al. 1992). The precise type(s) of collagen produced by these cells is not
known because 5' prolyl-4-hydroxylase is involved in the synthesis of all
types of collagen. Nevertheless, the presence of this enzyme suggests
ongoing and active production of the collagen molecule.

ALP (orthophosphoric-monoester phosphohydrolase) is a calcium-
and phosphate-binding protein and a phospho-hydrolytic enzyme with an
alkaline pH optimum (Coleman 1992). There are 4 forms of ALP, each
coded by separate genes (Bilezikian et al. 1996). The tissue-non-specific
ALP (TNSALP), the most widely studied form, is membrane-bound and
expressed not only in bone but also in liver and kidney (Coleman 1992).
ALP activity has been considered to be an important indicator for bone
formation and a marker enzyme of cells with osteoblastic phenotype (Rodan

ALP has been widely studied in periodontal tissues and cultured cells
derived from them. Histologically, ALP activity has been shown to be
heterogeneously distributed in the PL tissue (Nojima et al. 1990; Groeneveld
et al. 1993). The activity in this tissue appeared to be higher in regions
close to alveolar bone, whereas it was markedly lower in gingival connective
tissue compared with the PL (Yamashita et al. 1987; Groeneveld et al.
1993). Biochemical studies of ALP in cultured PL cells have also
established that the activity of this enzyme is higher than that in gingival
cells (Somerman et al. 1988; Piche et al. 1989; Nojima et al. 1990; Ogata et
al. 1995; Kuru et al. 1998a). Interestingly, PL cells exhibited even higher
levels compared with alveolar bone and bone marrow cells (Inoue et al.
1992; Ogata et al. 1995). On the other hand, normal gingival cells obtained
from different individuals have been found to exhibit widely differing levels of
ALP activity, from weak to moderate and even to high (Carnes et al. 1997).
In addition, cultured cells derived from inflamed gingiva and phenytoin-
induced hyperplasia also exhibited different ALP activity than normal
gingival fibroblasts (Hou and Yaeger 1995; Abe et al. 1996).

The results of the present study showed that higher basal levels of
ALP are present in the RT cells compared with the others (Kuru et al.
submitted). These findings suggest that RT cultures contain cells with
osteoblast-like characteristics. This is consistent with a previous study showing that new tissue formed beneath the membranes express ALP as well as bone-related proteins including osteonectin and bone sialoprotein (Amar et al. 1995). Other studies showing the formation of new cementum following GTR therapy also indicate the presence of hard connective tissue-forming cells (Gottlow et al. 1984; Gottlow et al. 1986). Although the basal ALP level of gingival cells was slightly higher than the PL cells on day 4, the latter showed higher basal activity on day 7. Recently, Carnes et al. (1997) demonstrated that human gingival cells had moderate to high ALP activity.

DEX is a synthetic glucocorticoid which has been reported to selectively stimulate the proliferation and differentiation of osteoprogenitor cells in vitro (Chen et al. 1997). Moreover, this hormone has also been shown to up-regulate ALP activity in PL cells but not in gingival cells (Kuru et al. 1998a). It has been previously suggested that mature PL tissue is likely to contain progenitor cells which not only give rise to PL fibroblasts but also to osteoblasts and cementoblasts (Lekic and McCulloch 1996). In the present study, DEX was added to the different types of periodontal cell cultures to examine whether this hormone would influence the in vitro differentiation of the cells. The results of these experiments showed that DEX up-regulated ALP activity substantially in the RT cells compared to moderate increase in the M and PL cells, possibly reflecting the presence of an increased proportion of osteoprogenitor cells in the newly-regenerated periodontal tissue. Although the reason why ALP activity was lower in the M cells compared with the RT cells is not known, it is possible that the membrane-associated cells may represent only a subset of those present in the RT. Moreover, as some fibroblast-like cells were also observed on the external side of membrane in addition to the internal side (Selvig et al. 1990), as shown in section 3.3.3, it is also possible that the M cell cultures may contain some contaminating cells from the gingival flap. In contrast, the RT cells were obtained directly from the regenerated periodontal tissue itself.

Both the M and PL cells but not the gingival cells were also found to have the ability to form mineralised nodules when induced by ascorbic acid
and β-glycerophosphate. Similarly, Wakabayashi et al. (1996; 1997) reported that cells isolated from GTR membranes formed nodules. However, the nodules formed by the M cells were considerably larger and their quantity was greater than those in the PL cultures. The finding that the M and PL cells exhibit high ALP activity and therefore may possess osteoblast-like properties was further supported by the findings of von Kossa staining as they were also able to form mineralised nodules.

Mature PL tissue has been suggested to contain progenitor cells which may differentiate and give rise to PL fibroblasts, osteoblasts and cementoblasts (Lekic and McCulloch 1996). Differentiation of the progenitors during periodontal wound healing, particularly in periodontal regeneration, has been considered to be regulated in vivo by a wide variety of ECM proteins, cytokines and growth factors which induce both selective and non-selective responses in cells involved (Pitaru et al. 1994). In this study, DEX as well as ascorbic acid and β-glycerophosphate have been used in vitro to stimulate the differentiation of osteoprogenitor cells involved in periodontal regeneration. The results of the present study provide evidence that the high ALP activity in the M and RT cultures, as well as nodule formation ability by the M cells may indicate that these cells have characteristics of osteoblast-like cells. Furthermore, the response of these cells to DEX, ascorbic acid and β-glycerophosphate suggest that increased numbers of differentiating osteoprogenitor cells are present in regenerating periodontium with normal PL tissue.

4.5 CONCLUSIONS

The results of this part of the study show that
i) cells associated with periodontal regeneration could be isolated and cultures of these could be obtained in vitro from retrieved membranes and underlying tissues;
ii) these cells exhibited some fibroblast-like characteristics similar to PL and gingival cells, including spindle-shaped morphology, presence of vimentin and 5' prolyl-4-hydroxylase and the absence of cytokeratin;
Chapter 4

iii) the M and RT cells also exhibit some osteoblast-like characteristics, and some may possibly be osteoprogenitor cells, as evidenced by ability to form mineralised nodules and high levels of ALP activity.
CHAPTER 5
EXTRACELLULAR MATRIX PRODUCTION
BY REGENERATIVE PERIODONTAL CELLS

5.1 INTRODUCTION

A principal role of the extracellular matrix (ECM) is considered to be as the substratum for the structural support of the cells embedded in it. In addition, the ECM contains a number of biologically active factors which play key roles during wound healing and regeneration by regulating the migration, proliferation, adhesion as well as differentiation of cells (Mariotti 1993; Pitaru et al. 1994). The regeneration of the periodontium is therefore likely to be controlled, at least partially, by the ECM. The specific components of the ECM which elicit these effects comprise structural, adhesive and chemotactic proteins such as collagen, decorin and fibronectin (Terranova et al. 1987; Somerman et al. 1989; Sage and Bornstein 1991; Bartold 1995; Giannopoulou and Cimasoni 1996) and also proteins which effect cell differentiation, such as osteopontin and bone sialoprotein (BSP) (MacNeil et al. 1995; D'Errico et al. 1997).

A number of immunohistochemical studies have provided information about the localisation of ECM proteins in the regenerating periodontium (Pritlove-Carson et al. 1994; Amar et al. 1995; Amar et al. 1997), but the expression of key ECM proteins by the cells associated with periodontal regeneration is not yet known. Therefore, in this part of the study, flow cytometry (FCM) was used to study certain functional features of the cultured cells, as well as their physical characteristics. The aims were:

i) to examine the size and internal granularity of GTR-associated M and RT cells in comparison with normal PL and gingival cells;
ii) to investigate the expression of ECM proteins by these cells and to determine possible differences in ECM production between the cells.
5.2 MATERIALS AND METHODS

5.2.1 Flow cytometry

Confluent cultures of the M (n=6), RT (n=4), PL (n=5) and gingival cells (n=9) were prepared for FCM, as described in 2.2.8.2. The following primary antibodies were used: mouse mAbs against fibronectin (Gibco), tenasin (Chemicon; Temecula, USA) and collagen type I (Sigma) (mAbs diluted 1:100); rabbit polyclonal antibodies against decorin, osteonectin (LF-BON-II), bone sialoprotein (LF-120) and osteopontin (LF-123), provided by Dr. L.W. Fisher (National Institute of Dental Research, Bethesda, USA) (Fisher et al. 1995), and osteocalcin (Biogenesis) (polyclonal antibodies diluted 1:1000). The size (forward scatter; FSC), granularity (side scatter; SSC) and fluorescence intensity of 10,000 individual cells were measured by FCM. The stored data were processed by CELLQuest Software which provides a statistical profile for each of the samples. The arithmetic mean of the FSC and SSC parameters and the geometric mean of the fluorescence intensity (average fluorescence intensity; AFI) were used as the unit for statistical analyses described below in 5.2.2. These parameters are presented as arbitrary units of scattered light and fluorescence.

In order to determine the effects of dexamethasone (DEX) on the cells, duplicate cultures of the M, RT, PL and gingival cells were incubated with 10^{-8} M DEX for 7 days. The cells were then immunostained for osteonectin, osteocalcin, BSP and osteopontin and examined by FCM.

5.2.2 Statistical analysis

The experiments described above were repeated twice under all conditions for each cell line of each culture of the M, RT, PL and gingival cells. The arithmetic mean of each of the duplicate experiments was calculated and considered as the statistical unit. The results of the cell size and granularity measurements were analysed using a parametric method, the one-way analysis of variance (ANOVA) test. However, the results of the fluorescence measurements of ECM expression were not distributed normally and therefore non-parametric tests were used for statistical analysis.
Firstly, the Kruskal-Wallis test was used to determine whether there were any differences between cell types overall. When statistically significant differences were found, the data were then further analysed using the Mann-Whitney U test to establish the specific differences between individual cell types. The differences between the DEX-treated and non-treated cells were analysed using the Wilcoxon Signed Rank test.

5.3 RESULTS

5.3.1 Cell size and cell granularity

The FSC and SSC of the cultured cells were measured by FCM to determine the size and granularity of the individual cells in the population. Figure 5.1 shows representative dot plots of cells in the M, RT, PL and gingival cultures. Each cell is represented by one dot, as detected by FCM. Although all plots appeared to be similar, it was notable that the M and PL cells had wider and more extended distributions compared with the RT and gingival cells.

Table 5.1 shows a summary of the size and granularity of the cultured cells obtained using a total of 24 cell lines in duplicate. These data show that the size of the M cells (FSC of 530 ± 26) was larger than that of the PL cells (498 ± 42), whereas the RT cells (494 ± 43) and the gingival cells (478 ± 20) were smaller than the PL cells. However, the differences in FSC parameters between the cell cultures were not statistically significant (p > 0.05).

The M cells were also found to be the most granular, having an SSC of 443 (± 27) compared with the RT cells (423 ± 19) and PL cells (432 ± 37). As with size, the gingival cells were also the least granular (365 ± 18). These differences were again not statistically significant (p > 0.05).
Figure 5.1. Dot plot profiles of 10,000 cells derived from the membrane (M), regenerated tissue (RT), periodontal ligament (PL) and gingival explants. Each cell is represented by one dot. The size and granularity were measured by FCM and presented as arbitrary units.
Table 5.1. Size (FSC) and granularity (SSC) of membrane-associated (M), regenerated tissue (RT), periodontal ligament (PL) and gingival cells measured by flow cytometry.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Parameter</th>
<th>M</th>
<th>RT</th>
<th>PL</th>
<th>Gingiva</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td></td>
<td>530 ± 26</td>
<td>494 ± 43</td>
<td>498 ± 42</td>
<td>478 ± 20</td>
<td>0.659</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(423 - 596)</td>
<td>(424 - 619)</td>
<td>(385 - 674)</td>
<td>(367 - 563)</td>
<td></td>
</tr>
<tr>
<td>SSC</td>
<td></td>
<td>443 ± 27</td>
<td>423 ± 19</td>
<td>432 ± 37</td>
<td>365 ± 18</td>
<td>0.130</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(344 - 543)</td>
<td>(397 - 482)</td>
<td>(354 - 599)</td>
<td>(291 - 436)</td>
<td></td>
</tr>
</tbody>
</table>

The values are arbitrary. Data are presented as the mean ± standard error of the means, the numbers in brackets show the range.

*Data were analysed using the one-way ANOVA test and p ≤ 0.05 is considered to be statistically significant.

5.3.2 Expression of connective tissue-associated proteins

Fluorescence profiles were obtained by FCM after immunostaining the M, RT, PL and gingival cells for the connective tissue proteins collagen type I, fibronectin, tenascin and decorin. Antigen levels expressed by the PL cells, representing the cell population derived from normal healthy PL tissue, was used as a baseline for comparison with the GTR-associated M and RT cells. A solid vertical line was drawn arbitrarily on the histograms presented in this chapter to indicate the mean of each of the PL profiles.

Nearly 50% of cells within the cultures examined were found to be positive for collagen I. The representative histograms (Figure 5.2) show that the expression of collagen I was narrow and uniformly distributed. This observation suggested that all the cells in each of the different cultures had very similar collagen levels, although there were differences in collagen expression between the different cell cultures. Thus, it was noted that both the M and RT profiles were shifted to higher fluorescence intensity units (to the right) of the vertical line, showing that the M and RT cells had higher levels than the PL cells. The results of a number of experiments are summarised in Table 5.2 and show that the differences in collagen I expression between all the cell types were statistically significant, using the
Kruskal-Wallis test (p < 0.05). In order to determine which two cell types were different from each other, the Mann-Whitney U test was carried out. The results of this statistical analysis revealed that, although the GTR-associated M and RT cells (AFIs of 75 and 73, respectively) had higher collagen levels than the normal PL cells (AFI of 66), these differences were not significant (p > 0.05). The M and RT cells also had elevated levels of this protein compared with the gingival cells (AFI of 40), but only the M cells were significantly higher (p < 0.01).

Table 5.2. Expression of soft connective tissue proteins by membrane-associated (M), regenerated tissue (RT), periodontal ligament (PL) and gingival (G) cells.

<table>
<thead>
<tr>
<th>Protein</th>
<th>M cells</th>
<th>RT cells</th>
<th>PL cells</th>
<th>G cells</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=6</td>
<td>n=4</td>
<td>n=5</td>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td>Collagen I</td>
<td>75</td>
<td>73</td>
<td>66</td>
<td>40</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>(56/118)</td>
<td>(31/129)</td>
<td>(42/92)</td>
<td>(25/53)</td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>290</td>
<td>272</td>
<td>188</td>
<td>97</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>(231/562)</td>
<td>(84/552)</td>
<td>(148/362)</td>
<td>(50/121)</td>
<td></td>
</tr>
<tr>
<td>Tenascin</td>
<td>62</td>
<td>63</td>
<td>53</td>
<td>34</td>
<td>0.154</td>
</tr>
<tr>
<td></td>
<td>(37/120)</td>
<td>(30/102)</td>
<td>(43/67)</td>
<td>(25/54)</td>
<td></td>
</tr>
<tr>
<td>Decorin</td>
<td>41</td>
<td>61</td>
<td>50</td>
<td>29</td>
<td>0.081</td>
</tr>
<tr>
<td></td>
<td>(35/65)</td>
<td>(40/116)</td>
<td>(45/65)</td>
<td>(23/48)</td>
<td></td>
</tr>
</tbody>
</table>

The values are arbitrary. Data are presented as the median and the numbers in brackets are the distribution of the data presented as 25/75 percentile.

*Data were analysed using the Kruskal-Wallis test and p ≤ 0.05 is considered to be statistically significant.

AFI = Average fluorescence intensity
Figure 5.2. Representative histograms of collagen I expression by M (bold blue line), RT (bold red line), PL (bold green line) and gingival cells (bold orange line). The corresponding negative control profiles are presented as dashed lines. The vertical black line shows the mean of the PL histogram, which is used to indicate the collagen I level expressed by the normal PL cells. Note high expression of collagen I in the M and RT cells, and low expression in the gingival cells relative to the PL cells. Fluorescence intensity units are arbitrary.
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As with collagen I, fibronectin expression in the M and RT cells was higher than in the PL cells, as shown in the representative histograms in Figure 5.3. Unlike collagen I, however, fibronectin distribution among the individual cells within each culture was relatively wide and, moreover, was not bell-shaped but skewed to levels of fluorescence which were greater than the mean. This non-uniform distribution observed with the M, RT and PL cultures is indicative of the presence of a heterogeneous cell population in the culture, i.e., the presence of sub-populations of cells expressing different levels of antigen. Detailed analysis of these fluorescence profiles was therefore carried out to examine whether the M, RT and PL cultures comprised distinct sub-populations of cells, as described in section 5.3.4 below.

The results of fibronectin expression are summarised in Table 5.2 and show that the differences in the expression of this antigen among the cell types were statistically significant, using the Kruskal-Wallis test \( p = 0.014 \). However, the GTR-associated M and RT cells expressed higher levels of fibronectin (AFIs of 290 and 272, respectively) than the cells derived from normal PL (AFI of 188), although these differences were not statistically significant \( p > 0.05 \) as determined by the Mann-Whitney U test. The M and RT cells also expressed fibronectin at levels 2.9- and 2.8-fold higher than the gingival cells, although only the difference between the M and gingival cells was statistically significant \( p \leq 0.001 \).
Figure 5.3. Representative histograms of fibronectin expression by M, RT, PL and gingival cultures. Histograms representing fibronectin-positive cells and the negative control cells are shown in bold and dashed coloured lines, respectively. The majority of the M and RT profiles are shifted to the right of the vertical black line, indicating that these cells express fibronectin at higher levels than the PL cells.
As with the expression of collagen type I and fibronectin, the representative profiles for tenasin expression by the M and RT cells demonstrated higher fluorescence levels than the PL cells, as shown in Figure 5.4. The histograms also showed that the GTR cells had a relatively wider distribution of tenasin compared to the PL cells. The observation that the M and RT profiles were skewed to the higher fluorescence levels suggested the presence of sub-populations of cells in these cultures.

The results summarised in Table 5.2 also confirmed that the GTR-associated cells expressed tenasin at higher levels (AFIs of 62 and 63 in the M and RT cells, respectively) than in the normal PL cells (AFI of 53). Gingival cells had an AFI of 34. Although both of the GTR-associated cells had approximately 1.2- and 2-fold higher tenasin levels than the PL and gingival cells, respectively, statistical analysis revealed that there were no significant differences between the cell types (p > 0.05). However, while 53% of the RT cells were positive for tenasin, only 32% of the normal PL cells were tenasin-positive. The proportions of tenasin-positive cells in the M and gingival cultures were also relatively low (34 and 43%, respectively).

The decorin profiles of all cell types (data not shown) appeared to be narrow and uniform, similar to collagen I profiles. The results in Table 5.2 show that the RT cells (AFI of 61) had the highest decorin expression among the cell types examined, although unlike the expression of the other connective tissue proteins examined, decorin was expressed at lower levels in the M cells (AFI of 41) than in the PL cells (AFI of 50). The gingival cultures again had the lowest AFI level, which was 29. Despite apparent differences in decorin AFI levels between the cell types, these were not statistical significant (p > 0.05).

Negative controls for the above experiments were the M, RT, PL and gingival cells treated with non-specific mouse IgG1 as primary antibody. Such cells had very low AFI levels, (10, 14, 8 and 6, respectively) and the profiles of these cells were very similarly narrow and uniform, as shown by the dashed lines in Figures 5.2-4.
Figure 5.4. Representative histograms of tenascin expression by M, RT, PL and gingival cultures. Histograms representing tenascin-positive cells and the negative control cells are shown in bold and dashed coloured lines, respectively. The vertical solid black line indicates the mean fluorescence intensity of the PL histogram. Note the higher levels and wider distributions of tenascin in the M and RT cells compared with the PL cells.
5.3.3 Expression of bone-associated proteins

In order to further characterise the GTR-associated cells, the expression of proteins known to be involved in the formation of hard connective tissue, such as bone and cementum, were investigated by FCM. The FCM profiles of osteonectin expression were found to be relatively narrow and uniformly distributed in all cultures (data not shown). However, the RT cells were found to have a higher level of osteonectin (AFI of 67) than the M and PL cells (AFIs of 45 and 56, respectively), as shown in Table 5.3. Gingival cells had an AFI of 38, although none of these differences were statistically significant (p > 0.05).

Differential expression of osteocalcin was evident in the representative histogram profiles, which showed that osteocalcin in the M, RT and PL cultures had a non-uniform distribution (Figure 5.5). These skewed profiles suggested the possibility that heterogeneous sub-populations of cells were present among the cells in these cultures, which were analysed further below (section 5.3.4). The mean fluorescence intensity of the PL cells, indicated by the vertical line in Figure 5.5, shows that the RT, but not the M cells, express higher osteocalcin levels than the PL cells. The differences in osteocalcin expression among the cell types are presented in Table 5.3 and were statistically significant (p < 0.05). However, the Mann-Whitney U test revealed that the 2.4- and 1.2-fold higher expression of osteocalcin in the RT cells was not statistically greater than in the M and normal PL cells, respectively (p > 0.05). The RT cells expressed this protein at a significantly higher level than the gingival cells (p < 0.05).

The BSP profiles of all the cell types were observed to be narrow and uniform (data not shown), although the RT cells (AFI of 56) had slightly higher levels than the M and PL cells (AFIs of 51 and 52, respectively) (Table 5.3). BSP in gingival cells was very low (AFI of 29). These differences were, however, not statistically significant (p > 0.05).

The representative osteopontin profiles of the M and RT cells exhibited markedly non-uniform distributions relative to the PL profile, as shown in Figure 5.6. Moreover, the profiles of both the GTR-associated cells appeared to contain more than one distinct peak, clearly indicating the
presence of heterogeneous sub-populations of cells. These were analysed below (section 5.3.4). In contrast, osteopontin distribution in the normal PL cells was uniform. Table 5.3 shows, however, that as with all other bone-associated proteins, the RT cultures expressed elevated levels of osteopontin (AFI of 58) compared with the other types of cells. Both the M and PL cultures expressed this protein at the same level (AFI of 46), while the gingival cells again expressed the lowest level (AFI of 30) among the cell types examined. However, statistical analysis showed that the differences were not statistically significant (p > 0.05).

Negative controls for the above experiments were the M, RT, PL and gingival cells which were incubated only with the FITC-conjugated secondary antibody. These cells had very low AFI levels (approximately between 5 - 10) and their fluorescence profiles were observed to be very narrow and uniform, as presented as dotted lines in Figures 5.5 and 5.6.

Table 5.3. Expression of bone-associated proteins by membrane-associated (M), regenerated tissue (RT), periodontal ligament (PL) and gingival (G) cells.

<table>
<thead>
<tr>
<th>Protein</th>
<th>M cells n=6</th>
<th>RT cells n=4</th>
<th>PL cells n=5</th>
<th>G cells n=9</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteonectin</td>
<td>45 (33/72)</td>
<td>67 (47/131)</td>
<td>56 (51/67)</td>
<td>38 (31/53)</td>
<td>0.113</td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>41 (27/67)</td>
<td>100 (79/173)</td>
<td>81 (73/98)</td>
<td>40 (31/56)</td>
<td>0.031</td>
</tr>
<tr>
<td>BSP</td>
<td>51 (28/103)</td>
<td>56 (34/104)</td>
<td>52 (47/63)</td>
<td>29 (17/45)</td>
<td>0.084</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>46 (33/63)</td>
<td>58 (35/109)</td>
<td>46 (43/62)</td>
<td>30 (21/48)</td>
<td>0.143</td>
</tr>
</tbody>
</table>

Data are presented as the median and the numbers in brackets represent the distribution as the 25/75 percentile.

*Data were analysed using the Kruskal-Wallis test and p ≤ 0.05 is considered to be statistically significant.

AFI = average fluorescence intensity
BSP = bone sialoprotein
Figure 5.5. Representative profiles of osteocalcin expression (solid lines) by M, RT and PL cells. The dotted lines represent the fluorescence profiles of negative control cells of each culture where the primary antibody was omitted. The vertical black line indicates the mean fluorescence intensity of osteocalcin in the PL cells. Note the relatively lower expression in the M cells and higher in the RT cells compared with the PL cells.
Figure 5.6. Representative profiles of osteopontin expression (solid lines) by M, RT and PL cells. The dotted lines represent the profiles of negative control cells. The vertical line indicates the mean fluorescence intensity of osteopontin in the PL cells. Note the higher expression in the RT cells than in the PL cells which are similar to the M cells.
5.3.4 Sub-populations of GTR-associated cells and normal PL cells

The fluorescence profiles of all the cultures were further analysed to examine possible heterogeneity among the cells. As noted above, the presence of a non-uniform, skewed distribution of fluorescence or the presence of more than one peak in the profiles is considered to indicate that sub-populations of cells are present in the cultures. Such profiles were identified and different levels of antigen expression were selected by gating. The cells within each arbitrary gate were then analysed for their size and granularity as well as their specific AFI level.

Among the soft-connective tissue proteins, the profiles of collagen I in all cell types were uniform, indicating that no subsets of collagen-expressing cells were present in the cultures. Similar results were obtained for decorin. The tenascin profiles of the M and RT cells demonstrated some skew but an apparent second peak was not evident. In contrast, the fibronectin profiles of the M, RT and normal PL cells were very broad, markedly skewed to higher fluorescence levels and appeared to have more than one peak of fibronectin expression. This suggested that a substantial proportion of the cells in the cultures have a higher level of fibronectin than other cells in the same culture. The profiles of fibronectin were therefore arbitrarily gated into sub-populations designated as fibronectin\textsuperscript{low} and fibronectin\textsuperscript{high} cells, as shown in Figure 5.7. The fibronectin\textsuperscript{low} cells were defined as having AFI values approximately between 4 and 560 and the fibronectin\textsuperscript{high} cells between 560 and 4500 AFI units.
Figure 5.7. Fibronectin\textsuperscript{low} and fibronectin\textsuperscript{high} cells in a culture of M cells. The vertical dotted lines represent the borders of the selected gates which are identified manually.

These regions, comprising the fibronectin\textsuperscript{low} and fibronectin\textsuperscript{high} cells in the M, RT and PL cultures were then 'back-analysed', as shown in Table 5.4, to determine the AFI levels of each of the cells as well as their size and granularity. The fibronectin\textsuperscript{low} cells comprised 69% and 44% of the total M and RT cells, respectively. These fibronectin\textsuperscript{low} GTR-associated cells were found to have markedly higher fluorescence intensity values (AFIs of 159 and 170, respectively) compared with the fibronectin\textsuperscript{low} PL cells (AFI of 43), which comprised 60% of total M cells. Similarly, the fibronectin\textsuperscript{high} M and RT sub-populations had much higher levels (1056 and 989, respectively) than the fibronectin\textsuperscript{high} PL cells (AFI of 289). Back-analysis also revealed that the fibronectin\textsuperscript{high} cells were larger and more granular than the fibronectin\textsuperscript{low} counterparts in all the M, RT and PL cultures, although there were no apparent differences in either the size or granularity between the fibronectin\textsuperscript{low}-expressing M, RT and PL cells or the fibronectin\textsuperscript{high}-expressing M, RT and PL cells. Figure 5.8 show the dot plots of the M cells from Figure 5.8 which were designated as fibronectin\textsuperscript{low} and fibronectin\textsuperscript{high}. Similar distributions were also observed for the RT and PL sub-populations (not shown).
### Table 5.4. Comparison of sub-populations in fibronectin-positive membrane-associated (M), regenerated tissue (RT) and periodontal ligament (PL) cells.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>AFI</th>
<th>Size</th>
<th>Granularity</th>
<th>% of total cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>159 ± 78</td>
<td>454 ± 76</td>
<td>390 ± 95</td>
<td>69 ± 11</td>
</tr>
<tr>
<td>RT</td>
<td>170 ± 89</td>
<td>448 ± 126</td>
<td>332 ± 143</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>PL</td>
<td>43 ± 19</td>
<td>487 ± 79</td>
<td>371 ± 75</td>
<td>60 ± 15</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard deviation. The average fluorescence intensity (AFI), size and granularity units are arbitrary.

![Dot plots of fibronectin\textsuperscript{low} and fibronectin\textsuperscript{high} M cells.](image)

**Figure 5.8.** Dot plots of the fibronectin\textsuperscript{low} and fibronectin\textsuperscript{high} M cells.
Among the bone-associated proteins, the fluorescence profiles of osteonectin and BSP were found to be uniform with no apparent second peak. In contrast, osteocalcin profiles of the M, RT and PL cells indicated heterogeneity of these cells in these cultures (see Figure 5.5). For back-analysis, the osteocalcin histograms were arbitrarily gated, as described above for fibronectin, and the cells within the gates designated as osteocalcin\textsuperscript{low} and osteocalcin\textsuperscript{high}. The osteocalcin\textsuperscript{low} sub-population in the RT cultures had a lower antigen level (AFI of 13) than that in the PL cultures (AFI of 35). In contrast, the osteocalcin\textsuperscript{high} sub-population in the RT cells was found to express this bone-associated protein at higher levels (AFI of 64) than that in the PL cells (AFI of 51). Osteocalcin expression in the M sub-populations, however, was somewhat lower than those of both the RT and PL cells.

The osteopontin histograms of the PL cells appeared to be wide with a single peak with no apparent second peak (see Figure 5.6). In marked contrast, the histograms of osteopontin-expressing cells in the M and RT cultures were observed to have two peaks which were subsequently gated, as shown in Figure 5.9, and designated as the osteopontin\textsuperscript{low} (AFI values between 3 - 22) and osteopontin\textsuperscript{high} (AFI values between 22 - 300) sub-populations. Further analysis showed that both the 'low' and 'high' RT sub-populations (AFIs of 15 and 50) expressed osteopontin very similar to the 'low' and 'high' M sub-populations (AFIs of 15 and 58, respectively). The osteopontin\textsuperscript{low} cells were smaller and less granular than their osteopontin\textsuperscript{high} counterparts in all cultures, similar to sub-populations expressing fibronectin at low and high levels (see Figure 5.7).
Figure 5.9. Osteopontin$^\text{low}$ and osteopontin$^\text{high}$ cells in a representative profile of the RT cells. The vertical dotted lines represent the borders of the selected gates.

5.3.5 Effects of DEX on cultured cells

In order to further examine the osteoblast-like properties of the cultured cells, they were treated with DEX, a hormone known to induce osteoblast differentiation (Chen et al. 1997). Thus, the change in the fluorescence profile of bone-associated proteins would indicate that DEX induced the osteoblastic differentiation of cells \textit{in vitro}. FCM analysis showed that treatment with DEX resulted in a slight decrease in both of the size and granularity in each of the types of cell relative to its non-treated counterpart (Figure 5.10), although these differences were not statistically significant (p > 0.05).
Figure 5.10. Changes in the size and granularity of the membrane-associated (M), regenerated tissue (RT), periodontal ligament (PL) and gingival (G) cells in response to DEX. The FSC and SSC parameters of DEX-treated cells are compared with those of corresponding non-treated cells, which are defined as 0. No statistically significant differences were found between the treated and non-treated cells (p > 0.05).

FCM analysis also revealed that treatment with DEX gave rise to a little change in the profiles of the PL and gingival cells compared with their non-treated counterparts. However, in addition to a slight shift to higher fluorescence levels, the M and RT cells exhibited some specific changes in the profiles for osteocalcin and osteopontin. The detailed analysis was therefore carried out only for the M and RT cells to examine the effect of DEX on the sub-populations in these cultures, as described below (section 5.3.6).

The results summarised in Figure 5.11 show that DEX increased the AFI levels of all of the antigens in all cultures except BSP, which declined. However, statistical analysis, using the Wilcoxon Signed Rank test, revealed that the differences between the non-treated cells and their DEX-treated counterparts in all cultures were not significant (p > 0.05).
Figure 5.11. Changes in the expression of osteonectin, osteocalcin, bone sialoprotein (BSP) and osteopontin by membrane-associated (M), regenerated tissue (RT), periodontal ligament (PL) and gingival (G) cultures in response to DEX. The expression of these proteins by non-treated cells is defined as 0 and compared with the DEX-treated cells. There were no statistically significant differences between the non-treated and DEX-treated cells (p > 0.05).

5.3.6 Sub-populations in DEX-treated M and RT cultures

5.3.6.1 Osteocalcin expression

Figure 5.12a shows a representative profile of osteocalcin expression in the non-treated M cells (solid profile), which have a relatively uniform distribution and an AFI of 24. After treatment with DEX, osteocalcin expression in the M cells (open profile) increased to a higher fluorescence level (AFI of 31), with no other apparent change in antigen distribution (Figure 5.12a).
Figure 5.12a. Osteocalcin expression by non-treated (blue) and DEX-treated (dark blue line) M cells.

Figure 5.12b shows representative profiles of the same experiments carried out with the RT cells. The non-treated RT cells (solid profile) exhibited a non-uniform distribution with an AFI of 27. The fluorescence profile had a main peak on the left (peak 1) and an apparent second peak (peak 2) of fluorescence which is skewed to higher fluorescence values on the right of the profile. The cells in regions corresponding to the peak 1 and peak 2 were gated and designated as osteocalcin\textsuperscript{low} and osteocalcin\textsuperscript{high}. Back-analysis revealed that the osteocalcin\textsuperscript{low} subset comprised 63% of the total cells and had an AFI of 12, whereas the osteocalcin\textsuperscript{high} subset comprised 37% of the total cells and had an AFI of 64. After treatment with DEX, neither the AFI of overall osteocalcin expression in the DEX-treated RT cells nor the AFI's of each of the subsets changed. However, the only difference between the DEX-treated RT cells and their non-treated counterparts was the change in the proportions of cells in each of the subsets. The proportion of osteocalcin\textsuperscript{low} cells decreased to 44% of the total cells while the proportion of cells in osteocalcin\textsuperscript{high} region increased to 56%. The osteocalcin\textsuperscript{high} cells were also larger and more granular (FSC and SSC of 351 and 274, respectively) than the osteocalcin\textsuperscript{low} cells (FSC and SSC of 384 and 327, respectively).
5.3.6.2 Osteopontin expression

As shown in Figure 5.13a, the osteopontin profile of the non-treated M cells (solid profile) had a single peak (peak 1) and relatively uniform distribution with an AFI of 17. However, the DEX-treated M cells (open profile) was shifted to a higher fluorescence value (AFI of 22) and had a substantially different antigen distributions. Thus, in addition to the peak 1, which appeared to be in the same place as the single peak of the non-treated cells, a second peak (peak 2) became evident. The DEX-treated M cells in the regions corresponding to peak 1 and peak 2 were gated and designated as osteopontin$^{\text{low}}$ and osteopontin$^{\text{high}}$ subsets, respectively. Approximately 75% of the DEX-treated M cells were osteopontin$^{\text{low}}$ and had far lower osteopontin expression (AFI of 15) compared with the osteopontin$^{\text{high}}$ subset (AFI of 58). The cells in the osteopontin$^{\text{low}}$ subset were also found to be smaller and less granular than the osteopontin$^{\text{high}}$ subset of M cells.
Figure 5.13a. Osteopontin expression by M cells cultured in the absence (solid blue) and presence (dark blue line) of DEX. The profile of the DEX-treated cells shifted to right of the non-treated profile, and exhibited a new peak (peak 2) in addition to peak 1.

As shown in Figure 5.13b, the representative osteopontin profile of the non-treated RT cells exhibited a distribution with two apparent and similar peaks (peak 1 and peak 2). As described previously, the cells in each of the regions were gated and designated as osteopontin<sub>low</sub> and osteopontin<sub>high</sub> cells. The cells in each region accounted for approximately 50% of the total RT cells. However, after treatment with DEX, the proportion of the osteopontin<sub>low</sub> cells decreased to 36%, whereas the cells in the osteopontin<sub>high</sub> subset comprised 64% of the total DEX-treated RT cells. Back-analysis also revealed that the osteocalcin<sub>low</sub> cells with an AFI of 11 were smaller and less granular than the osteocalcin<sub>high</sub> cells which had an AFI of 60. These observations may suggest that some cells in the M and RT population differentiated in the presence of DEX and consequently expressed substantially higher levels of osteocalcin, whereas other cells remained unaffected by DEX and, thus, their osteocalcin level remained unchanged.
Figure 5.13b. Osteopontin expression by RT cells cultured in the absence (yellow profile) and presence of DEX (red line). Two peaks present in both the profiles are designated as peak 1 and peak 2. Note the differences in each of the peaks between the profiles of non-treated and DEX-treated cells.

5.4 DISCUSSION

The present study investigated a number of features of the GTR-associated M and RT cells using, for the first time, the FCM technique. This procedure enables certain physical and chemical properties of individual cells to be measured simultaneously as they pass through a laser beam (Shapiro 1988). The scattered light is detected by sensors and is relative to the size and granularity of the cells, while detectors of emitted fluorescence light measure the relative level of antigens which had been immunostained using fluorochrome-conjugated monoclonal or polyclonal antibodies (Ormerod 1994). The actual data obtained are relative since antigen levels measured by FCM are arbitrary units depending on the electronic input and settings of the specific cytometer. However, as reported by Sumner et al. (1991), the relative values obtained by FCM are proportional to the actual number of molecules present in and/or on the cells. Thus, the relative level of fluorescence intensity represents the actual level of the corresponding molecule. FCM differs fundamentally from the qualitative immunocytochemical staining technique of cultured cells, as used in Chapter
Chapter 5

4, because it provides quantitative data. An additional major advantage of FCM lies in being able to measure the properties for each individual cell in a population rather than measuring the cell population as a whole (as in the cell-ELISA technique). Consequently, the real advantage of FCM is the ability to analyse distinct subsets of cells in a heterogeneous population without the need to physically separate the cells. Applications of FCM are widespread and include measurement of cell-associated proteins (Kuru et al. 1997b; 1997c; 1998a; Parkar et al. submitted), cell-cycle analysis (Lopes et al. 1998), determination of blood cell populations (Orfao et al. 1995), malignancy and microbial biology (Ormerod 1994).

The FCM procedure carried out in this study measured relative levels of intracellular as well as cell surface-associated antigens in each individual cell. The analysis of intracellular as well as cell surface antigens was possible for two reasons. Firstly, EDTA was used instead of trypsin to detach the cells from the plastic culture dishes (Bou-Gharios et al. 1994; Lopes et al. 1998; Kuru et al. 1998a), so that surface antigens remained associated with the cell. Secondly, saponin was used to permeabilise the cell membrane, allowing the entry of the antibodies and consequently enabling the intracellular levels of proteins to be measured in addition to surface-associated antigens as previously reported (Abraham et al. 1991).

The results of the present study demonstrates that the GTR-associated M and RT cells appear to have certain morphological and functional properties which differ from the normal PL cells and also from normal gingival cells. Although all cell cultures were observed to be spindle-shaped when examined under phase-contrast microscopy (see Chapter 4), some differences were noted when the size and granularity of the cultured cells were compared using the FCM technique. The presence of an extensive rough endoplasmic reticulum in the cells associated with the ePTFE membrane, as observed by TEM (see Chapter 3), is consistent with the finding of this chapter that these cells are more granular than the other cells. The size and granularity of the RT cells, however, were very similar to those of the PL cells. The gingival cultures consisted of the smallest and least granular cells among the cell cultures, consistent with the observation
of Kuru et al. (1998a) that gingival cells were less granular compared with
the PL cells, which appear to contain large aggregates of glycogen (Rose et
al. 1987).

Since the ECM has a regulatory role and fibroblast-like cells control
the production and turnover of the ECM, this study examined and compared
the expression of certain key ECM proteins by cells in the M, RT, PL and
gingival cultures. Collagen type I, fibronectin and tenascin were found to be
expressed by all the cell types examined, as observed previously in
immunohistochemical studies of the normal as well as healing periodontium
(Terranova et al. 1989a; Pritlove-Carson et al. 1994; Matsuura et al. 1995;
Amar et al. 1997). However, in the present study these antigens were, for
the first time, quantitatively measured and found to be expressed at higher
levels in the GTR-associated M and RT cells than in the PL and gingival
cells, suggesting that they play an important part in the healing periodontium
following GTR surgery.

The M cells were found to express collagen and other major soft
connective tissue proteins at higher levels than the normal PL cells.
Because the M cells were associated with ePTFE membranes in vivo, these
membranes do not appear to have an inhibitory effect on the ECM
production, in contrast to a previous study of the down-regulation of collagen
by gingival fibroblasts grown directly on the membranes in vitro (Locci et al.
1997). However, Locci et al. (1997) used gingival cells rather than the
ePTFE-associated cells. Nevertheless, the present findings indicate that the
levels of collagen type I, fibronectin and tenascin are all up-regulated in
periodontal regeneration.

The expression of bone-associated proteins osteonectin, osteocalcin,
BSP and osteopontin, and their corresponding mRNA transcripts, have
previously been demonstrated in normal PL (Matsuura et al. 1995; Nohutcu
et al. 1996; Amar et al. 1997), in agreement with the present finding that
these proteins are normally expressed by cells derived from normal PL,
although at relatively low levels. This is not unexpected since the PL cell
population comprises not only PL fibroblasts but also hard connective tissue
Chapter 5

cells, including cementoblasts, osteoblasts as well as their progenitors (Berkovitz et al. 1995).

Although localisation of these bone-associated proteins in regenerating periodontium has been demonstrated immunohistochemically in previous in vivo studies (Amar et al. 1995; Matsuura et al. 1995; Ho et al. 1995; Xiao et al. 1996; Amar et al. 1997), the present study is the first to show that human RT cells, derived from newly-formed soft tissue beneath the ePTFE membrane, express relatively high levels of osteonectin, osteocalcin, BSP and osteopontin compared with the cells derived from normal PL tissue. It has been suggested that the cellular origin of the regenerating periodontium after wounding was the cells present in the adjacent healthy unwounded PL tissue (Gould et al. 1980). The paravascular cells (McCulloch 1995) and the cells migrating from the endosteal spaces (McCulloch et al. 1987) might also have contributed to the RT population. During healing, proliferative and synthetic activities of these RT cells may be induced by a number of biologically active factors, including growth factors. Therefore, the cells present in the RT may become more productive during early stages of regeneration compared to the cells present in the normal PL, and express high levels of hard connective tissue-related proteins which are likely to contribute directly to the new formation of hard tissues of the periodontium. Thus, the presence of these proteins could be a major molecular feature of the formation of new alveolar bone and new cementum following GTR, which has been demonstrated in previous histological studies both in animals and in humans (Nyman et al. 1982a; Nyman et al. 1982b; Gottlow et al. 1984; Caffesse et al. 1988; Sander and Karring 1995). This finding was also in agreement with Lekic et al. (1997), who recently showed that the elevated levels of osteopontin and BSP during periodontal regeneration was accompanied by the formation of cementum and alveolar bone in rat.

The hormone DEX has been previously shown to promote osteoblast differentiation (Chen et al. 1997), and the present experiments showed that DEX increased the levels of osteonectin, osteocalcin and osteopontin in the GTR-associated M and RT cells as well as in the PL cells. Although these
changes were not found to be statistically significant, they nevertheless indicate the presence of DEX-responsive differentiated cells which are likely to be the osteoprogenitor cells in the GTR-associated and normal PL cell populations.

Although DEX induced the overall up-regulation of osteonectin, osteocalcin and osteopontin expression by cultured M, RT and PL cells, the failure of DEX to elicit elevated levels of BSP in these cultures may be due to the short incubation period (7 days), since this protein is mainly expressed during mineralisation by fully differentiated osteoblasts (Lekic et al. 1996).

There is much evidence to suggest that, in addition to differences in fibroblast phenotype and function between anatomically different tissues, fibroblast populations within individual tissues are not homogeneous but may consist of heterogeneous sub-populations (McCulloch and Bordin, 1991; Abraham et al. 1991; Fries et al. 1994; Atherton et al. 1994; Pagliarini et al. 1995). Such observations have previously been made with cultured cells from both the gingiva (Schor and Schor 1987; Hou and Yaeger 1993) and the PL (Hassell and Stanek 1983; Somerman et al. 1988; Mariotti and Cochran 1990; Hou and Yaeger 1993; Irwin et al. 1994a; McCulloch 1995; Lekic and McCulloch 1996; Kuru et al. 1998a). In the present study, a fibronectin$^{\text{high}}$ sub-population, indicated by an apparent second peak, was identified within the M and RT cultures as well as in the PL cultures. The RT cultures also exhibited fluorescence profiles which indicated sub-populations of osteocalcin$^{\text{high}}$ and osteopontin$^{\text{high}}$ cells. Moreover, the specific changes observed in the osteocalcin and osteopontin profiles of the M and RT cells after treatment with DEX suggest that this hormone not only altered the overall expression but also selectively affected some cells in these populations, possibly osteoprogenitor cells. Thus, the present study provides evidence, for the first time, that the cells associated with periodontal regeneration also contain functionally distinct sub-populations.

The cells derived from the PL tissue did not exhibit specific changes in the osteocalcin and osteopontin profiles when treated with DEX. This may be due to the very small numbers of the osteoprogenitor cells present in the normal PL tissue compared to the RT. Therefore, the PL cells might have
lacked a distinct second peak in response to DEX and only expressed a slight increase in bone-associated proteins overall.

Although the M and RT cells are both very likely to be associated with periodontal regeneration, certain functional differences between them were nevertheless observed. The relatively elevated levels of the soft connective tissue-associated proteins in the M cells may reflect the presence of an elevated proportion of fibroblast-like cells, whereas the RT population may comprise more osteoblast-like cells since the RT cell cultures express relatively high levels of bone-associated proteins. This differential production of soft and hard connective tissue antigens suggests that the RT is more committed to cementum and bone formation, as also indicated by the relatively elevated levels of ALP activity compared with the M cells (see Chapter 4).

There were also differences in the response of both the M and RT cells to DEX. Thus, while treatment with DEX increased the overall expression of osteocalcin in the M cells, the hormone produced a distinct, new second peak of osteocalcin-expressing cells in the RT cultures. Moreover, when the osteopontin profiles of the DEX-treated M and RT cells were examined, two subsets of cells (the osteopontin^low and osteopontin^high sub-populations) were observed in both M and RT cultures, although the proportion of osteopontin^high RT cells was greater than that of osteopontin^high M cells. This observation suggests again that possible subsets in the M cells are functionally different than the subsets in the RT cells.

When the normal PL and gingival cultures were compared, the PL cells were found to have relatively higher levels of collagen I, fibronectin, tenascin and decorin than gingival cells, in agreement with previous studies (Caffesse and Quinones 1993; Hou and Yaeger 1993; Hou et al. 1995; Kuru et al. 1996; Kuru et al. 1998a). However, the PL cells also expressed higher levels of osteonectin, osteocalcin, BSP and osteopontin compared with the normal gingival cells which have only very low levels of these proteins. Carnes et al. (1997) also recently demonstrated that the cultured gingival cells had osteoblast-like characteristics, such as high ALP activity, whereas
Lekic et al. (1997) have not detected the bone-associated proteins in normal gingiva.

5.5 CONCLUSIONS

The experiments carried out in this part of the study have suggested that:

i) the cultured M, RT, PL and gingival cells are similar in size and granularity,

ii) the GTR-associated M and RT cells express higher levels of collagen I, fibronectin and tenascin than the normal PL cells;

iii) the RT cells express higher levels of bone-associated proteins than the PL as well as the M cells;

iv) the GTR-associated cells may contain subsets of cells which express fibronectin, osteocalcin and osteopontin at different levels and may also differentially respond to DEX, indicating that these cells are heterogeneous.

In summary, the present investigation has isolated, identified and characterised the GTR-associated cells which may play a crucial and essential role in the regenerative process that takes place during healing following GTR surgery.
6.1 INTRODUCTION

Early studies on periodontal wound healing have led to the development of GTR, a treatment modality based on guiding the tissue components of the periodontium during healing after surgery (Nyman et al. 1982a; Nyman et al. 1982b). Although significant clinical improvements have been reported using GTR therapy (Gottlow et al. 1993; Cortellini et al. 1993a), the molecules involved in regenerative periodontal wound healing are still not known. In the first part of this chapter, the intraosseous periodontal defects in humans were treated by GTR using ePTFE membranes and their healing over a 6 month period was assessed clinically. This was to ensure that the gingival crevicular fluid (GCF) samples, which were collected from these defect sites and subjected to further analysis described in the second part of this chapter, would represent the fluid of a regenerating periodontal wound. Clinical measurements were also recorded for the healthy periodontal sites which were adjacent to the periodontal defect and included in the GTR surgery, because the GCF samples obtained from these sites would represent the wound fluid of a normal healthy site undergoing healing. In addition, the intraosseous periodontal defects treated by conventional flap (CF) surgery were also evaluated clinically, since the GCF samples collected from these sites would represent the wound fluid of a site which healed mainly by repair rather than regeneration.

The aim of the first part of this chapter was therefore to report the clinical findings of the periodontal sites which provided GCF samples that were investigated in the second part of this chapter.

6.2 MATERIALS AND METHODS

6.2.1 Periodontal sites and clinical measurements

Periodontal sites were divided into 3 groups with respect to their periodontal status and the treatment to be received: i) the GTR test sites
which were periodontal defect areas treated by GTR using ePTFE membrane, ii) the GTR control sites which were healthy sites in the same surgical field but one complete unit away from the defect, and iii) the CF sites which were periodontal defect areas treated by CF surgery. Initially 24 GTR and 12 CF subjects participated in this study, although only the data of 17 GTR and 11 CF subjects who completed this study are presented in this chapter.

Clinical measurements, including probing pocket depth (PPD), clinical attachment level (CAL) and marginal recession of gingiva (REC), were recorded for all the sites at the baseline (before surgery) and 6 months post-operatively, as described in 2.1.3. Intra-surgical clinical measurements, including the distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (CEJ-BC), the distance from the CEJ to the base of the defect (CEJ-BD) and defect depth (DD), were recorded for only GTR test sites before membrane placement. In addition, the distance from the CEJ to the newly-regenerated tissue (CEJ-RT) was also measured for these sites after membrane removal.

6.2.2 Statistical analysis

The clinical data were found to be not normally distributed, and therefore, non-parametric statistical tests were required to be used. For the analysis of the GTR test and CF sites, 1 site per patient with the deepest baseline PPD was chosen as the statistical unit. However, there were no differences in the statistical outcome when the mean value of 2-4 sites per patient was used as the statistical unit. Therefore, only the data of sites with the deepest PPD were presented here. For the analysis of GTR control sites, 1 value per patient (the mean value of 2-4 sites) was used as the statistical unit.

For each group, the differences between the baseline and post-surgery clinical measurements were analysed by the Wilcoxon Signed Ranks test. The differences in clinical measurements between the GTR test and CF sites were analysed by the Mann-Whitney U test.
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6.3 RESULTS

6.3.1 Clinical characteristics of GTR test sites

Six males and 11 females (mean age of 39.6 ± 10 years; range 24 to 59) participated in this part of the study. Intraosseous defects located at 5 incisors, 4 premolars and 8 molars were treated by placement of ePTFE membrane. Five procedures were performed by lecturers, 2 by consultants, 2 by registrars and 8 by postgraduate students.

Table 6.1 summarises the clinical measurements of the GTR test sites. The median PPD was found to be reduced from a baseline value of 8.0 mm to 3.0 mm at 6 months post-surgery. The median CAL was also found to be reduced from 9.0 mm to 5.0 mm post-surgery. When the pre- and post-surgery measurements were compared using the Wilcoxon paired test, a median PPD reduction of 4.0 mm and a median CAL gain of 4.0 mm were found to be statistically significant (p < 0.001). Although the median REC (1.0 mm) was the same for both baseline and 6 months examinations, a statistically significant difference was found between these (p < 0.05). This was due to the differences in the distribution of REC data between the baseline and post-surgery (see the 25/75 percentile values in Table 6.1).

Table 6.1. Clinical measurements (mm) of the GTR test sites at the baseline (pre-surgery) and 6 months post-surgery, and the changes (baseline-6 months).

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Baseline</th>
<th>6 months</th>
<th>Change</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPD</td>
<td>8.0</td>
<td>3.0</td>
<td>4.0</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>(6.0/9.0)</td>
<td>(2.0/5.0)</td>
<td>(4.0/6.0)</td>
<td></td>
</tr>
<tr>
<td>CAL</td>
<td>9.0</td>
<td>5.0</td>
<td>4.0</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>(8.0/10.0)</td>
<td>(4.0/7.0)</td>
<td>(3.0/5.0)</td>
<td></td>
</tr>
<tr>
<td>REC</td>
<td>1.0</td>
<td>1.0</td>
<td>0.0</td>
<td>0.046</td>
</tr>
<tr>
<td></td>
<td>(0.0/2.0)</td>
<td>(1.0/3.0)</td>
<td>(-2.0/0.0)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as the median values and the numbers in brackets are the 25/75 percentile.

PPD = probing pocket depth
CAL = clinical attachment level
REC = recession of the gingival margin

* Data are analysed using the Wilcoxon Signed Ranks test and p ≤ 0.05 is considered to be statistically significant.
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The intra-surgical clinical measurements recorded during the first and second surgical steps of GTR are presented in Table 6.2. At the first step surgery, prior to membrane placement, the median for the total bone defect (CEJ-BD) was 11.0 mm. This was made up of a median crestal loss (CEJ-BC) of 4.5 mm and a median intraosseous defect (DD) of 4.5 mm. At the second step surgery, immediately after membrane removal, all the periodontal defects were observed to be occupied with a soft newly-regenerated tissue (RT) which had a dark red colour and jelly-like texture characteristic of granulation tissue. The distance from the CEJ to the newly-formed RT (CEJ-RT) was observed to be 3.0 mm. The amount of RT was calculated as (CEJ-BD) - (CEJ-RT) and defined as the RT formation the median value of which was 7.0 mm. Furthermore, the distance between the bone crest and the regenerated tissue (BC-RT) was calculated as (CEJ-BC) - (CEJ-RT) and found to be 1.0 mm. Thus, this new tissue regenerated a total of 7.00 mm which was 1 mm above the level of the alveolar bone crest.

Table 6.2. Intra-surgical clinical measurements (mm) of periodontal defects treated by GTR.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Median</th>
<th>(25/75 percentile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEJ-BC</td>
<td>4.5</td>
<td>(3.0/7.5)</td>
</tr>
<tr>
<td>CEJ-BD</td>
<td>11.0</td>
<td>(8.5/12.0)</td>
</tr>
<tr>
<td>DD</td>
<td>4.5</td>
<td>(0.5/7.0)</td>
</tr>
<tr>
<td>CEJ-RT</td>
<td>3.0</td>
<td>(2.0/5.0)</td>
</tr>
<tr>
<td>RT formation</td>
<td>7.0</td>
<td>(5.0/8.5)</td>
</tr>
<tr>
<td>BC-RT</td>
<td>1.0</td>
<td>(0.0/6.0)</td>
</tr>
</tbody>
</table>

CEJ-BC = distance from the CEJ to the bone crest
CEJ-BD = distance from the CEJ to the base of the defect
DD = defect depth
CEJ-RT = distance from the CEJ to the regenerated tissue
RT formation = (CEJ-BD) - (CEJ-RT)
BC-RT = (CEJ-BC) - (CEJ-RT)
6.3.2 Clinical characteristics of GTR control sites

Table 6.3 demonstrates the clinical measurements of GTR control sites (mean of 2-4 sites per subject) at baseline and 6 months post-operatively. The PPD measurement exhibited a median reduction of 0.5 mm, from a baseline median PPD of 2.3 mm to a median of 1.6 mm post-surgery, and this difference was statistically significant ($p < 0.01$). However, the CAL remained almost the same (medians of 2.3 and 2.5 mm pre- and post-operatively, respectively) and the difference was not significant ($p > 0.05$). The median REC measurements were observed to be 0 and 0.4 mm at baseline and 6 month post-surgery, respectively, and a median change of 0.3 mm was found to be statistically significant ($p < 0.01$). Thus, this surgical procedure resulted in significant reduction in PPD and recession of marginal gingiva of the sites which were periodontally healthy.

Table 6.3. Change in clinical measurements (mm) of GTR control sites, 6 months post-surgery.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Baseline</th>
<th>6 months</th>
<th>Change</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPD</td>
<td>2.3 (1.8/2.8)</td>
<td>1.6 (1.3/2.0)</td>
<td>0.5 (0.3/0.9)</td>
<td>0.003</td>
</tr>
<tr>
<td>CAL</td>
<td>2.3 (2.0/2.9)</td>
<td>2.5 (2.0/2.7)</td>
<td>0.0 (-0.2/0.3)</td>
<td>0.776</td>
</tr>
<tr>
<td>REC</td>
<td>0.0 (-0.5/0.4)</td>
<td>0.4 (0.0/1.4)</td>
<td>-0.3 (-0.7/0.5)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Data are presented as the median values and the numbers in brackets are the 25/75 percentile.

PPD = probing pocket depth
CAL = clinical attachment level
REC = recession of the gingival margin

* Data are analysed using the Wilcoxon Signed Ranks test and $p \leq 0.05$ is considered to be statistically significant.

6.3.3 Clinical characteristics of CF sites

Periodontal defects in 4 males and 7 females (mean age of $36.5 \pm 8$ years; range 23 to 48) were treated by CF surgery. Changes in clinical measurements of these defects are shown in Table 6.4. The median PPD
was found to be reduced from baseline 6.5 mm to 4 mm at 6 months post-operatively. The median CAL also reduced from a baseline value of 7.5 mm to 6 mm post-operatively. The changes in pre- and post-operative measurements of PPD (3.5 mm) and CAL (3.0 mm) were found to be statistically significant ($p \leq 0.01$). The baseline median REC of 1 mm increased to 1.5 mm post-operatively, thus a median recession of 1.0 mm in marginal gingiva was statistically significant ($p < 0.05$).

### Table 6.4. Change in clinical measurements (mm) of periodontal defects treated by conventional flap surgery over 6 months.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Baseline</th>
<th>6 months</th>
<th>Change</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPD</td>
<td>6.5</td>
<td>4.0</td>
<td>3.5</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>(6.0/9.0)</td>
<td>(2.0/5.0)</td>
<td>(2.8/4.0)</td>
<td></td>
</tr>
<tr>
<td>CAL</td>
<td>7.5</td>
<td>6.0</td>
<td>3.0</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>(6.8/9.0)</td>
<td>(4.0/6.0)</td>
<td>(1.5/3.2)</td>
<td></td>
</tr>
<tr>
<td>REC</td>
<td>1.0</td>
<td>1.5</td>
<td>-1.0</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>(0.0/1.0)</td>
<td>(0.8/2.0)</td>
<td>(-1.3/0.0)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as the median values and the numbers in brackets are the 25/75 percentile of the distribution.

PPD = probing pocket depth
CAL = clinical attachment level
REC = recession of the gingival margin

* Data are analysed using the Wilcoxon Signed Ranks test and $p \leq 0.05$ is considered to be statistically significant.

### 6.3.4 Comparisons of clinical measurements of defects treated by GTR and CF surgery

The baseline clinical measurements of the GTR test sites were found to be not significantly different from those of the CF sites, using the Mann-Whitney U test ($p > 0.05$). Similarly, there was no statistically significant differences when the measurements at 6 months were compared between these two groups ($p > 0.05$). However, when the changes in these measurements were compared, the reduction in PPD of the GTR sites was greater than the CF sites and this difference was statistically significant ($p < 0.05$). Although gain in CAL was also greater in the GTR sites, the
difference was not significant \((p = 0.08)\). Similarly, change in REC in the GTR sites was not significantly different from that in the CF sites \((p > 0.05)\). Thus, although the baseline characteristics of periodontal defects were similar, the PPD reduction in defects treated with GTR surgery was statistically significantly different from that in defects treated with CF surgery.

### 6.4 DISCUSSION

The clinical outcome of GTR therapy was evaluated in this part of the study. The treatment using GTR resulted in a significant PPD reduction of 4 mm at 6 months. Similar findings have been reported (Sander et al. 1994), although smaller (Kersten et al. 1992; Proestakis et al. 1992; Brägger et al. 1992; Teparat et al. 1998) or larger reductions in PPD also have been shown (Cortellini et al. 1993a; Becker and Becker 1993; Cortellini et al. 1995; Cortellini et al. 1996a; Kiliç et al. 1997). A significant gain in CAL of 4 mm was achieved in the present study. Similar gains in CAL were reported also by Cortellini et al. (1993a; 1995), Becker and Becker (1993) and Kiliç et al. (1997) for human intrabony defects treated by non-resorbable membranes. However, CAL gain in the present study was higher than the results of several studies where vertical bone defects were treated by GTR (Brägger et al. 1992; Kersten et al. 1992; Proestakis et al. 1992; Sander et al. 1994; Nowzari et al. 1995; Teparat et al. 1998). Gottlow et al. (1992) defined the sites which had gained 2 mm or more CAL after 6 months of GTR surgery as ‘successfully treated’. Based on this definition, the clinical outcome of nearly all periodontal defects (94%) treated by GTR in the present study was considered as successful.

Since the early case report of Gottlow et al. (1986) clinical trials using GTR have shown considerable variation in outcome. The amount of residual periodontal support, defect depth and configuration, membrane exposure and contamination, coverage of the newly-formed tissue by the gingival flap, compliance with a supportive periodontal maintenance program and smoking habit have all been suggested as factors which may effect the results of GTR therapy (Selvig et al. 1992; Selvig et al. 1993;
One or more of these factors may thus have been the cause of differences between the present study and previously published studies.

The surgical procedures in previous GTR trials have been performed by experienced investigators, which has an impact on the clinical outcome of GTR therapy (Cortellini et al. 1993a; Cortellini et al. 1996a). However, the present study was not planned as a well controlled clinical trial and therefore, it represents more realistic conditions in a routine clinic where the operations are performed by a number of dental surgeons who possess different levels of experience, knowledge and practical skill. Nevertheless, the results of this study indicated that GTR therapy resulted in significant clinical improvements, including reduction in PPD and gain in CAL.

The periodontal defects treated with CF surgery in the present study resulted in a PPD reduction of 3.5 mm and a gain in CAL of 3 mm. These results were consistent with those obtained by Cortellini et al. (1995; 1996a) and indicated that this treatment also results in significant clinical improvements, as reported by Lindhe et al. (1982). CF surgery displayed significantly less PPD reduction than the GTR therapy, in agreement with the study of Cortellini et al. (1995) and Kiliç et al. (1997). However, despite larger gains in CAL for the GTR compared to the CF group, there was no significant difference between these two treatment modalities in CAL gain, in contrast to the studies of Cortellini et al. (1995; 1996a).

Recession of marginal gingiva after GTR surgery is an expected side effect (Gottlow et al. 1986; Cortellini et al. 1993a). However, in the present study there was very limited recession of the marginal gingiva not only in the GTR test sites, but also in the adjacent GTR control sites and the defects treated by conventional surgery. Therefore, it can be assumed that the REC measured in the GTR group was a normal outcome of the periodontal surgical procedure and may not be directly related to the membrane placement.
The finding that the RT exceeded the crestal alveolar bone level indicated that this therapy promoted total defect fill by soft regenerated tissue, which was red and jelly-like in appearance but firm and resistant to probing forces. The amount of the RT observed in the present study was within the range of previously published reports which showed a mean RT ranging from 4 to 8 mm (Tonetti et al. 1993; Tonetti et al. 1996).

In addition to the periodontal defect sites treated using ePTFE membranes, the clinical measurements of healthy sites adjacent to these defect sites were also monitored in this study. While a 0.5 mm reduction in PPD was observed, GTR surgery did not result in any change in CAL. This finding is consistent with the study by Stein et al. (1993) who reported a mean CAL loss of 1 mm which was not significantly different from the healthy sites that were covered by a non-resorbable membrane.

### 6.5 CONCLUSIONS

Based on the findings of this part, it was concluded that

i) the periodontal defects treated with GTR show significant clinical improvements, including significant reduction in PPD and clinical attachment gain, at 6 months after surgery and therefore they may represent the periodontal sites which heal by regeneration.

ii) The finding that the periodontal defects treated with CF also exhibit clinical improvements suggest that these sites may heal by both repair and regeneration processes.

iii) The GTR control sites also show some changes in clinical measurement after GTR surgery.

In summary, the periodontal defects treated by GTR healed by regeneration, and therefore the GCF samples collected from these sites represent the fluid of a regenerating periodontal wound and are consequently used to investigate certain molecules described in the next part below.
PART II: GROWTH FACTORS AND THEIR RECEPTORS IN PERIODONTAL REGENERATION

6.6 INTRODUCTION

Growth factors are biologically active polypeptide molecules and thought to play important roles in wound healing by regulating the activities of fibroblasts, epithelial cells and endothelial cells (Deuel et al. 1991; Oates et al. 1993; Graves and Cochran 1994; The American Academy of Periodontology 1996; Howell et al. 1996). Although certain growth factors have been applied onto surgical periodontal wounds in animals and in a few human studies in order to promote regeneration (Lynch et al. 1991; Terranova 1993; Wang et al. 1994b; Howell et al. 1997), their presence during periodontal wound healing, thus far, have not been investigated. GCF is an extracellular fluid that is considered to reflect the ongoing processes in surrounding periodontal tissues. Therefore, the GCF samples from healing periodontal sites were collected in order to detect the growth factor that may be involved in periodontal regeneration, but which growth factor? In order to answer this question, the expression of certain growth factor receptors were studied first in the newly-regenerated tissue, because the growth factors have been shown to co-localise with the corresponding receptors during wound healing (Green et al. 1997; Gold et al. 1997). The aims of this part of the study were therefore:

i) to investigate the expression of growth factor receptors in regenerating periodontal tissue (RT), in comparison with normal PL and gingival tissues;

ii) to determine the level of growth factors, whose receptors were up-regulated in the RT, in GCF samples collected from sites undergoing GTR and CF surgery.
6.7 MATERIALS AND METHODS

6.7.1 Immunohistochemical staining

An indirect immunohistochemical staining technique described in 2.2.1.4 was used to investigate the expression of the following growth factor receptors: platelet-derived growth factor (PDGF) receptor α (PDGFR-α) (Biogenesis), PDGF receptor β (PDGFR-β) (Biogenesis), TGF-β receptor (TGF-βR) (Chemicon), epidermal growth factor receptor (EGFR) (Chemicon), fibroblast growth factor receptor (FGFR) (Chemicon) and insulin-like growth factor-I receptor (IGF-IR) (Biogenesis). The tissues used for these investigations comprised 6 samples of RT, 4 of PL and 4 of gingiva which were previously collected (see section 2.1.4.1).

6.7.2 Collection and analysis of GCF and blood samples

GCF samples were collected from sites (2-6 sites per patient) related to the surgery using absorbent paper strips, as described in 2.1.4.3. The samples were obtained just before surgery (week 0) and during a period of 6 months after surgery (see Figure 2.4). In addition, 8 subjects who underwent GTR surgery also provided samples of venous blood. The volume of GCF collected was measured by weighing, as described in 2.1.4.3. Because strips with any visible sign of saliva or blood contamination were discarded, the number of paper strips differed from one subject to another and also from one time point to another for each individual subject. Thus, where samples from several sites of similar status were pooled, the average GCF volume per site was calculated using the following equation:

average GCF volume per site = total GCF volume / number of strips

GCF and blood samples were prepared for the subsequent analyses as described in sections 2.2.9.1 and 2.1.4.4, respectively.

In a preliminary experiment, the levels of PDGF-AB were investigated in the GCF samples of 4 GTR subjects using a sandwich ELISA assay, as described in 2.2.9.2. Since this assay was unable to detect this growth factor in the majority of samples, the remaining GCF samples were not
tested. The levels of TGF-β1, however, were investigated in GCF samples of 17 GTR and 11 CF subjects using a sandwich ELISA assay, as described in 2.2.9.3. TGF-β1 was also examined in a total of 16 blood samples taken on two different occasions from 8 of the GTR subjects.

6.7.3 Statistical analysis

The data of GCF were found to be not normally distributed, and therefore non-parametric statistical methods were used. The Wilcoxon Signed Ranks test was used to test the differences in GCF volumes collected at weeks 2, 4, 6, 7, 12 and 26 with the GCF volume collected at week 0, separately for GTR test, GTR control and CF sites. Differences in GCF volumes between these three sites were tested using the Mann-Whitney U test. The same statistical analyses were performed for the TGF-β1 data.

6.8 RESULTS

6.8.1 Expression of growth factor receptors

The expression of growth factor receptors in the RT was examined and compared with those in the normal PL and gingival tissues in order to determine whether any of the growth factor receptors are differentially expressed and therefore its corresponding factor may be involved in periodontal wound healing. A summary of the results derived from these experiments is presented in Table 6.5. In the present study, PDGFR-α was not detected in any of the tissues examined (not shown). PDGFR-β was observed to be expressed in isolated areas throughout the connective tissue of RT (Figure 6.1a) stronger than in PL tissue which showed a moderate expression (Figure 6.1b). Biopsy samples of gingiva also showed a moderate positive expression in connective tissue, whereas this antigen was absent in gingival epithelium (Figure 6.1c).

Histological observation of the RT sections stained for the receptor of TGF-β revealed that this antigen was also heterogeneously distributed (Figure 6.2a). The intensity of this receptor appeared to be elevated in the
RT compared with PL (not shown). It was also relatively lower in gingival connective tissue, whereas gingival epithelium was negative (Figure 6.2b).

Expression of the receptor for EGF by the RT was found to be restricted with some isolated patchy areas only in the epithelium (not shown). Nevertheless, the intensity of the staining in the RT seemed to be stronger than in the gingival epithelium, whereas PL and gingival connective tissues did not express this receptor (not shown).

Staining for the receptors of FGF and IGF-I was very weak and variable in the RT sections, whereas it was negative in both the gingival and PL sections (not shown). Control sections treated with non-specific IgG as the primary antibody were all negative (not shown).

The results of this immunohistochemical study suggest that expression of PDGFR-β and TGF-βR are up-regulated in the RT samples. Therefore, it is also likely that their corresponding growth factors may be present and involved in periodontal regeneration. Based on this hypothesis, the presence of the growth factors, PDGF-AB and TGF-β, were examined in GCF samples collected from GTR test, GTR control and CF sites, as described below (sections 6.8.3 and 6.8.4).

Table 6.5. Comparison of the expression of growth factor receptors in the regenerated tissue (RT), periodontal ligament (PL) and gingival tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>PDGFR-α</th>
<th>PDGFR-β</th>
<th>TGF-βR</th>
<th>EGFR</th>
<th>FGFR</th>
<th>IGF-IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-/+</td>
<td>-/+</td>
</tr>
<tr>
<td>PL</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gingival EP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gingival CT</td>
<td>-</td>
<td>+</td>
<td>-/+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

EP = epithelium
CT = connective tissue
++ strong positive
+ moderate positive
-/+/ weak and variable
- negative
Figure 6.1. Expression of PDGFR-β by RT (a), PL (b) and gingival tissues (c). Note high level of expression in isolated areas (arrows) in (a) and less intense but rather homogeneous expression in (b). While gingival connective tissue (CT) in (c) expressed this antigen less intense than the RT [see (a)], gingival epithelium (EP) was negative, as observed by the absence of staining (i.e., absence of brown colour) (original magnifications x 200).
Figure 6.2. TGF-βR expression in RT (a) and gingival tissues (b). Note heterogeneous distribution of this receptor in (a). While gingival connective tissue (CT) had a very weak positive expression, gingival epithelium (EP) was negative (original magnifications x 200).
6.8.2 GCF volume

6.8.2.1 GCF volumes before and after surgery

As described in section 6.7.2, the GCF samples were collected from GTR test, GTR control and CF sites and their volumes were determined in order to calculate the concentration of any molecule to be detected. Table 6.6 shows the median and percentiles of the average volumes of GCF samples collected from subjects before surgery, which was considered as the baseline (week 0), and for a period of 26 weeks after periodontal surgery. For the GTR test sites, the median GCF volume increased from a baseline value of 0.3 µl to 1.5 µl at 2 weeks post-surgery and this difference was highly statistically significant (p < 0.001). The volume of GCF remained relatively high throughout the period when the membrane was retained in situ, i.e. until week 6. One week following the membrane removal (week 7 post-operatively), the median GCF volume (1.0 µl) was still high and significantly different from the baseline level (p ≤ 0.01). However, the median GCF volume declined to below baseline levels at 12 and 26 weeks post-operatively and these differences were not statistically significant (p > 0.05).

For GTR control sites, the volume of GCF collected showed a statistically significant increase (p ≤ 0.01) from 0.1 µl before surgery to 0.6 µl at 2 weeks post-surgery. After this time point, the median GCF volumes remained similar with some minor fluctuation from week 4 to week 12. By week 26, the volume of GCF (0.06 µl) was significantly lower than the baseline level (p ≤ 0.01).

For CF sites, the median baseline GCF volume (0.3 µl) increased substantially following surgery (1.1 µl) and the difference between these values was found to be statistically significant (p ≤ 0.01). GCF volume showed a decrease until 26 weeks post-surgery and volumes over this period were not significantly different from the baseline (p > 0.05).
Table 6.6. Volume of GCF collected from GTR test, GTR control and conventional flap (CF) sites.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Volume of GCF (μl) collected at weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>GTR-test</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>(0.1/0.6)</td>
</tr>
<tr>
<td>GTR-control</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>(0.1/0.2)</td>
</tr>
<tr>
<td>CF</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>(0.2/0.4)</td>
</tr>
</tbody>
</table>

Data are presented as the median and the numbers in brackets are the 25/75 percentile.

* p ≤ 0.05 ** p ≤ 0.01, ***p ≤ 0.001 compared to volume at week 0 for the same site, using the Wilcoxon Signed Ranks Test.

6.8.2.2 Comparison of GCF volumes between groups

The data regarding the GCF volumes of GTR test, GTR control and CF surgery groups are shown diagrammatically in Figure 6.3. Before periodontal surgery, very similar amounts of GCF samples were collected from GTR test and CF sites and there was no significant difference between them (p > 0.05). In contrast, the GCF volume collected from the GTR control sites was less than half of the GTR test and CF sites, and statistically significantly different from the GCF volume of the CF sites (p ≤ 0.05) but not the GTR test site (p = 0.056). All sites showed a marked increase in GCF volume after surgery. Throughout the retention period of the membrane, GCF volume of the GTR test sites was higher and significantly different from that of the GTR control sites. Moreover, the GCF volume was 3-fold higher and significantly different from that of CF sites at 6 weeks post-surgery (p < 0.05).

Although GCF volume decreased in both GTR test and control sites after membrane removal, GTR test sites still showed elevated levels of GCF volume (nearly 5-fold) compared to the GTR control sites at 7 weeks post-
surgery, and this difference was highly significant ($p < 0.001$). By weeks 12 and 26, GCF volumes of GTR test sites were still higher and significantly different from the GTR control sites ($p < 0.01$ and $p < 0.05$, respectively) but not from the CF sites ($p > 0.05$). However, it was notable that at 26 weeks post-surgery the GCF volumes decreased to nearly half the baseline volume in all three groups.

![Graph showing GCF volume over time](image)

**Figure 6.3.** Volume of GCF collected from GTR test (GTR-t), GTR control (GTR-c) and conventional flap (CF) sites before and over 26 weeks after surgery.

- * $p < 0.05$
- ** $p < 0.01$
- *** $p < 0.001$ compared to GTR control at the same time point
- * $p < 0.05$ compared to CF at the same time point

### 6.8.3 Detection of PDGF-AB in GCF samples

The presence of the growth factor PDGF-AB in a total of 54 GCF samples collected from both test and control sites of 4 GTR patients were examined using an ELISA assay. A standard curve, as shown in Figure 6.4, was prepared for the experiment. The concentrations of PDGF-AB in each of the GCF samples were then calculated from this curve. In addition, the total amount of PDGF-AB was calculated by reference to the original volume of GCF collected using the equation below:

\[
\text{total amount} = \text{concentration} \times \text{volume of GCF}
\]
PDGF-AB was detected in 37% of GTR test samples, while only 26% of GTR control samples had detectable levels of this growth factor. Due to the missing values, no statistical analyses were performed. While PDGF-AB was detected at almost every stage in one subject, it was not detected at all in another subject and was sporadic in the remaining 2 subjects with more positive samples at 6 weeks and beyond. In contrast, the control sites showed more positive detection across subjects (3 out of 4 at baseline) but only one positive sample beyond 6 weeks. The concentrations of PDGF-AB ranged from 2.3 to 150.4 ng/ml in GTR test samples and from 8.9 to 315.5 ng/ml in GTR control samples. As shown in Figure 6.5, the amounts of PDGF-AB demonstrated a wider range in GCF samples collected from test sites (5 to 128 pg) compared with those of control sites (ranging from 2 to 79 pg). In addition, 70% of test sites samples had this growth factor with the amount >30 pg, whereas only 28% of samples obtained from control sites had this amount of PDGF-AB.
Figure 6.5. A scatter diagram of amounts of PDGF-AB (pg) in GCF samples collected from GTR test (solid) and GTR control sites (open) of 4 subjects.

6.8.4 TGF-β1 in samples of GCF and serum

A sandwich ELISA assay was used to measure the growth factor TGF-β1 in a total of 258 GCF samples collected from test and control sites of 17 GTR subjects and 11 CF subjects. Serum samples (n=16) collected from 8 of the GTR subjects were also examined. A standard curve, as shown in Figure 6.6, was prepared for each individual experiment. Concentrations of TGF-β1 in samples were then calculated from this curve. The total amount of TGF-β1 in each sample was also calculated by reference to the original volume of GCF using the equation as described previously for PDGF-AB (section 6.8.3).
As shown in Table 6.7, most of the GCF samples were found to have levels of TGF-β1 which could be detected by the assay method used. The TGF-β1 was also detected in all serum samples tested. Over the 6 month period, 95% of GCF samples of GTR test sites and 88% of samples of GTR control sites were positive for TGF-β1 while only 75% of GCF samples of CF sites had detectable levels of this growth factor.

Both the GTR test and control sites had the same proportion of TGF-β1-positive samples at the baseline, whereas the proportion was lower in CF samples. It was notable that all 3 sites regardless of the type of surgery had the highest proportion of TGF-β1-positive samples (100%) at week 2, which was the first visit after periodontal surgery. At 6 weeks post-surgery and beyond a larger proportion of samples of test sites had detectable TGF-β1 compared to the control sites of the same patients. The lowest proportions of positive GCF samples, however, were observed in CF sites.
Table 6.7. Proportions of GCF samples with detectable levels of TGF-β1.

<table>
<thead>
<tr>
<th>Sites</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>7</th>
<th>12</th>
<th>26</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTR test</td>
<td>94</td>
<td>100</td>
<td>91</td>
<td>94</td>
<td>93</td>
<td>92</td>
<td>100</td>
</tr>
<tr>
<td>GTR control</td>
<td>94</td>
<td>100</td>
<td>91</td>
<td>77</td>
<td>87</td>
<td>85</td>
<td>88</td>
</tr>
<tr>
<td>CF</td>
<td>73</td>
<td>100</td>
<td>-</td>
<td>89</td>
<td>-</td>
<td>56</td>
<td>60</td>
</tr>
</tbody>
</table>

CF = conventional flap

6.8.4.1 Concentration of TGF-β1 in GCF samples

Table 6.8 shows the median and percentile values for TGF-β1 concentration in GCF samples obtained from GTR test, GTR control and CF sites for a period of 26 weeks. The median baseline concentration of this growth factor was detected as 20.7 ng/ml in the GTR test samples. The concentrations were found to decrease by weeks 2 and 4, but the differences from the baseline level were not significant (p > 0.05). However, by weeks 6 and 7 the TGF-β1 concentrations (8.6 and 9.9 ng/ml, respectively) were significantly lower than the baseline level (p < 0.05). The concentration of TGF-β1 increased at weeks 12 and 26, but was not significantly different from the baseline concentration (p > 0.05).

The median baseline concentration of TGF-β1 in GTR control samples was 104.1 ng/ml. The level declined drastically at week 2 (24.2 ng/ml) and then some fluctuations were observed until week 26 when the level rose again (62.0 ng/ml). None of these concentrations were found to be significantly different from the baseline concentration (p > 0.05).

The median concentration of TGF-β1 in baseline samples from CF sites was detected to be 26.7 ng/ml. The concentration decreased at 2 and 6 weeks post-surgery followed by an increase at weeks 12 and 26. However, statistical analysis showed that the differences in TGF-β1 concentration between the baseline and any other time points were not significant (p > 0.05). Thus, these results show that the TGF-β1 concentrations decreased just after surgery followed by an increase in all
three groups, although only the concentrations in GTR test samples at weeks 6 and 7 were significantly lower than their respective baseline value.

Table 6.8. Concentration of TGF-β1 in GCF samples of GTR test, GTR control and conventional flap (CF) sites.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Concentration of TGF-β1 (ng/ml) at weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>GTR-test</td>
<td>20.7</td>
</tr>
<tr>
<td>GTR-control</td>
<td>104.1</td>
</tr>
<tr>
<td>CF</td>
<td>26.7</td>
</tr>
<tr>
<td></td>
<td>(10/56)</td>
</tr>
</tbody>
</table>

Data are presented as the median and the numbers in brackets are the 25/75 percentile.

*p < 0.05 compared to the corresponding baseline value, using the Wilcoxon Signed Ranks test.

The Mann-Whitney U test was used to test the differences in TGF-β1 concentrations between the GTR test, GTR control and CF samples, at each time point. While the baseline concentrations in samples of both the GTR test and CF groups were similar, the GTR control samples had a higher baseline level. However, there were no statistically significant differences between them (p > 0.05). The TGF-β1 concentrations in the GTR test samples were found to be significantly different from those in the GTR control samples at weeks 4 and 6 (p < 0.01) and from CF samples at week 26 (p < 0.05).

6.8.4.2 Amount of TGF-β1 in GCF samples

The total amount of TGF-β1 in GCF samples was calculated as described in section 6.8.4, and the median and the percentile values for GTR test, GTR control and CF samples are shown in Table 6.9. While the
median baseline amount of GTR test sites was 34.4 pg, nearly a 2-fold increase was observed at week 2, and this difference was statistically significant (p < 0.05). The median amount of TGF-β1 showed a gradual decrease from week 4 throughout the study period until week 26 which was 15.2 pg and significantly lower than the baseline level (p > 0.05).

Unlike the GTR test sites, the median baseline amount (54.4 pg) of GTR control sites decreased to 32.9 pg by week 2, but this difference was not statistically significant (p > 0.05). The TGF-β1 amount remained lower than the baseline throughout the study period and the levels at weeks 7 and 12 were statistically significantly lower than the baseline (p < 0.05).

The TGF-β1 amount in the CF samples showed an increase from a baseline value of 34.4 pg to 48.9 pg at the first visit after surgery and this difference was not statistically significant. The amount decreased throughout the study period, only the amount at week 26 was significantly lower than the baseline (p < 0.05). These results demonstrated that during early stages of healing only the GTR test samples had significantly higher amounts of TGF-β1 compared to the baseline amount.

### Table 6.9. Amount of TGF-β1 in GCF samples of GTR test, GTR control and conventional flap (CF) sites.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Absolute amount of TGF-β1 (pg) at weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>GTR-test</td>
<td>34.4</td>
</tr>
<tr>
<td></td>
<td>(19/65)</td>
</tr>
<tr>
<td>GTR-control</td>
<td>54.4</td>
</tr>
<tr>
<td>CF</td>
<td>34.4</td>
</tr>
<tr>
<td></td>
<td>(17/49)</td>
</tr>
</tbody>
</table>

Data are presented as the median and the numbers in brackets are the 25/75 percentile.
*p < 0.05 compared to the corresponding baseline, using the Wilcoxon Signed Ranks test.
When the TGF-β1 amount was compared between the sites, the baseline amounts in the GTR test and CF samples were found to be the same, while the GTR control group had a higher baseline level. However, 2 weeks after surgery, the amount in the GTR test samples was observed to be 1.8- and 1.2-fold higher than those in the GTR control and CF samples, respectively. Furthermore, the amount of TGF-β1 in the GTR test samples remained 1.3-, 1.5- and 1.8-fold higher than the amount in the GTR control samples at weeks 4, 6 and 7, respectively. In addition, the GTR test samples also had 1.9-fold higher TGF-β1 amount than the CF group at week 6. At weeks 12 and 26 the amount of TGF-β1 was similar for all 3 groups. Although statistical analysis showed that there were no significant differences in the amount of TGF-β1 between any of the groups at any time point (p > 0.05), it was notable that the amounts in the GTR test sites were higher than the other sites between weeks 2 and 7.

**6.8.4.3 TGF-β1 in serum samples**

The concentrations of TGF-β1 in serum samples were also investigated in order to determine the systemic level of this growth factor. The median baseline TGF-β1 concentration of 8 blood samples was 37.1 ng/ml. The samples obtained from the same patients at week 6 had a very similar concentration (37.3 ng/ml) and this was not statistically significantly different from the baseline concentration (p > 0.05).

**6.9 DISCUSSION**

In this part of the study, for the first time, expression of growth factor receptors in the RT tissue obtained from regenerating periodontal defects and their corresponding factors in GCF samples collected from these defect sites were investigated.

Samples of GCF were collected using paper strips from the sites, where clinical measurements were recorded, before and at intervals over a period of 26 weeks after different modalities of periodontal surgery. The baseline GCF volumes obtained from periodontally healthy sites (GTR
Chapter 6

control sites) were far less than those of the sites with periodontitis (GTR test sites and CF sites). This finding is consistent with previous studies which have found a correlation between the amount of GCF obtained and the periodontal status of the site where GCF had been collected (Löe and Holm-Pedersen 1965; Giannobile et al. 1995; Griffiths et al. 1998). The finding that, the GCF volume of samples collected from periodontal defect sites in GTR and CF groups were the same, may suggest that these defects were very similar.

The majority of GCF studies are cross-sectional. The samples of GCF have been collected from sites with different clinical status (which were either clinically healthy or exhibited signs of gingivitis or periodontitis) and then tested for a ‘biochemical marker’. However, little is known about the effect of periodontal treatment on the volume of GCF. Although a few studies have reported that GCF volume collected from sites with periodontitis decreased after the initial non-surgical periodontal therapy compared to the pre-treatment level (Eley et al. 1995), thus far, no longitudinal study exist to investigate the influence of periodontal surgery, particularly GTR surgery. The present study, therefore, represents for the first time data on this subject.

The results of this study showed that the volume of GCF obtained from sites treated by periodontal surgery increased significantly immediately after operation. This marked change may reflect the inflammatory stage of periodontal wound healing, as it has been shown that this process is accompanied by enhanced permeability of gingival blood vessels, which results in increased amounts of fluid passing through the vascular walls into the extravascular space (Egelberg 1966). This finding is also in agreement with previous studies which have shown that a single episode of non-surgical treatment of periodontal pockets resulted in an increase in GCF volume 2 days after treatment (Talonpoika and Hämäläinen 1992; Talonpoika et al. 1993; Talonpoika and Hämäläinen 1993). In the present study, the first time point that GCF was obtained was not 2 days, but 2 weeks after surgery. This was the first visit of patients following the surgery and also when the sutures were removed. Nevertheless, median GCF
volume of GTR test samples was 2.5 and 1.4 times higher than those of GTR control and CF samples at this time point.

The finding, that the GCF volumes collected from GTR test sites throughout the membrane retention period was markedly and statistically significantly higher compared to the adjacent control sites as well as the CF sites at week 6, suggests that the ePTFE membrane caused additional inflammation of the surrounding periodontal tissues. While the GCF volume reduced to a level similar to baseline after 4 weeks in GCF control sites, the GCF volume of GTR test sites took 12 weeks to reach levels similar to baseline. In addition, the tissue forming beneath the membrane was found to reflect a granulation tissue when removed at 6 weeks after GTR surgery, as shown in Chapter 3. Therefore, the high levels of GCF could also be explained by ongoing healing process of the newly-regenerating tissue.

It has been established that the biological functions of growth factors are mediated by receptors on the target cells (Sporn and Roberts 1991). Each growth factor receptor contains 3 regions: i) an extracellular domain that includes the ligand binding domain(s); ii) a transmembrane domain that transverses the cell membrane and iii) an intracellular domain that is cytoplasmic. Binding of the ligand (i.e., the corresponding growth factor) to the extracellular domain of the receptor initiates a cascade of signalling events which lead to specific cellular responses.

In the present study, PDGFR-β but not PDGFR-α was detected in the RT sections (Kuru et al. 1998b). The absence of the PDGFR-α might have resulted from the effect of the TGF-β possibly being present in the healing periodontium, because TGF-β has been shown to decrease the numbers of PDGFR-α in the PL cells in vitro (Oates et al. 1995). Recently, De Paulo Eduardo et al. (1998) have shown that elevated levels of the PDGFR-β, but not the PDGFR-α, were expressed in affected gingiva obtained from patients with drug-induced gingival hyperplasia. Green et al. (1997) have studied immunolocalisation of PDGF A and B chains and corresponding α and β receptors in healing human gingival wounds. They have found that expression of PDGFR-β but not PDGFR-α was up-regulated 3 to 7 days
after wounding. Although the RT samples were obtained approximately 6 weeks after surgery, the presence of numerous inflammatory cells and newly-formed blood vessels, as shown in Chapter 3, suggests that the healing process of the periodontal wound was still going on at that time point and therefore RT samples could be considered to represent the specimens obtained from a healing wound area.

The growth factor PDGF has been shown to co-localise with the corresponding receptors (Green et al. 1997). Therefore, it could be assumed that the up-regulation of the PDGFR-β in the RT might coincide with the presence of the corresponding ligands, namely PDGF-AB and -BB. Therefore, PDGF-AB was investigated in the wound fluid samples of the periodontium where active healing process was taking place following periodontal surgical procedures.

Although GCF has been shown to originate from the serum in surrounding blood vessels, it could not be considered purely a transudate of serum because of differences in the concentrations of their organic and inorganic components (Weinstein et al. 1967). GCF has been also shown to reflect the ongoing processes in surrounding periodontal tissues, including inflammation, connective tissue turnover and breakdown, and alveolar bone turnover and resorption (Talonpoika and Hämäläinen 1992; Embery and Waddington 1994; Nakashima et al. 1994; Yavuzyilmaz et al. 1995; Giannobile et al. 1995; Griffiths et al. 1998). Since the products of inflammatory responses during periodontal disease can be found in GCF (Wilton et al. 1993; Yavuzyilmaz et al. 1995; Haerian et al. 1995; Alexander et al. 1996), it was hypothesised that the products of healing processes, one of which is PDGF, could also be present.

The present study is the first to show that PDGF-AB is present in GCF samples following GTR surgery. This finding is consistent with the role of PDGF-AB in wound healing, which has been shown to stimulate mitogenesis (Oates et al. 1993). However, only 17 out of 54 GCF samples had detectable levels of this factor, while the remaining samples had either no PDGF-AB or the levels were below the detection limits of the assay used.
Perhaps quite low physiologic levels of PDGF is enough for its effect on wound healing, since this growth factor has been reported to have a synergistic effect on PL cells when combined with other growth factors including IGF-I and TGF-β (Matsuda et al. 1992; Rutherford et al. 1992). Salcetti et al. (1997) collected GCF samples from stable and failing implant sites and analysed for PDGF, but it was not reported which dimer of PDGF was tested. However, they have found that PDGF concentrations were higher at stable implant sites than failing implant sites. The concentrations of PDGF detected in GCF samples of GTR sites were comparable with the study of Salcetti et al. (1997). Low levels of PDGF (approximately 0.1 ng/ml) have been also reported to be present in wound fluids of human skin (Cooper et al. 1994; Ono et al. 1995a; Ono et al. 1995b), in contrast it was not detected in fluids of skin graft donor site wounds in humans (Grayson et al. 1993).

The present results of immunohistochemistry revealed that the receptor of TGF-β was present and up-regulated in the regenerating periodontium compared to normal mature PL and gingiva (Kuru et al. 1998b). This finding is consistent with the role of TGF-β, such as in ECM formation, cell proliferation, differentiation, migration and angiogenesis (Lynch et al. 1989; Barnard et al. 1990). The presence of the receptors determines the responsiveness of the cells to the corresponding ligands. Gold et al. (1997) have shown, using immunohistochemistry, that the expression of TGF-β is co-localised with its receptors in wounded skin in animals. However, TGF-β has been found 1 to 5 days before its corresponding receptors, suggesting that the presence of the growth factor may up-regulate the presence of its receptor. Frank et al. (1996) analysed the mRNA levels of TGF-β isoforms and their receptors during skin wound healing in rats and have found a strong correlation between expression of the ligands and their receptors. Taken together, this growth factor was considered likely to be involved in the regenerative healing process of the periodontium and therefore wound fluid of the healing periodontium was examined, for the first time, for the presence of TGF-β1.
The majority of GCF samples were found to have detectable levels of TGF-β1. In contrast, Salcetti et al. (1997) could not detect this growth factor in any of the GCF samples obtained from implant sites in humans, using a similar commercially available ELISA kit. These investigators, however, did not treat the samples with acid in order to enable them to detect the active form of TGF-β1. Therefore, no TGF-β1 has been detected in any of the samples, since the kit used was able to detect only the acid-activated form of this growth factor. In the present study, however, the GCF samples were acidified according to manufacturer’s instructions, and all 16 serum samples and 229 of 258 GCF (approximately 89%) samples were found to posses detectable levels of TGF-β1. The lack of detectable amounts of TGF-β1 in some samples could be both due to small volumes of GCF and to the level of this factor being below detection limits. However, Cooper et al. (1994) have collected wound fluids from human chronic pressure ulcers and detected TGF-β1 in only 3 of 20 samples. On the other hand, detectable levels of this growth factor have been reported by Ono et al. (1995a; 1995b) in skin donor site wound fluid obtained from 24 patients and in blisters of skin burns in 12 patients as well as by Ludwicka et al. (1995) in bronchoalveolar lavage fluid of 15 scleroderma patients and 14 healthy individuals.

During healing after periodontal treatment, new synthesis of ECM proteins, including collagen types I and III and fibronectin, occurs as evidenced by their increased concentration in GCF (Talonpoika and Hämäläinen 1992; Talonpoika and Hämäläinen 1993; Talonpoika et al. 1993). TGF-β1, a growth factor shown to stimulate the production of ECM proteins (Matsuda et al. 1992; Howell et al. 1996), was detected in large amounts in the GTR-treated sites compared with the CF-treated sites and GTR control sites 2 weeks after surgery. The wound fluid collected from GTR test sites at 4 weeks still had elevated amounts of this factor compared with the samples of GTR control sites. This finding suggests that TGF-β1 is involved in early healing and regenerative processes of the periodontium.
following placement of a membrane, whereas in sites with healthy periodontium the duration of TGF-β1 involvement might be shorter.

Although TGF-β1 has been reported to stimulate ECM production, proliferation of PL cells and osteoblast chemotaxis when used in vitro (Hughes et al. 1992; Dennison et al. 1994; Howell et al. 1996), the in vivo topical application of this factor combined with IGF and bFGF onto surgically created defects in dogs did not result in increase in bone regeneration (Selvig et al. 1994). The finding of the present study, nevertheless indicate the presence and possible involvement of this factor during healing of periodontal wounds. However, TGF-β1 has been shown to modulate other growth factors (Caffesse and Quinones 1993) and therefore the effect of this factor, as shown in in vitro studies, may also depend on the presence and level of other growth factors in the wound area.

The normal physiological levels of growth factors in normal and healing periodontium have previously not been reported. However, both TGF-β and PDGF have been added into culture media in previous in vitro studies at concentrations ranging from 0.01-1000 ng/ml (Anderson et al. 1998; Boyan et al. 1994). For the in vivo studies, the application time, a suitable carrier system and proper dose for the use of a growth factor or combination of growth factors are yet to be identified (Howell et al. 1996). In addition, as soon as these factors are applied they might be washed away by the fluids present in the surrounding environment. These may necessitate the use of high doses of growth factors clinically. Indeed, Howell et al. (1997) applied 150 µg/ml each of the growth factors PDGF-BB and IGF-I to periodontal osseous defects in humans and observed significantly greater bone formation compared with the defects treated by conventional flap surgery only.

TGF-β inhibits the proliferation of epithelial cells in porcine skin wounds in vivo (Lynch et al. 1989) and of gingival epithelial cells in vitro (Lu et al. 1997). Since rapid proliferation and downgrowth of gingival epithelium in early phases of periodontal healing have been shown to prevent formation of new attachment (Aukhil et al. 1987; Nyman et al. 1980), topical
application of TGF-β may delay this process and at the same time with its stimulatory effect on fibroblasts and osteoblasts, this factor may accelerate periodontal regeneration. PDGF-B, on the other hand, has already been shown to promote fibroblast proliferation and formation of new bone and cementum in animals (Wang et al. 1994b; Lynch et al. 1991) and also bone regeneration in humans (Howell et al. 1997) when applied to periodontal wounds in combination with IGF-I. In addition, TGF-β enhanced the mitogenic response of PL cells to PGDF and also the synthesis of PDGF by osteoblasts in vitro (Oates et al. 1993; Bilezikian et al. 1996). Several different combinations of growth factors have been applied on periodontal wounds in animals (The American Academy of Periodontology 1996). PDGF-AB and TGF-β1, as evidenced in the present study, are likely to play crucial roles in early phases of periodontal regeneration and may also have a potential therapeutic use. While PDGF-AB may increase the number of fibroblasts and osteoblasts in the wound area, TGF-β1 may prevent epithelial proliferation and promote ECM synthesis by fibroblast and osteoblasts as well as angiogenesis. They may also trigger the synthesis of endogenous growth factors via autocrine control (Tipton and Dabbous 1998), thereby enhancing the cascade of healing events following periodontal surgery. However, the effect of the combination of PDGF-AB and TGF-β1 on periodontal healing remains to be tested.

6.10 CONCLUSIONS

Based on the results of this second chapter, it was concluded that,

i) the periodontal surgery causes an increase in the volume of GCF during the immediate post-operative phase and the presence of a membrane as a part of GTR surgery has a more pronounced effect on the GCF volume compared to CF surgery;

ii) the finding that PDGFR-β and TGF-βR were up-regulated in newly-regenerated soft tissue forming underneath the non-resorbable membranes used for treatment of periodontal defects indicates that these are likely to have important roles in periodontal regeneration;
iii) the growth factor PDGF-AB was detected in fluid of periodontal wounds and may be involved in periodontal healing;

iv) the result that higher amounts of TGF-β1 in GCF obtained from sites treated with GTR suggest that this factor is also likely to be involved in regenerative healing of the periodontium and therefore may be used as a therapeutic tool along with PDGF-AB.
CHAPTER 7
SUMMARY AND FUTURE STUDIES

7.1 SUMMARY AND GENERAL CONCLUSIONS

It has become widely recognised during the past 15 years that regeneration of the periodontium is biologically possible. An increasing number of studies have demonstrated the restoration of diseased periodontal tissues to a healthy and functional form using the principles of GTR (Caffesse 1988; Gottlow 1993; Cortellini et al. 1995; Cortellini et al. 1996; Garrett 1996). However, GTR therapy still achieves only a limited degree of success and is still unpredictable, particularly in the treatment of Class III furcation defects and horizontal bone loss (Pontoriero et al. 1989). In order to obtain an improved clinical outcome, new techniques and a wide range of barrier membranes have been developed, tested and used to treat human periodontal defects (Scantlebury 1993). However, the key for achieving complete periodontal regeneration lies in a better and more comprehensive understanding of the cellular and molecular mechanisms which underlie GTR (Terranova and Wikesjö 1987; McCulloch and Bordin 1991; Pitaru et al. 1994; McCulloch 1995). This study was undertaken to characterise the types of cells and their functional activities which are associated with regenerative periodontal healing in order to develop more successful strategies.

The ePTFE membranes retrieved from subjects who underwent GTR surgery were initially investigated by RT-PCR and TEM to determine whether any intact cells remained attached to the membranes. These experiments showed that both surfaces of the middle and apical portions of the membranes contained inflammatory cells and fibroblast-like cells which were closely associated with collagen fibres. A limited number of samples of the newly-regenerated soft periodontal tissue (RT) underlying the membranes was also obtained and found to be similar to granulation tissue, containing not only fibroblast-like cells and numerous inflammatory cells but also newly-formed blood vessels within a loosely arranged matrix.
It was of interest that epithelial cells, whose exclusion is an important clinical objective of GTR, were nevertheless observed to be present in RT samples in vivo, as revealed by immunohistochemical examination. These cells were also found to migrate out of the RT explants in vitro. Although their origin remains uncertain, this finding indicates that these cells were present in the regenerating periodontium beneath the barrier membranes. However, the epithelial cells did not appear to hinder the regenerative healing of the periodontium, since the RT samples were obtained from sites where excessive tissue formation beyond the normal bone contour was evident.

Cells associated with the barrier membranes in vivo (M cells) and those derived from the RT (RT cells) were both successfully cultured in vitro. They were observed, using phase-contrast microscopy, to have a similar spindle-shaped morphology as the cultured cells derived from periodontal ligament (PL) and gingiva. Also like these normal cells, the GTR-associated M and RT cells examined by immunocytochemistry were found to express vimentin, suggesting their mesenchymal origin. In addition, flow cytometry (FCM) analysis showed that the M, RT, PL and gingival cells were similar in size and granularity.

However, when the functional activities of these cells were investigated using, for the first time, the technique of FCM, it was found that collagen type I, fibronectin and tenascin, major constituents of soft connective tissue, were expressed by the GTR-associated M and RT cells at higher levels than the normal PL cells. Furthermore, the RT cells were also found to express the bone-associated proteins osteonectin, osteocalcin, BSP and osteopontin at relatively higher levels than the normal PL cells. These results indicate the relative up-regulation of these antigens in the M and RT cells specifically and their important role in establishing the new extracellular environment of the regenerating soft and hard periodontal tissues during the wound healing process.

The RT cells also had significantly higher basal levels of ALP activity than the normal PL cells. The results of von Kossa staining showed that the M cells had the ability to form mineralised nodules in vitro and in greater
numbers than the nodules formed by the PL cells. These findings suggest that both the M and RT cultures contain cells which have osteoblast-like characteristics.

After cultures of RT cells were treated with DEX, which has previously been shown to induce the differentiation of osteoprogenitor cells (Bellows et al. 1990), the levels of bone-associated proteins as well as ALP activity were found to be markedly up-regulated. In M cell cultures, incubation with DEX in the presence of ascorbic acid and β-glycerophosphate, also increased the formation of mineralised nodules by these cells in vitro. These findings provide evidence for the presence of osteoprogenitor cells in the RT and M cultures.

Examination of FCM profiles revealed that the M, RT and PL cells not only exhibited differences in the expression of ECM proteins but also contained apparently distinct subsets of cells which differentially expressed these antigens. Although fibroblast heterogeneity in normal PL tissue has previously been reported (Phipps 1997), the present study strongly suggests that GTR-associated cells also comprise heterogeneous sub-populations with different functional activities. These sub-populations of GTR cells, which remain to be more precisely delineated, are likely to have unique and essential roles in the regeneration of the PL, alveolar bone and cementum during wound healing following GTR surgery.

Growth factors regulate a number of cellular activities, which are of critical importance during wound healing, including proliferation, migration, differentiation and synthesis (Howell et al. 1996). Although these factors either alone or in combination have been applied topically onto periodontal wounds to enhance regeneration (Howell et al. 1997; Wikesjö et al. 1998), little is known about their precise role in periodontal wound healing. Since the function of growth factors depend on their binding to specific receptors on target cells (Sporn and Roberts 1991), in the present study the expression of these receptors in normal and regenerating periodontium was examined using immunohistochemical procedures. Two of these, the receptors for PDGF and TGF-β were found to be apparently up-regulated in
the RT samples. This observation suggests that their respective growth factors are also likely to be involved in human periodontal regenerative healing.

To determine whether PDGF and TGF-β were an important feature of GTR, GCF samples collected from periodontal defect sites were used as representative fluid of a periodontal wound undergoing regenerative healing after GTR surgery. Clinical measurements confirmed that this therapy elicited significant clinical improvements. However, it was found that the growth factor PDGF-AB could be detected in only a small proportion of the GCF samples, probably because of the limited sensitivity of the detection procedure. In marked contrast, the growth factor TGF-β1 was readily measured in the majority of GCF samples. The significant elevation of the amount of this factor in the GTR test samples immediately after surgery, compared with the GTR control and conventional flap samples, indicates that TGF-β1 is likely to play an important role in the regenerative periodontal healing process.

7.2 FUTURE STUDIES

The results of these studies indicated that both the M and RT cells are very likely to be closely associated with periodontal regeneration. However, differences in cell proliferation rate, expression of soft and hard connective tissue proteins and ALP activity were nevertheless observed. The RT cells most probably more closely correspond to the regenerative cells in vivo since these were derived directly from the RT tissue. Although a substantial proportion of regenerative cells are undoubtedly also present among the M cells, M cell cultures may also contain some cells derived from the gingival flap, which was associated with the external surface of the membrane, as well as cells from alveolar bone, which was in contact with the internal apical surface of the membrane in situ. In further studies of periodontal regenerative cells, it is therefore considered that RT tissue should be utilised as the most representative source of material. However, because of the limited availability of the RT tissue, the retrieved non-
resorbable membranes also provides a suitable, if less than perfect, source of cellular material.

In the present study, the normal PL cells served as the baseline cell type for comparison purposes, because the GTR-associated cells were considered to originate primarily from the remaining healthy PL tissue adjacent to the periodontal defect area. However, previous studies have shown that the osteoblasts derived from the adjacent alveolar bone as well as the cells present in endosteal spaces of this bone contribute to regenerative periodontal healing (McCulloch et al. 1987; Melcher et al. 1987). It would therefore be of value, in future studies, to compare the functional activities of alveolar bone cells with those of the GTR-associated cells.

In order to promote the adhesion, proliferation and differentiation of PL cells, several bioactive molecules, such as ECM proteins and growth factors, have previously been used both in vitro and in vivo (Smith et al. 1987; Lynch et al. 1991; Oates et al. 1993; O'Neal et al. 1994; Oates et al. 1993; O'Neal et al. 1994; Nishimura and Terranova 1996). Testing of such agents on the GTR-associated cells in vitro instead of normal PL cells may be of real benefit, since the M and RT cells were found to be functionally different from the PL cells. Optimal concentrations and the precise functional effects of these agents could thus be evaluated in vitro prior to animal studies and subsequent clinical use. In addition, these pharmacologically active reagents could also be applied in combination in tissue culture, as multiple factors undoubtedly participate in wound healing in vivo.

Since the GTR-associated cells were found to be heterogeneous, the isolation and study of the sub-populations, for example their differential response to growth factors and production of ECM components, would be of value. Although such cells have a limited life span in vitro, long-term cell lines could be obtained by retroviral transduction with temperature-sensitive SV40 large T antigen, as previously described for periodontal cells (Parkar et al. submitted). This would enable to obtain immortal cell lines, to clone the individual cells and to study them further in detail. Moreover, because this T antigen is inactivated at high temperature, the cells would continue to
proliferate at 34°C but would become growth-arrested at 39°C. The withdrawal of the cells from the cell cycle at this high temperature is known to be associated with the expression of differentiated cell functions. Such experiments would therefore enable the differentiation of progenitor cells to be studied as well as the functions of differentiated cells in the M and RT cultures.

The FCM technique was found to be invaluable for studying the phenotypes and function of the different types of cultured periodontal cells. This technique could be used further to find out whether, for example, the osteopontin-expressing cells also express another bone protein at the same time. This could be achieved by double immunostaining of cultured cells (i.e., staining the same cell using two different antibodies).

GCF could be a potentially valuable source of molecular 'markers' of periodontal regeneration. The present study shows that it is possible to obtain and measure specific components in GCF samples collected from the periodontal defect sites treated by GTR. While this study shows that TGF-β1 could readily be detected in GCF samples, the limited sensitivity of the assay prevented the detection of PDGF. However the growth factor PDGF, as a potential marker, could be further investigated in GCF samples if sensitivity of the currently available ELISA technique could be improved, using for example the avidin-biotin complex.
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APPENDIX 1

EXPLANATORY STATEMENT (Periodontal flap surgery)

Following initial periodontal (gum) treatment we would now recommend gum surgery in areas where there is still some disease.

We are currently investigating how gums heal after surgery and which substances are involved during the healing period. In order to help us in our research, we wish to collect some samples from you before surgery and during your normal follow up visits after surgery. This will give us important information about how to achieve better healing.

Using strips of blotting paper, we would like to collect samples of fluid that comes out from between the teeth and the gum tissues. This fluid reflects the healing occurring locally around the gum and bone tissue. The fluid is also influenced by general factors as a result of blood circulating to the area. Therefore, we would like to collect 10 ml of blood (equivalent to two teaspoons) before you start treatment and six weeks after surgery. The blood samples will be compared with the gum fluid samples in order to assess the amount of local as opposed to general factors involved in healing. We would also like to perform clinical measurements which will allow us to determine the progress of healing of your gums. The procedures are routine, rapid and painless.

If you do not wish to take part in this study, this will not in any way affect the treatment provided for you at this hospital. You are also free to withdraw from the study at any stage without giving a reason and without it affecting the treatment being provided.

If you have any concerns and enquiries regarding this study please contact:
Dr. Gareth Griffiths (Tel) 0171 915 1103
Mrs. Leyla Kuru (Tel) 0171 915 1280
EXPLANATORY STATEMENT (GTR Subjects)

Following initial periodontal (gum) treatment we would now recommend gum surgery in areas where there is still some disease. A small thin barrier membrane will be placed around a tooth in an attempt to restore your damaged gums and underlying bone. We are currently investigating how gums heal after surgery and which substances and cells are involved during the healing period. In order to help us in our research, we wish to collect some samples from you before surgery and during your normal follow up visits after surgery. This may give us information about how to achieve better healing in the future.

There are a number of samples we wish to collect. At each visit, strips of blotting paper will be used to collect samples of the fluid that comes out from between the teeth and the gum tissues. This fluid reflects the healing occurring locally around the gum and bone tissue. The fluid is also influenced by general factors as a result of blood circulating to the area. Therefore, we would like to collect 10 ml of blood (equivalent to two teaspoons) before you start treatment and six weeks after surgery. The blood samples will be compared with the gum fluid samples in order to assess the amount of local as opposed to general factors involved in healing. In addition, we will perform some clinical measurements to determine the clinical progress of your gum and bone healing.

The barrier membrane needs to be removed 6 weeks after the initial surgery. The membrane is normally thrown away, whereas we wish to keep it to examine which cells have been involved in the healing process. In some cases, after membrane removal, too much gum material has been reformed and must be removed to keep the correct shape of the gums and bone. We would like to keep this material to help in our study of the cells and substances involved in the healing process.

If you do not wish to take part in this study, this will not in any way affect the treatment provided for you at this hospital. You are also free to withdraw from the study at any stage without giving a reason and without it affecting the treatment being provided. If you have any concerns and enquiries regarding this study please contact:

Dr. Gareth Griffiths (Tel) 0171 915 1103
Mrs. Leyla Kuru (Tel) 0171 915 1280
CONSENT FORM

I, ..................................................
of ..................................................
hereby fully and freely consent to undergo the procedures involved in the
clinical research investigation outlined on the explanatory statement, a copy
of which is attached to this form. I understand that the investigation is a
contribution to medical knowledge and does not form part of any treatment I
may currently be receiving. I note that I may withdraw my consent at any
stage in the investigation, without this affecting the provision of treatment.

I confirm that the nature and purpose of the study have been explained to
me, and I have read the written explanation.

(signed) ....................... Date ......................

Investigator’s ....................... Date ......................

Signature