CELLULAR AND MOLECULAR PROCESSES IN PERIODONTAL GROWTH AND RENEWAL

Thesis submitted by
Mohamed H. Parkar

for the degree of
DOCTOR OF PHILOSOPHY

Eastman Dental Institute
for Oral Health Care Sciences,
University College London (UCL)
University of London

2000
Chronic inflammatory periodontal diseases (CIPDs) are considered to be mediated by cytokines in response to dental plaque, involve destruction of connective tissue, bone breakdown and ultimately tooth loss. In contrast, the repair and regeneration of periodontal tissues are wound healing processes characterised by active cell growth and the production of new extracellular matrix (ECM). In addition, in gingival hyperplasia (GH), a pathological condition resulting from the administration of certain immunosuppressants, anti-epileptic drugs and calcium channel-blocking agents, there is enlargement of the gingival connective tissue and excessive accumulation of ECM. As in other hyperplastic lesions, GH has been associated with growth factors and anabolic steroid hormones, which are known to regulate cell proliferation, differentiation and the synthesis of ECM components. The actions of these factors are mediated through specific cell surface and intracellular receptors, although their role in periodontal tissues has not been well defined.

This study has examined the expression of androgen and growth factor receptors in gingival fibroblasts (GF) and in periodontal ligament (PDL) cells. Analysis using the reverse-transcribed polymerase chain reaction (RT-PCR) established that the androgen receptor (AR) is expressed by both types cell. However, the levels of AR were found to be similar in both normal and GH tissues, while AR levels in normal GF in vitro were unaffected by the drugs which cause GH in vivo. In contrast, the activity of the AR may be important in GH, since androgens were found to down-regulate the interleukin-6 (IL-6), a cytokine involved in connective tissue turnover.

Growth factor receptor expression was analysed by flow cytometry (FCM) and the results showed that although the β-receptor for platelet-derived growth factor (PDGF) was expressed by all the cells, receptors for transforming growth factor (TGF-β) type I and II, fibroblast growth factor (FGF) and insulin-like growth factor (IGF) were differentially expressed by GF and PDL cells. In contrast, neither the GF nor PDL cells expressed either the PDGF receptor α or the epidermal growth factor (EGF) receptor.
The cellular and molecular events associated with periodontal wound healing may be similar to those occurring during embryonic development, in which the ECM protein fibronectin (FN) plays an important role. This aspect of the study investigated the expression of FN molecular isoforms in periodontal tissues. All embryonic isoforms were found to be expressed in healthy gingiva, periodontal ligament and GH tissues whereas CIPD tissues generated only the low molecular weight adult isoforms. Furthermore, certain growth factors elicited pronounced up-regulation of the embryonic FN isoforms in PDL cells, which may be of particular relevance to periodontal regeneration.

In order to develop in vitro models for the investigation of periodontal disease and regeneration, long-term cell lines of GF and PDL cells were obtained by retroviral transduction of primary cells with a temperature-sensitive oncogene, the SV40 large T antigen. This was carried out in order to provide a continuous source of cells for the investigation of certain key proteins which have a fundamental role in periodontal wound healing.

The results of this study have demonstrated that the activity of the AR may have an important role in periodontal growth. Furthermore, the periodontal cells differentially express key growth factor receptors and the PDL cells produce embryonic FN isoforms in response to certain growth factors which are likely to be involved in periodontal growth and renewal.
INDEX OF CONTENTS

Abstract 2
Index of contents 4
List of tables 9
List of figures 10
List of abbreviations 13
Acknowledgements 15
Declaration 16
Publications as a result of this thesis 17

CHAPTER 1

INTRODUCTION

1.1 The structure and cells of the periodontium 20
   1.1.1 Gingiva 20
   1.1.2 Periodontal Ligament 20
   1.1.3 The cells of the periodontium 21
      1.1.3.1 Epithelial cells 21
      1.1.3.2 Cementoblasts 23
      1.1.3.3 Osteoblasts 23
      1.1.3.4 Fibroblasts 24
   1.1.4 Immortalisation of mammalian cells 26

1.2 Periodontal Diseases 27
   1.2.1 Periodontal breakdown: chronic inflammatory periodontal disease (CIPD) 29
      1.2.1.1 The role of cytokines in CIPD 29
         1.2.1.1.1 Interleukin-1 (IL-1) 30
         1.2.1.1.2 Interleukin-6 (IL-6) 31
   1.2.2 Gingival overgrowth: drugs-induced gingival hyperplasia (GH) 32
      1.2.2.1 Histopathology 33
      1.2.2.2 Possible mechanisms of GH 34
1.3 Sex hormones and periodontal diseases
  1.3.1 Sex hormones and cytokines
  1.3.2 Sex hormones and growth factors in relation to hyperplasia
  1.3.3 Sex hormone receptors
  1.3.4 Sex hormone receptors in periodontal tissues
1.4 Periodontal healing, repair and regeneration
  1.4.1 Growth factors and their receptors
    1.4.1.1 Platelet-derived growth factor (PDGF)
    1.4.1.2 Transforming growth factor-β (TGF-β)
    1.4.1.3 Epidermal growth factor (EGF)
    1.4.1.4 Fibroblast growth factor (FGF)
    1.4.1.5 Insulin-like growth factor (IGF)
  1.4.2 The role of growth factors and their receptors in GH
1.5 Extracellular matrix (ECM)
  1.5.1 Collagen
  1.5.2 Fibronectin
  1.5.3 Osteocalcin
  1.5.4 Osteonectin
  1.5.5 Osteopontin
1.6 Statement of the problems and aims

CHAPTER 2

MATERIALS AND METHODS

2.1 Methods
  2.1.1 Tissue samples
  2.1.2 Cell culture
2.2 Treatment of cells
  2.2.1 Treatment with sex hormones
  2.2.2 Treatment with growth factors and cytokines
  2.2.3 Drug treatment
2.3 RNA isolation, reverse-transcribed polymerase
2.3.1 RNA extraction 56
2.3.2 RT-PCR 56
2.3.3 Northern blot analysis 58

2.4 Determination of IL-6 secretion by enzyme-linked immunosorbent assay (ELISA) 58

2.5 Flow cytometry (FCM) 59

2.6 Immortalisation of gingival and PDL cells 61
2.6.1 Retroviral transfection 61
2.6.2 Cloning procedure 61
2.6.3 Growth characteristics of immortalised cells 61
2.6.4 Immunocytochemical staining of T antigen 62
2.6.5 Alkaline phosphatase (AP) assay 62

CHAPTER 3
THE ROLE OF SEX HORMONE RECEPTORS IN PERIODONTAL DISEASE AND GROWTH

3.1 RT-PCR analysis of oestrogen and androgen receptor expression in periodontal tissues and cells 68
3.1.1 Introduction 68
3.1.2 Materials and methods 69
3.1.3 Results 69

3.2 The effects of androgens on gingival and PDL cells 71
3.2.1 Introduction 71
3.2.2 Materials and methods 72
3.2.3 Results 73
3.2.3.1 Cell proliferation 73
3.2.3.2 Measurement of AR mRNA in normal and GH tissues 76
3.2.3.3 Measurement of AR protein in GF by FCM 76
3.2.3.4 Regulation of IL-6 expression by androgen in GF and PDL cells 88

3.3 Discussion 94
transduced cells

6.3.2 FCM analysis of transduced cells
   6.3.2.1 Size and granularity of transduced cells
   6.3.2.2 FCM analysis of ECM components

6.3.3 AP expression in transduced cells

6.4 Discussion

CHAPTER 7
SUMMARY AND FUTURE STUDIES
REFERENCES
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1</td>
<td>Sequence of oligonucleotide primers and predicted sizes of PCR products</td>
<td>66</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>PCR amplification annealing conditions</td>
<td>67</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>Size and granularity of gingival fibroblasts treated with drugs as measured by FCM</td>
<td>84</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Growth factor receptor density (AFI) on GF and PDL cells</td>
<td>111</td>
</tr>
<tr>
<td>Table 6.1</td>
<td>Size and granularity of transduced PDL and gingival cells grown at 34°C and 39°C</td>
<td>142</td>
</tr>
<tr>
<td>Table 6.2</td>
<td>Comparison of ALP levels in PDL and gingival cells</td>
<td>144</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Structure of the periodontium and the tooth</td>
<td>22</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Schematic representation of hormone-receptor Interaction</td>
<td>40</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Schematic representation of reverse-transcriptase polymerase chain reaction (RT-PCR)</td>
<td>64</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>The laser light applied to a cell in the flow cytometer is reflected at various angles</td>
<td>65</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>RT-PCR analysis of ER and AR expression in periodontal tissues and cells</td>
<td>70</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Effects of DHT on DNA synthesis in GF</td>
<td>74</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Effects of DHT and growth factors on DNA synthesis in GF</td>
<td>75</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Agarose gel of GAPDH PCR for determination of optimal amplification cycle</td>
<td>77</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Agarose gel of AR PCR for determination of optimal amplification cycle</td>
<td>77</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>Kinetic analysis of GAPDH and AR mRNA levels in GF</td>
<td>78</td>
</tr>
<tr>
<td>Figure 3.7</td>
<td>Relative levels of AR mRNA with respect to GAPDH in normal GT and GH</td>
<td>79</td>
</tr>
<tr>
<td>Figure 3.8</td>
<td>Representative FCM dot-plot profile of GF</td>
<td>81</td>
</tr>
<tr>
<td>Figure 3.9</td>
<td>Representative histogram plot showing AR expression in MCF7 and GF</td>
<td>82</td>
</tr>
<tr>
<td>Figure 3.10</td>
<td>Comparison of AR expression by normal and GH derived fibroblasts and MCF7 cells cultured under drug-free conditions</td>
<td>83</td>
</tr>
<tr>
<td>Figure 3.11</td>
<td>Effects of CsA on AR expression by normal GF</td>
<td>85</td>
</tr>
</tbody>
</table>
Figure 3.12 Effects of CsA on AR expression by fibroblasts derived from GH tissue 86
Figure 3.13 Effects of PHT and NIF on AR expression by normal GF 87
Figure 3.14 Effects of DHT on IL-6 production by normal GF 89
Figure 3.15 Effects of DHT on IL-6 production by diseased gingival fibroblasts and normal PDL cells 90
Figure 3.16 Effects of anti-androgenic drugs on IL-6 production by normal GF 92
Figure 3.17 RT-PCR analysis of the effects of DHT on IL-6 mRNA levels with respect to GAPDH 93
Figure 4.1 Representative histograms of growth factor receptor expression by GF and PDL cells 104
Figure 4.2 Comparison of growth factor receptor expression by GF and PDL cells 105
Figure 4.3 Representative histogram showing the effects of DHT on growth factor receptor expression by GF 107
Figure 4.4 Representative histogram showing the effect of DHT on IGF-R expression by GF 108
Figure 4.5 Effects of DHT on IGF-R expression by normal GF 109
Figure 4.6 Representative histogram showing the effects of CsA on growth factor receptor expression by GF 110
Figure 5.1 The generalised structure of FN protein 118
Figure 5.2 RT-PCR analysis of alternative splicing of FN mRNA in healthy PDL and CIPD tissues 120
Figure 5.3 RT-PCR analysis of alternative splicing of FN mRNA in GH tissue 121
Figure 5.4 Effects of growth factors on FN splicing in cultures of PDL cells 123
Figure 5.5 Effects of the inflammatory cytokine IL-1β on FN splicing in cultures of PDL cells 124
Figure 6.1  Phase contrast micrographs of transduced PDL cells cultured at 34°C and 39°C 133
Figure 6.2  Expression of the tsT antigen in transduced PDL cells 134
Figure 6.3  Growth of tsT-transduced PDL and gingival cells 136
Figure 6.4  The expression of mRNA by Northern blot and RT-PCR analysis of OC, OP, COLI and AP in transduced cells 138
Figure 6.5  Representative histogram of the FSC of PDL cells grown at 34°C and 39°C 139
Figure 6.6  Representative histogram of the SSC of PDL cells grown at 34°C and 39°C 140
Figure 6.7  FCM analysis of OC, ON, OP and COLI expression in transduced PDL cells grown at 34°C and 39°C in the absence and presence of DEX and D3 143
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Symbol / abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>aFGF</td>
<td>acidic fibroblast growth factor</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>αMEM</td>
<td>alpha minimum essential medium</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>AFI</td>
<td>average fluorescence intensity</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>CIPD</td>
<td>chronic inflammatory periodontal disease</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy deoxyribonucleic acid</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporine A</td>
</tr>
<tr>
<td>dATP</td>
<td>2'-deoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>2'-deoxycytosine 5'-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>2'-deoxyguanidine 5'-triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>2'-deoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DES</td>
<td>diethylstilbestrol</td>
</tr>
<tr>
<td>DEX</td>
<td>dexamethasone</td>
</tr>
<tr>
<td>D3</td>
<td>vitamin D3</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>oestrogen receptor</td>
</tr>
<tr>
<td>FN</td>
<td>fibronectin</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FCM</td>
<td>flow cytometry</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter</td>
</tr>
<tr>
<td>GF</td>
<td>gingival fibroblasts</td>
</tr>
<tr>
<td>GH</td>
<td>gingival hyperplasia</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IgG₁</td>
<td>immunoglobulin G₁</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NIF</td>
<td>nifedipine</td>
</tr>
<tr>
<td>oligo-dT</td>
<td>oligodeoxythymidine</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDL</td>
<td>periodontal ligament</td>
</tr>
<tr>
<td>PLF</td>
<td>periodontal ligament fibroblasts</td>
</tr>
<tr>
<td>PHT</td>
<td>phenytoin</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse-transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SSC</td>
<td>side Scatter</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my supervisor Dr. I. Olsen for his guidance throughout this thesis. I am particularly grateful to Prof. H. Newman for giving me the opportunity to initiate this study and for his encouragement and support in completing the thesis. I would also like to thank all my colleagues in the Department of Periodontology for their valuable advice and for providing tissue samples.

Finally, I wish to express my sincere thanks and appreciation to my family for their patience and constant support to fulfil my dream.
DECLARATION

This thesis is the result of my own original investigations, except where otherwise stated. The initial retroviral transduction of periodontal cells was performed in the containment III facility by Dr. M. O'hare, Breast Cancer laboratory, Department of Surgery, University College London.
PUBLICATIONS AS A RESULT OF THIS THESIS

PAPERS


CHAPTER 1
INTRODUCTION

1.1 The structure and cells of the periodontium

The periodontium is a complex and highly specialised organ comprising the tissues responsible for the attachment of the teeth to the jaw. It consists of soft tissues, the periodontal ligament (PDL) and the gingiva, and the hard connective tissues, cementum and alveolar bone (Ten Cate, 1989) (Fig 1.1). Although anatomically these tissues are linked, each tissue has a unique structure and function. It may be considered one of the most dynamic structures with a continuous high rate of cell turnover (Hassell, 1993).

1.1.1 Gingiva

The most peripheral layer of the soft periodontal tissues, the gingiva, is perhaps the most studied clinically (Schroeder and Page, 1990). It begins at the gingival margin and extends to the mucogingival junction. It is composed of dense fibrous connective tissue also called the lamina propria, covered by keratinised stratified squamous epithelium, of which there are three types: the oral gingival epithelium, the oral sulcular epithelium and the junctional epithelium (Schroeder, 1991). The gingival epithelium provides a protective sheath for the underlying periodontal tissues. The gingival connective tissue is highly organised and accounts for the major proportion of the gingiva. In healthy gingiva, collagenous proteins constitute approximately 65% of the total protein of which type I collagen is the major component. Additionally, a heterogeneous group of noncollagenous proteins play an important role in the structural integrity and function of the gingiva (Mariotti, 1993).

1.1.2 Periodontal ligament

The PDL is a highly cellular soft connective tissue linking the alveolar bone and cementum (Berkovitz et al., 1995). The PDL contains a number of different types of cell, both differentiated and precursors, which play an important role in the homeostasis of the periodontium with its capacity for repair
and regeneration. Although the precise origins of each of these cells is not known, they are principally located in paravascular sites, i.e. in close proximity to blood vessels or in vascular channels of the alveolar bone. Although the main of function of the PDL is tooth anchorage, it also plays a major role in the development and maintenance of fibrous and calcified tissue, in the synthesis and resorption of alveolar bone and the transport of nutrients and metabolites (Pitaru et al., 1994).

1.1.3 The cells of the periodontium

The main cell types of the periodontium are epithelial cells, cementoblasts, osteoblasts and fibroblasts.

1.1.3.1 Epithelial cells

The main epithelial cell population of the periodontium is located in the gingival epithelium and is responsible for the important function of protecting underlying connective tissue. It consists of four layers: basale, spinosum, granulosum and corneum (Schroeder and Listgarten, 1997). The basale layer cells are mainly cuboidal and some of them have the ability to migrate through the epithelium on to the outer surface, differentiating and eventually keratinising and are known as keratinocytes. Immediately adjacent to the stratum basale is the stratum spinosum which consists of cells that have stopped synthesising basal lamina components and contain an increased level of cytoplasmic filaments. On top of the spinosum lies the stratum granulosum a flattened cell layer. The outermost layer of the epithelium is the stratum corneum where the cells lack nuclei and have completed the process of keratinisation. Furthermore, these cells have lost various organelles including mitochondria and endoplasmic reticulum and are therefore unable to maintain normal metabolic activity. The oral sulcular epithelium shows the same four layers as gingival epithelium but does not keratinise and is therefore more permeable.
Fig 1.1 Diagrammatic representation of the principal structures of the periodontium (periodontal ligament, gingiva, cementum, alveolar bone).
The junctional epithelium not only serves as a protective layer but it also provides the outermost attachment mechanism to the tooth. The cells are mainly in the basal lamina the width of which is only few cell layers. These have a particularly high turnover continually renewing itself throughout life and unlike the other epithelia lacks the three other strata (Hassell, 1993).

1.1.3.2 Cementoblasts

Cementoblasts are fibroblast-like cells found in the periodontal ligament and are often indistinguishable from periodontal fibroblasts (Berkovitz et al., 1995). These cells are responsible for secreting the organic matrix of cementum. When active they appear as a distinct layer of cells on the root surface similar to osteoblasts but the turnover among cementoblasts is slow compared with that of osteoblasts. Induction of cementoblasts in periodontal ligament takes place throughout life as evidenced by the continual rate of cellular cementum deposition. As cellular cementum formation proceeds, the cementoblasts are trapped within the cementum matrix and are then referred to as cementocytes. It is difficult to culture cementoblasts or cementocytes but they are thought to be derived from progenitors in vascular spaces of the periodontal ligament or endosteal space of bone (McCulloch et al., 1987; Pitaru et al., 1994).

1.1.3.3 Osteoblasts

Osteoblasts are cuboidal specialised bone-forming cells of mesenchymal origin (Arnett and Stevenson, 1993). The function of the active osteoblast is to secrete type I collagen which is the main component of the bone matrix, as well as other matrix proteins including osteonectin, osteocalcin, bone sialoprotein, osteopontin and proteoglycans (Arnett, 1990). When the cells are most active they show strong basophilic cytoplasm and prominent rough endoplasmic reticulum (RER) characteristic of protein-secreting cells. The cells are situated on the surfaces of alveolar bone and as bone deposition proceeds, osteoblasts become incorporated in the matrix as osteocytes (Berkovitz et al., 1995).
1.1.3.4 Fibroblasts

The predominant cell type in both the gingival connective tissue and the periodontal ligament is the fibroblast, which accounts for about 5.6% of their volume. Fibroblasts are characterised by their spindle or stellate morphology, presence of vimentin filaments and synthesis of extracellular matrix (ECM). These cells play a pivotal role in normal tissue turnover and in pathology (McCulloch and Bodin, 1991). Periodontal fibroblasts are responsible for the overall production and turnover of most of the extracellular matrix. Although fibroblasts from different tissues appear similar microscopically, there is growing evidence to suggest that these cells are phenotypically and functionally heterogeneous even within a tissue type (Fries et al., 1994; Phipps et al., 1997). Similar phenotypic heterogeneity has been observed in gingival fibroblast (GF) and PDL cell populations (Hakkinen and Larjava, 1992; Irwin et al., 1994) and there is considerable evidence from work in rodents to suggest that this heterogeneity is generated due to lineage hierarchy present during the development and maintenance of the periodontium. This suggests that the system of fibroblast lineage present in periodontal connective tissues resemble that of other cell renewal systems in mammals, including the haematopoietic system and the gut epithelium (Pitaru et al., 1994; McCulloch, 1995). There are limited data available regarding the origin of the GF, but it has been suggested that these cells derive mainly from perifollicular mesenchyme in the dental follicle and there is some evidence to indicate that they may partly originate from the periodontal ligament itself (McCulloch and Bodin, 1991).

Many studies have supported not only the notion that gingival and periodontal fibroblasts are distinct, but there is a growing body of literature to suggest that each fibroblast population comprises subsets of cells, each with unique phenotype and function. Whilst the main function of GF is to maintain the integrity of the gingival connective tissue, in contrast the PDL cells have a more specialised function in that it is involved not only in the formation and maintenance of the PDL but is also responsible for the repair, remodelling and regeneration of the periodontium (Berkovitz et al., 1995). The evidence for the existence of heterogeneous fibroblast populations has been based on a
number of studies, including cell morphology, turnover, proliferative capacity and differential response to biomodulators. It has also been shown that the PDL cells have characteristics of osteoblasts and can synthesise osteonectin (Nohutco et al., 1996) bone gla protein (Nojima et al., 1990) and alkaline phosphatase (AP) (Kawase et al., 1988) at higher levels than the GF. Similarly, both GF and PDL cells respond differentially to various biomodulators such as parathyroid hormone (Piche et al., 1989), prostaglandin (Ogata et al., 1995), ECM proteins (Giannopoulou and Cimasoni, 1996) and various growth factors (Dennison et al., 1994). The studies of Lineback et al. (1983) showed that clones of PDL cells differed from each other not only in their total collagen synthesis but also in the type of collagen produced. Similarly, other work (Roberts et al., 1978; Rose et al., 1987) indicated subsets of PDL cell populations based on morphology. Several other studies have also reported phenotypic and functional differences within the GF population (Hakkinen and Larjava, 1992; Hou and Yager, 1993). Studies in animal models have also suggested functional differences between gingival and PDL derived fibroblasts. It was shown that when the roots were covered with PDL cells which had been cultured in vitro and then re-implanted in vivo, they acted as progenitor cells and gave rise to new PDL connective tissue (Van Dijk et al., 1991; Lang et al., 1995). In contrast, GF failed to produce new tissues (Boyko et al., 1981).

Although a number of strategies have been used to investigate fibroblast heterogeneity, most of these studies have been performed on a short-term bulk cultures which tend to be phenotypically unstable. In order to study fibroblast subpopulations it is important to isolate clonal cell lines. A cloned cell population is one derived from a single parental cell. One method of obtaining such clones is by limited dilution plating, where a heterogeneous culture of cells is plated at very low dilution, followed by clonal expansion. However, due to a limited life-span of primary cultures the cells lose their proliferative capacity after a few generations and therefore this approach is not feasible to obtain sufficient number of cells for a further study. This problem can be circumvented by immortalisation of primary cells using oncogenic proteins.
1.1.4 Immortalisation of mammalian cells

Normal mammalian cells in culture have a limited life-span before reaching a point at which growth is irreversibly arrested, a phenomenon known as senescence (Hayflick, 1965). Several factors can overcome this phenomenon and enable conversion of cells into an immortal phenotype. Although spontaneously immortalised mouse cells have been obtained in culture, normal human cells have rarely spontaneously immortalised in vitro (Gonos and Spandidos, 1992).

Immortalisation is a primary step in tumourogenesis and cancer development which can be achieved by the synergistic effect of oncogenes, growth factors and cellular genetic alterations (Spandidos, 1985; Hunter, 1991; Gonos and Spandidos, 1992). The precise mechanism of cell immortalisation is not yet fully understood, however, immortalisation of cells may not necessarily mean an increase in proliferative potential but rather decrease in the differentiated state. This has been supported by the observation that cell proliferative capacity decreases as cells differentiate and tumour arises because tumour cells are unable to differentiate (Harris, 1990). In most cases oncogenes seem to act by preventing the cells from reaching the differentiated state.

Several viral oncogenes are capable of immortalising primary cells and these include Adenovirus E1A, SV40, Polyoma large T-antigen and the E7 gene of the papilloma virus. Similarly, cellular oncogenes also have the capacity to establish continuous proliferation of primary cells in culture (Jat and Sharp, 1986; Kelekar and Cole, 1987). The DNA viral gene, SV40 T-antigen has been used to establish immortalised human fibroblasts. The expression of the SV40 T-antigen extended their proliferative capacity beyond the point of senescence (Stein, 1985). This oncogenic protein stimulates mitogenesis, eliminating the function of cell cycle-regulating tumour suppressor genes such as p53 and the retinoblastoma (Rb) gene (DeCaprio et al., 1988; Stamps et al., 1992) and thereby allowing the proliferation of normally growth-limited cells. These cells showed progressive loss of differentiated capacity due to chromosomal instability. This problem was overcome by the use of a
replication defective recombinant amphotropic retroviral vector system containing the SV40 T-Ag gene. However, many phenotypic characteristics as well as functional activities of cells are not manifest until after they have undergone differentiation from proliferating precursor cells. Transduction with viral construct encoding a temperature-sensitive T (tsT) has therefore been used to obtain long-term cultures in which cells continue to divide at low (permissive, 34°C) temperature but become arrested at a high (non-permissive, 39°C) temperature at which the T antigen is inactivated (Jat and Sharp, 1989). These cells remained stable during extensive passaging and cloning. Wright et al., (1989) a proposed two stage mechanism to explain immortalisation of human cells. In their model, senescence is described as mortality stage 1 (M1) and SV40 T antigen-induced crisis as mortality stage 2 (M2). Both stages are under the control of several genes and are caused by independent mechanisms. Introduction of T-antigen inactivates the M1 genes and extends the growth phase by 20-30 divisions until the cells enter stage M2. T-antigen binds to a number of cell cycle-regulating proteins including tumour suppressor genes such as p53 and the Rb gene (Fanning and Knippers, 1992). Binding of these proteins by T-antigen may play an important role in overcoming M1 functions since it has been shown that T-antigen mutants that are unable to bind these proteins are defective in immortalising cells. Initiation of M2 causes a phenomenon known as ‘crisis’, in which most cells die in culture. Only when the genes controlling the function of M2 are inactivated by mutation and cells escape from crisis is immortalisation achieved (Shay et al., 1991).

1.2 Periodontal diseases

The diseases affecting the periodontium can be classified broadly into two groups. The first group include inherited diseases that arise from defects in the gene structure of proteins such as those caused by mutations and deletions. These inherited diseases include connective tissue and metabolic disorders as well as defects in the immune cell function which affect a broad range of tissues including the periodontium. Conditions affecting the matrix proteins include the Ehlers-Danos syndrome caused by mutations in the
collagen genes (Uitto et al., 1986). Various forms of this syndrome cause cementum defect as well as periodontal destruction. Hypophosphatasia is a group of diseases characterised by reduced levels of serum alkaline phosphatase which cause premature loss of deciduous teeth in children (Beumer et al., 1973). Defects in the immune cell functions such as Chediak-Higashi syndrome, leukocyte adhesion deficiency (LAD) and chronic granulomatous disease (CGD) lead to inadequate immune response in which periodontal destruction is a common feature. Chediak-Higashi syndrome is a rare autosomal recessive genetic disease resulting in impaired killing of certain microorganisms leading to severe gingivitis, extensive loss of alveolar bone and premature loss of tooth (Temple et al., 1972). CGD is also a rare disease, which has both autosomal and X-linked genetic forms. The defect in this disease is due to inability of cells to produce the enzyme NADPH oxidase resulting in no oxidative killing capacity (Smith and Curnette, 1991). LAD syndrome results in deficiency of cell surface receptors on granulocytes, monocytes and lymphocytes which are involved in cell adhesion activities. As a consequence, individuals exhibit a variety of disorders including severe chronic periodontitis (Waldrop et al., 1987). Gingival overgrowth which has a genetic component includes idiopathic or familial hereditary hyperplasia.

The second group of diseases are acquired and include chronic inflammatory periodontitis and drug-induced gingival overgrowth. Chronic inflammatory periodontal diseases (CIPDs) are induced by cytokines and other inflammatory mediators in response to dental plaque and are characterised by soft connective tissue degradation and bone damage. In contrast, gingival hyperplasia (GH) involves the enlargement of gingival epithelial as well as connective tissue and excessive ECM accumulation. Although GH is a common complication in patients who take drugs such as the immunosuppressive cyclosporin, the anti-epileptic drug phenytoin and calcium channel-blocking agent nifedipine, it may also represent an exaggerated phase of growth during regenerative process following CIPDs.
1.2.1 **Periodontal breakdown: chronic inflammatory periodontal disease**

The CIPDs are the most common of all the inflammatory lesions affecting the human adult population (Loe, 1993). The CIPDs have been divided into two main groups, gingivitis and periodontitis (Ranney, 1993). It is generally accepted that the initial, early and established lesions refer to the onset and development of gingivitis. It is recognised that the established lesion is very often stable and never progresses and develops into periodontitis. However, in a susceptible host the continuing accumulation of subgingival plaque will cause the lesion to deteriorate. This will result in chronic periodontitis involving the deeper tissues of the periodontium leading to loss of periodontal ligament and the alveolar bone and ultimately tooth loss.

One of the first consequences of bacterial challenge is the inflammatory process triggered by activation of the local host response and the subsequent release of various mediators. These mediators including cytokines are released by the infiltrating inflammatory cells, mainly polymorphonucleocytes, macrophages and lymphocytes, as well as by connective tissue cells including fibroblasts. Although microbial components initiate an inflammatory response, their continued presence is required for the progression of periodontal pathology (Page, 1991). The release of certain cytokines and growth factors from the inflammatory cells and resident fibroblasts play a vital role in tissue homeostasis. Tissue homeostasis represents a delicate balance between anabolic and catabolic activities.

### 1.2.1.1 The role of cytokines in CIPDs

Cytokines are multifunctional polypeptides and glycoproteins produced by both lymphoid and non-lymphoid cells that act locally or systemically by binding to their respective receptors. They play a central role in a broad range of biological activities, including the regulation of immunity, inflammation, tissue remodelling and embryonic development. Individual cytokines are capable of stimulating the production of other cytokines generating a complex cytokine network which elicits many different cell responses (Duff, 1994). Although the functions of fibroblasts are regulated by various cytokines, fibroblasts also
produce cytokines themselves. This indicates that fibroblasts can also be included in the cytokine network and may play an important role in local inflammatory responses. Under normal conditions the production of cytokines is transient and strictly controlled, uncontrolled production of cytokines gives rise to a myriad of pathological conditions. Many cytokines have been identified (Henderson and Poole, 1994), however, not all are relevant to pathogenesis of periodontal diseases but there is considerable evidence to suggest that the pro-inflammatory cytokines IL-1 and IL-6 are involved in periodontal pathogenic processes (Page, 1991; Birkedaal-Hansen, 1993).

1.2.1.1 Interleukin-1

IL-1 is predominantly synthesised by macrophages and monocytes but also produced by various normal and neoplastic cells (Durum et al., 1990). Two distinct polypeptides exist (IL-1α, IL-1β) and they are the products of distinct genes (Lomedico et al., 1986). They bind to the same receptor, which is found on many cell types at varying densities, but gingival fibroblasts have higher levels than other fibroblasts (Okada and Murakami, 1998). IL-1α and IL-1β share only 27% homology but have similar biological activities which include lymphocyte and macrophage activation, leukocyte infiltration, prostaglandin synthesis, fever induction, acute phase protein release and tissue destruction (Durum et al., 1990; Dinarello, 1994). The activities of these molecules can be suppressed by a natural antagonist protein (IL-1RA) which functions by blocking the binding of IL-1 to the receptor. Both forms of IL-1 are produced as precursors which are then processed to the mature form by a unique enzyme, pro-IL-1 converting enzyme (ICE) (Mitzutani et al., 1991). Under normal conditions IL-1 has its main effect on connective tissue cells to stimulate the synthesis of ECM, however, the local excessive production of the cytokine seems to induce the production of other cytokines, prostaglandins and matrix-degrading enzymes that may be responsible for connective tissue destruction. There is a large body of evidence implicating IL-1β in the pathogenesis of bone diseases and periodontitis (Kornman et al., 1997). A number of studies have demonstrated increased levels of IL-1β in gingival crevicular fluid (GCF) from
patients with gingivitis, early onset and adult periodontitis (Honig et al., 1989; Hou et al., 1995). Similarly other authors have reported higher levels of IL-1β messenger RNA in chronic periodontitis tissues compared with normal tissues (Tokoro et al., 1996).

1.2.1.1.2 Interleukin-6

IL-6 is a multifunctional cytokine synthesised by many cells including T and B cells, monocytes, endothelial cells and fibroblasts (Kishimoto, 1989). Although it may be produced by most cells it can be induced in certain cell types by other cytokines and it has been implicated as a mediator of the effects of IL-1. Its expression can be controlled by negative regulators such as glucocorticoids and retinoic acid. Unlike IL-1, it is a glycoprotein with varying molecular size due to post-transcriptional modification. IL-6 has a broad spectrum of biological activities and plays a major role in B-cell differentiation but is also a prominent mediator of inflammation and tissue injury (Ishimi et al., 1990; Hughes and Howell, 1993). IL-6 has also been shown to affect bone resorption by its ability to stimulate the recruitment as well as the formation of osteoclasts. This cytokine is also involved in the pathogenesis of a number of autoimmune disorders, such as rheumatoid arthritis and psoriasis (Houssiau et al., 1988; Grossman et al., 1989; Horii et al., 1989) since elevated levels of serum IL-6 have been detected in these conditions. Although IL-6 has been shown to be produced by both normal and inflamed GF (Bartold et al., 1991; Takahashi et al., 1994), the level of this cytokine in GCF has been correlated with adult periodontitis (Gemmell and Seymour, 1993; Reinhardt et al., 1993; Geivelis et al., 1993). However, serum levels in patients with CIPDs are not elevated (Takahashi et al., 1994). Furthermore, the work of Dongari-Bagtzoglou and Ebersole (1998), using flow cytometry (FCM), suggested that the increased cytokine synthesis by GF in adult periodontitis may be due to a higher cytokine synthetic capacity of sub-sets of cells. In contrast to IL-1, IL-6 may counteract the catabolic effect of IL-1 by enhanced production of tissue inhibitors of metalloproteinases (TIMPs) (Irwin and Myrillas, 1998). A number of studies have also shown that increased IL-6 expression may be involved in
other oral pathologies including denture induced fibromatosis (Nakao et al., 1995), oral submucous fibrosis (Haque et al., 1998). In addition, this cytokine has been shown to positively affect the growth and metabolism of connective tissue cells such as fibroblasts and thus has been suggested to play a pathogenic role in fibrotic diseases including gingival hyperplasia (Williamson et al., 1994; Morton and Dongari-Bagtzoglou, 1999). However, the precise function and regulation of IL-6 in periodontal tissues in health and disease is still not clear.

1.2.2 Gingival overgrowth: drug-induced gingival hyperplasia (GH)

Hyperplasia refers to an enlargement of tissue caused by an increase in number of cells. Gingival overgrowth may be acquired due to a fibrous and granulomatous inflammatory response associated with the use of certain systemic drugs. Although gingival inflammation is a feature of GH similar to CIPDs, however, whilst matrix components are degraded in CIPDs they accumulate in GH. Furthermore, in CIPD there is a mixture of destruction, repair and regeneration and the presence of hyperplasia may correspond to an exaggerated phase of repair. GH is a common, serious and recurrent side-effect associated with the use of certain type of drugs (Brown et al., 1991; Seymour et al., 1996; Nishikawa et al., 1996). Although reported first in 1939 (Kimball, 1939) in patients being treated with phenytoin (PHT) for epilepsy, a number of other drugs subsequently have been identified as causing this lesion, including the immunosuppressive cyclosporin A (CsA) (Thomasson et al., 1993; O'Valle et al., 1994; Seymour et al., 1997) prescribed for patients who have undergone organ transplants to prevent rejection. Nifedipine (NIF), belonging to a family of calcium channel-blockers, has been extensively used in the treatment of cardiovascular conditions including hypertension, angina and cardiac arrhythmia is also implicated in GH (Nishikawa et al., 1991; Fujii et al., 1994; Ishida et al., 1995).
1.2.2.1 Histopathology

Although all three types of drugs have different pharmacological properties, they nevertheless all elicit a similar clinical gingival appearance and histologically they are almost indistinguishable. Generally, GH occurs within three months of the start of drug therapy and affects predominantly the buccal and interdental gingiva of the upper and lower anterior teeth (Thomasson et al., 1996) and often regressing after discontinuation of the drug. Several studies have shown that one of the features of GH is enhanced proliferation of normal fibroblasts, which show well-developed RER characteristic of protein synthesis and secretion, the cells resembling myofibroblasts (Yamasaki et al., 1987). The cells showed an increased connective tissue components of the ECM but quantitative analysis of different ECM proteins showed differences using histocytochemical analysis, with type I and type III collagens, and FN showing strong expression in the NIF group than in other periodontal pathologies (Bonnaure-Mallet et al., 1995; Seymour et al., 1996). The epithelium of the enlarged gingiva tends to be parakeratinised and there is usually marked epithelial down-growths into the underlying connective tissue, with elongated rete pegs. There is also considerable evidence to indicate that plaque and gingival inflammation influence the initiation and progression of GH (Thomason et al., 1993). Inflammatory cells are present in the connective tissue, mainly plasma cells and lymphocytes, the amount depending on the extent of inflammation. Clinically, these lesions may become so extensive that the teeth are displaced and/or their crowns covered with overgrown gingival tissue. Despite extensive studies, the pathogenesis of GH is uncertain and treatment is mainly confined to surgical removal of the overgrown tissue and to the maintenance of adequate oral hygiene.

A number of factors influence the occurrence and severity of the disease. Several clinical studies using PHT and CsA have suggested that children and adolescents are more prone to GH than adults, but there are no data available on the use of calcium-channel blockers in children as this group of drugs is used only for treating the adult population (Seymour et al., 1996). However, animal studies have supported an age-related effect of calcium-
channel blockers (Nishikawa et al., 1996). Epidemiological studies have shown that not all patients receiving these drugs develop GH. The prevalence rate for patients treated with PHT is approximately 50%, whilst those medicated with NIF and CsA show incidences of 30% and 20% respectively (Seymour et al., 1987; Barclay et al., 1992). The drugs appear to have a synergistic effect on gingival tissue when used in combination (Thomasson et al., 1993). The terms ‘responders’ and ‘non-responders’ have been proposed to describe patients who are either susceptible or not susceptible to the drugs (Modeer et al., 1990).

1.2.2.2 Possible mechanisms of GH

Many hypotheses have been proposed to explain GH. It has been suggested that functional heterogeneity exist in GF since these cells are known to show a markedly heterogeneous response to various stimuli (Cockey et al., 1989). Studies with GF obtained from monozygous and dizygous twin pairs also indicated genetic variation in terms of proliferation rates and protein synthesis (Cockey et al., 1987). Cells from dizygotic twins showed greater variation than those from monozygotic twins in response to PHT, further confirming the influence of genetic factors in the pathogenesis of GH. A common feature of most of the drugs that cause GH is that they are calcium (Ca++) antagonists. Calcium is essential in many of the biological processes and influx of Ca++ leads to activation of a cascade of biochemical reactions including a phenomenon known as apoptosis. This is a form of programmed cell death that occurs under numerous developmental and physiological conditions that require elimination of cells from tissues and organs. It is a mechanism that plays an important role in the control of tissue overgrowth by eliminating unnecessary cells in a controlled manner without inducing an inflammatory response. Therefore, one possible mechanism of GH could be the abrogation of the apoptic programme as result of the blockage of Ca++ influx (McConkey and Orrenius, 1977).

Many studies have demonstrated an increased synthesis of ECM proteins in GH but these are degraded during CIPD. This increase in ECM may be due to either the matrix overproduction or to reduction in the degradation of
ECM. Degradation is a highly regulated process, however, it can become excessive during inflammation, and may be impaired in hyperplasia. Several metabolic pathways are associated with matrix changes. Degradation of matrix by a family of enzymes called metalloproteinases (MMPs) is one pathway involved in remodelling of ECM (Birkedal-Hansen, 1993). These enzymes are expressed at low levels in normal adult tissues but are up-regulated in normal and pathological remodelling processes such as embryonic development, tissue repair, inflammation and metastasis. They degrade all known ECM proteins and have overlapping substrate specificities. Their activity is further regulated by tissue inhibitors of metalloproteinases (TIMPs) which bind to the enzymes forming non-covalent complexes (Birkedal-Hansen, 1993). Both PTH and CsA have been shown to affect collagenase (MMP-1) and TIMP activity (Hassell, 1982; Tipton et al., 1991).

As mentioned previously, plaque-induced inflammation may be an aggravating factor in GH suggesting that cytokines may have a role in the progression of the disease. However, the significance of these cytokines remains to be determined. Whilst several cytokines including IL-1β and IL-6 have been shown to play a significant role in CIPD, very few studies have examined these factors in GH. The effects of IL-1 on androgen metabolism have been investigated in gingival tissues and the cytokine caused increased conversion of androgens to the active metabolite dihydrotestosterone (DHT) (Kasasa and Soory, 1995). This finding may be relevant in GH as DHT has been shown to affect matrix synthesis in connective tissues. However, Iacopino et al. (1997) examined the effects of CsA and PHT on IL-1 expression in macrophages, both in vitro and in vivo, and found no significant up-regulation of the cytokine at the mRNA and protein levels. This indicated that, although IL-1 is up-regulated in inflammatory conditions causing tissue destruction, it may not be directly associated with GH. Similarly, Williamson et al. (1994) analysed IL-6 expression, by in situ hybridisation and radioimmunoassay, in gingival tissue from CsA-induced GH patients and compared it with normal tissue. These workers showed an increase in IL-6 expression, at both the mRNA and protein levels, in GH compared to the normal tissue. This result is in contrast to a
report by Yashimura et al. (1991) which showed that CsA significantly reduced serum IL-6 suggesting that the drug may inhibit IL-6 production in peripheral mononuclear cells whereas it may stimulate local synthesis of IL-6 by GF.

1.3 Sex hormones and periodontal diseases

Hormones have considerable effects on body physiology and a number of studies have suggested that sex hormones, particularly in women experiencing hormone changes, may have an important role in inflammatory periodontal disease (Amar and Chung, 1994). It has been observed that gingival inflammation and hyperplasia are frequent during puberty, pregnancy and menstruation (Löe, 1965; Hugoson, 1970; Nyman, 1971; Sutcliffe; Mombelli et al., 1989) and the former is also commonly seen in women taking oral contraceptives or receiving hormone replacement therapy (Kalkwarf, 1978; Pankhurst et al., 1981). The production of female sex hormones changes drastically at menopause as a result of decreasing ovarian function. A pronounced decrease in oestrogen is thought to be responsible for the development of osteoporosis in such women. Although Groen et al. (1968) found that the incidence of periodontitis correlated with signs of generalised osteoporosis, Kribbs et al. (1989) could not find a positive cause and effect relationship between tooth loss from periodontitis and osteoporosis. El Attar and Hugoson (1974) showed the formation of the biologically more active metabolite of oestrogen, 17β-estradiol, in human gingival tissues and also increased oestrogen metabolism in inflamed gingiva. In addition, androgens have been found to influence the activity of gingival tissue. Ziskin (1941) studied the effects of androgens on gingiva in rhesus monkeys and humans and found that the gingival connective tissues showed evidence of hyperplasia. There is evidence to suggest that males are more susceptible than females to GH (Ishida et al., 1995; Nishikawa et al., 1996). It has been reported that the minimum drug concentration required is lower in males than females (Barak et al., 1987; Ishida et al., 1995). Metabolic studies have suggested that gingiva might be a target tissue for androgens since numerous publications have demonstrated that these hormones are metabolised into their biologically active
form, DHT, in gingival tissues (Vittek et al., 1979; Ojanotko et al., 1980). Furthermore, several studies have shown that the levels of DHT in fibroblasts derived from GH patients were considerably higher than in control patients (Soory et al., 1988; 1990). Animal model studies also suggest gender differences in susceptibility to GH. Nyska et al. (1994) studied the degree of GH induced by the calcium channel-blocker oxodipine in a group of castrated male dogs and compared it with normal dogs. They observed that the histology in the castrated dogs was similar to that of normal gingiva. Similarly, Nishikawa et al. (1996) reported that male rats were more susceptible than females to NIF-induced gingival overgrowth. Recent study by Dayan et al. (1998) using normal and castrated beagle dogs further confirmed the role of testosterone in GH induced by calcium channel antagonist, oxodipine. They showed that there was a good correlation between of serum levels of testosterone and GH in normal dogs treated with the drug. However the castrated dogs did not develop GH and, furthermore, when the castrated dogs were treated with testosterone and the drug the same dogs developed GH.

1.3.1 Sex hormones and cytokines

A number of studies have suggested that sex hormones modulate the expression of cytokines, which in turn may influence the pathogenesis of periodontal diseases. Pacifici et al. (1991) have shown that peripheral blood mononuclear cells from recently oophorectomised women demonstrated elevated production of IL-1, which was decreased by oestrogen therapy. Two other studies, (Payne et al., 1993; Reinhardt et al., 1994), have also shown elevated IL-1 levels in GCF of oestrogen-deficient patients. IL-1 and IL-6 are known to play an important role in a number of other diseases. IL-6 has been shown to be important in the pathogenesis of autoimmune diseases including rheumatoid arthritis (RA) (Houssiau et al., 1988). Furthermore, the incidence and the development of the disease is sex associated, with females more susceptible than males. Moreover testosterone therapy of male RA patients, who tend to have lower testosterone levels than normal males, alleviates several aspects of disease pathology (Ansar et al., 1988). Other studies have
shown that androgen deficiency in male hypogonadism and in castrated males, and oestrogen deficiency in females are associated with bone loss, a process in which IL-6 has been reported to play a central role (Manolagas et al., 1995). IL-6 deficient mice have been shown to be protected from any change in either bone mass or bone remodelling caused by oestrogen deficiency (Poli et al., 1994). Thus, IL-6 appears to be a potent osteotropic factor that may play an important role in conditions associated with increased bone resorption.

1.3.2 Sex hormones and growth factors in relation to hyperplasia

A number of studies have implicated sex steroids, particularly androgens, in the development and progression of hyperplasias and their effects have been shown to be mediated through modulation of growth factors and/or their receptors. Androgen is known to modulate epidermal growth factor receptor (EGF-R) and insulin-like growth factor receptor (IGF-R) expression in androgen sensitive human prostate hyperplastic cell lines (Fiorelli et al., 1991; Xin-Hua et al., 1993; Ashton et al., 1995). There is also an evidence to suggest that EGF and sex hormone receptors may be involved in normal and neoplastic growth of gastrointestinal mucosa (Polimeno et al., 1994). The study of Sciarra (1995) also showed up-regulation of EGF in prostatic cells in response to androgen treatment, whereas the anti-androgenic drug flutamide, decreased production of the growth factor. Using similar cell culture studies, Levine et al. (1992) demonstrated up-regulation of EGF and fibroblast growth factor (FGF) when cells were treated with DHT. A mammary carcinoma cell line MCF7, known to express all the steroid hormone receptors, has been extensively used to examine the mechanism of steroid hormone-induced cell growth (Lippman and Dickson, 1989). These studies have shown that both oestrogens and androgens modulate the synthesis of various growth factors including transforming growth factor-β (TGF-β), IGF and FGF. In addition, these studies also showed that the sex steroids had effects on growth factor receptor expression, particularly EGF and FGF receptors. Oestrogen receptors have been shown to be present in osteoblast-like cells and oestrogens have been shown to increase the production of IGF and TGF-β in these cells (Ernst...
et. al., 1989; Hering et al., 1995). It is therefore clear that growth factor and growth factor receptor expression can be altered by steroid hormones, and that sex steroids may exert their growth stimulating effects on cells through an autocrine or paracrine mechanism involving growth factor/growth factor receptor expression pathways. Thus, it is possible that the capacity of growth factors to stimulate wound healing, regeneration and GH could be enhanced by steroid hormones.

1.3.3 Sex hormone receptors

The sex hormones exert their influence by binding to specific intracellular receptors which belong to a superfamily of ligand-activated transcription factors that regulate cell growth, differentiation and development (Evans, 1988; Beato, 1989; Brinkman, 1994; Zhou et al., 1994). The receptors are zinc finger proteins sharing a highly conserved DNA binding domain which interact with hormone response element (HRE) and modulate the transcription of target genes (Fig 1.2). HRE sequences have been identified in the promoters of many genes, including adhesion molecules, cytokines, growth factors, degradative enzymes and the ECM components (Cavailles et al., 1988; Weisz et al., 1990; Freeman et al., 1991; Fox et al., 1991). The formation and activity of sex hormone-receptor complexes thereby play an important part in cell interactions, inflammation and connective tissue turnover. Thus, while it is generally considered that the main targets of oestrogen and testosterone are sex-associated tissues, they are also capable of influencing gene activities in the cells of many other tissues (Yron et al., 1979; Fujimoto et al., 1994; Miyake et al., 1994; Morales et al., 1995).
Fig 1.2 Schematic representation of hormone-receptor interaction in target cell. The hormone interacts with the receptor inducing conformational change which then binds to DNA sequence known as hormone response elements (HRE) and enhance transcriptional activity.

1.3.4 Sex hormone receptors in periodontal tissues

The potential responsiveness of cells to the action of sex hormones depends on the expression of their receptors. Several studies have attempted to demonstrate the presence of oestrogen and androgen receptors (ER and AR, respectively) in periodontal tissues. Formicola et al. (1970) injected radioactive oestradiol into female rats and observed that the labelled hormones were retained not only in the genital tract but also in the gingiva. This suggested that the gingival tissue contained some type of binding sites for oestrogens. However, the results of experiments using ligand-binding analysis (Southren et al., 1978) and autoradiography (Vittek et al., 1982; Hernandez et al., 1981; Aufdemorte and Sheridan, 1981) have been equivocal, as have more recent investigations using immunocytochemistry (Ojanotko-Harri et al., 1992; Forabosco et al., 1992). Using ligand-binding method to measure the receptor content of human and rabbit gingiva, Southren et al. (1978) demonstrated the
presence of both the AR and the ER. Other authors attempted to detect these receptors by autoradiography (Vittek et al., 1982; Hernandez et al., 1981; Aufdemorte and Sheridan, 1981), but non-specific labelling impeded interpretation of the results. Ojanotko-Harri et al. (1992) detected the AR but were unable to detect any ER in the gingival tissues, while Forabosco et al. (1992) were able to demonstrate the presence of the ER in some of the gingival tissues by immunohistochemistry.

1.4 Periodontal healing, repair and regeneration

Wound healing following an acute tissue injury involves a complex, highly regulated and co-ordinated process of cellular and biochemical interactions that lead to the formation of new tissue components (Clark and Henson, 1988). After an acute injury, the disruption of the wound vasculature leads to fibrin formation and platelet aggregation. Activated platelets at the wound site release several growth factors involved in tissue growth, repair and regeneration including PDGF, TGF-β, FGF, IGF and EGF. In addition, the cells adjacent to the injured site also are induced to release growth factors within a few hours after injury. Following tissue injury, neutrophils accumulate with subsequent migration of macrophages into the wound site several days later and provide another source of numerous growth factors. The release of soluble growth factors from these cells initiate orderly migration, proliferation and differentiation of mesenchymal cells in the wound and subsequent synthesis of a wide variety of ECM proteins including fibronectin and collagen (Kiristy and Lynch, 1993). Due to the chronic nature of CIPD the amount of growth factors may be limited in this lesion. Thus, the addition of appropriate growth factors may accelerate and enhance wound healing and regeneration. Several growth factors have been used topically, singly or in combination, in animal studies to promote periodontal regeneration (Graves and Cochran, 1994). Most forms of hyperplasia seem to be associated with excessive growth factor stimuli. TGF-β and bFGF have been implicated in a variety of fibrotic and hyperplastic diseases. For example, TGF-β is involved in pulmonary fibrosis (Finkelstein et al., 1994), hepatic fibrosis (Annoni et al., 1992) and
TGF-β and bFGF have been associated with thyroid hyperplasia (Logan et al., 1994; Becks et al., 1994). It is thus possible that the events occurring during fibrosis are similar to those in normal wound healing and regeneration but are greatly exaggerated. Thus, the unregulated secretion of growth factors could account for the hyperplastic cellular response.

1.4.1 Growth factors and their receptors

Growth factors are naturally occurring molecules that have the potential to initiate and mediate many of the complex biological responses associated with inflammation and wound healing (Clark and Henson, 1988). Growth factors when first characterised were thought to have growth stimulatory activity, but it is now recognised that they are multifunctional molecules and have both stimulatory and inhibitory properties. Growth factors either have an autocrine, where the cells that produce them are also affected by them, or paracrine action in which case the production of a growth factor by one cell type affects the function of a different cell type. These factors exert their effects by binding to specific trans-membrane receptors on target cells which then generate a cascade of intracellular signals which, for example, stimulate chemotaxis, cell proliferation, differentiation and production of ECM. As these activities relate to healing, repair and regeneration, they are essential processes for the maintenance of the periodontium. The nature and the role of some of these growth factors are discussed below.

1.4.1.1 Platelet derived growth factor

PDGF, produced in response to vascular damage, is a potent mitogen for cells of mesenchyme origin (Westermark, 1990). It derives from two different polypeptides encoded by two distinct but related gene products, PDGF-A and PDGF-B. The active protein is formed by dimerisation of A-chain and B-chain polypeptides to produce three isoforms, AA, BB, and AB. These isoforms have unique binding affinities for the PDGF receptor subunits, α and β, found on cell membranes. Dimerisation of the receptor chains is also required for biological activity. The αα dimer of the receptor binds to PDGF-
AA, -AB and -BB, the αβ dimer to PDGF-AB, -BB and ββ only to PDGF-BB. Thus the capacity of a cell to respond to these growth factors will be determined by the expression of receptor phenotypes. PDGF has a variety of effects on PDL cells including chemotaxis, proliferation and matrix synthesis (Cho et al., 1995). It also enhances cell migration into the healing wound area. PDGF has been used in a number of in vivo periodontal regeneration studies either alone or in combination with other factors with varying degrees of success (Lynch, 1994).

1.4.1.2 Transforming growth factors-β

TGF-βs belong to a superfamily of proteins that regulate cell growth, differentiation, chemotaxis and ECM formation (Balkwill, 1993). Five different genes have been identified that encode the TGF-β polypeptides. TGF-βs are secreted as large inactive latent molecules consisting of two homodimeric polypeptides held together by covalent bonds that require activation. Only the activated dimers bind to receptors. Six receptors (TGF-βRI- TGF-βRVI) have been identified for these proteins which also constitute a superfamily but only the first three have been extensively investigated. There is a high degree of homology between the receptors but they bind the different isoforms with differential affinities. TGF-β has been shown to have variable effects on cells. It can either stimulate or inhibit proliferation of different cell types, or of the same cell type, depending upon the in vitro conditions (Centrella et al., 1987; Wrana et al., 1988; Keski-Oja et al., 1988). In addition, it decreases the synthesis of MMPs and therefore results in a decrease in connective tissue matrix destruction (Alvares et al., 1995). TGF-β has also been shown to regulate both PDGF-A and -B chain formation as well as PDGF receptor expression (Oates et al., 1995).

1.4.1.3 Epidermal growth factor

EGF was first purified from mouse submaxillary glands. This tissue contains the highest concentrations of EGF detected in any tissue, and can induce premature eyelid opening in newborn mice and accelerating incisor
tooth eruption (Carpenter and Wahl, 1990). EGF is a polypeptide consisting of 53 amino acids with three intra-chain disulphide bonds essential for biological activity. It is synthesised as a large precursor molecule of 1207 amino acids from which the amino terminal domain is cleaved by proteases. Many other proteins contain regions of homology to EGF including coagulation factors, plasminogen activator, fibronectin and a related protein (EGF-α) identified in a variety of virus-transformed cells. Both EGF and EGF-α exert their biological effects by binding to the EGF receptor. The receptor is a glycoprotein found on a variety of cells (Cho et al., 1991). It has intrinsic tyrosine kinase activity and has the capacity to phosphorylate itself as well as other substrates. EGF can stimulate epithelial, endothelial and mesenchymal cells and promote epithelial keratinisation. It is mitogenic for PDL cells and has growth stimulating activity for gingival cells in vitro (Matsuda, 1992 and 1993). EGF receptor has been shown to be localised on rat PDL cells during differentiation and the receptor turnover has been known to be increased in human gingival fibroblasts when treated with PHT (Modeer et al., 1990).

1.4.1.4 Fibroblast growth factor

FGFs were initially isolated from neural tissue extracts, but since then have been detected in a large number of normal and neoplastic tissues and also found in bone matrix (Hefti, 1993). The first member of this class to be isolated had an isoelectric point (pl) of 9.6 and was given the name basic fibroblast growth factor (bFGF). A related protein was later isolated with an acidic pl and termed acidic fibroblast growth factor (aFGF). Each is a single polypeptide chain of 140 amino acids and share 55% sequence homology. FGF receptors have been identified on a variety of cells and both growth factors bind to the same receptor (Hefti, 1993). Both FGFs have been shown to be involved in the proliferation and differentiation of a wide variety of cells including the PDL cells (Terranova, 1989; Blom, 1994). Although FGFs have no effect on mature osteoblasts, these growth factors are potent stimulators of endothelial cell migration and proliferation which is critical for the
vascularisation of bone (Takayama, 1997) and thus may promote hard tissue regeneration.

1.4.1.5 Insulin-like growth factor

IGFs are growth-promoting proteins that share extensive homology with proinsulin (Humble, 1990). There are two different IGFs (IGF-1, IGF-2) which are independently regulated, IGF-1 has about 40% homology to insulin and about 60% homology to IGF-2. Both are found in large amounts in bone. IGFs are produced by osteoblasts and stimulate bone formation by inducing cell proliferation, differentiation and ECM synthesis, particularly they enhance type I collagen synthesis. In addition to its ability to stimulate collagen synthesis, IGF-1 decreases the rate of collagen degradation. Although both factors have similar effects, a number of studies suggest that IGF-1 is the most potent. IGF-1 has been shown to be chemotactic for PDL cells increasing proliferation and protein synthesis (Blom et al., 1992). IGF-1 binding studies have shown the presence of the receptors on the PDL cell surface. The effects of IGF-1 on PDL cells in combination with other growth factors such as FGF, PDGF and TGF-β are synergistic.

1.4.2 The role of growth factors and their receptors in GH

It has been speculated that a variety of growth factors and their receptors may be involved in the pathogenesis of GH. Modeer et al (1990) investigated the effects of PHT on the EGF receptor and found that EGF receptor metabolism was down-regulated in responder fibroblasts and up-regulated in nonresponder fibroblasts and that EGF caused stimulation of DNA synthesis. The association of GH with growth factors has been studied both in vitro and in vivo. Dill et al (1993) investigated the effects of PHT in vitro on PDGF-BB expression in macrophages and demonstrated that growth factor gene expression was up-regulated. In another study the expression of the same growth factor was measured, both at the mRNA and protein level using CsA-induced gingival hyperplastic tissue, again showing up-regulation of PDGF (Nares, et al., 1996). Studies of Plemons et al. (1996) in gingival
hyperplastic human tissues confirmed the findings of Nares et al. (1996) on the CsA-induced up-regulation of PDGF-BB and extended the study to identify macrophages as the source of the growth factor. Moreover, in vitro and in vivo studies on gene expression of PDGF-BB producing cells in normal gingiva and both PTH and CsA-induced GH (Iacopino et al., 1996) also supported the idea that enhanced PDGF-BB expression in macrophage is associated with GH. Cell culture studies using rat macrophages and human blood monocytes (Dill et al., 1997) showed that PHT increased the expression of the gene for PDGF-BB. These studies demonstrated that macrophages are probably the predominant inflammatory cell type present in GH connective tissue and therefore may be the major source of growth factors. TGF-β and bFGF have also been implicated in GH. Saito et al., (1996) demonstrated that in drug-induced gingival overgrowth connective tissues, there were a greater number of cells positive for TGF-β and bFGF and their receptors. Similarly, a recent study by Sasaki et al. (1998) indicated that bFGF may be one of the factors involved in PHT-induced GH, since they observed a significant rise in serum FGF level in the drug treated patients. As described previously, macrophages and monocytes play a key role in connective tissue turnover through the release of anabolic growth factors such as PDGF, TGF-β and FGF. These growth factors are potent chemotactants for fibroblasts and increase fibroblastic proliferation as well as stimulate the production of ECM components, which are the characteristic abnormalities of GH. Thus, unregulated hyperplasia may be associated with the abnormal secretion of growth as a result of drug therapy.

1.5 Extracellular matrix

As mentioned earlier, fibroblasts produce a variety of macromolecules, collectively called matrix proteins, which are secreted, and deposited in the vicinity of the cells to form an organised structure referred to as the ECM (Mariotti. 1993). The secreted molecules include structural proteins such as collagens, and glycoproteins including fibronectin, osteonectin, osteopontin and osteocalcin. These molecules play important roles in cell-cell, cell-matrix interactions and act as repositories for growth factors, hormones and other
bioactive molecules, thereby having a significant role not only in embryonic development but also in the maintenance of periodontal tissue organisation, repair and regeneration (Aukhil, 1992; Amar and Chung, 1994). The ECM molecules interact with the cell via specific receptors known as integrins. Wound repair and regeneration comprise a complex process involving a number of steps including cell migration, proliferation and matrix synthesis. The initial response to tissue injury involves the infiltration of inflammatory cells to form a fibrin clot at the site of the wound, followed by provisional matrix synthesis. The newly formed matrix contributes significantly to recruitment of fibroblasts and provides signals to bring about the new phase of formation and organisation of fully functional matrix (McCarthy and Turley, 1993). The composition and organisation of ECM in turn affects cell function, primarily due the ability of the matrix to concentrate, stabilise and enhance the interaction of growth factors and the cell, thereby modulating its function (Flaumenhaft and Rifkin, 1991). The ECM molecules themselves can act as growth signals. For example, fibronectin can induce DNA synthesis while collagen may provide signals to terminate proliferation. A number of studies have suggested that some of the ECM proteins exist in several different isoforms including fibronectin (FN), one of the most widely distributed and perhaps the best characterised of the isomeric ECM proteins because of its role in embryonic development, wound healing, regeneration and normal tissue turnover. Many of the growth factors reviewed are regulators of the formation and destruction of ECM. These growth factors control the expression of either the genes for matrix themselves, for the α- and β-chains of integrins, as well as the expression of certain key enzymes which regulate the degradation of matrix molecules. Therefore, the production and maintenance of the ECM components are critical for the overall physiological well-being of the periodontium. The nature and function of some of these molecules are discussed below.
1.5.1 Collagen

The most abundant ECM component of the periodontium is collagen. It is composed of three distinct polypeptide chains, called α chains, which intertwine with each other to form the triple helical and the globular domains of the collagen molecule. The collagen triple helices have a characteristic repetitive amino acid sequences (Gly-X-Y), where X is often proline and Y hydroxyproline. This sequence is required for the formation and stability of the protein (Van der Rest and Garrone, 1991). There are many genes which code for at least 14 different collagen molecules, of which type I and III are the commonest in PDL and gingival connective tissue, whereas types IV, V and VI represent minor forms (Prockop et al., 1984; Becker et al., 1993; Bartold et al., 1995). Type IV is found mainly in the gingival epithelium, and type V in the matrix of the lamina propria (Romanos et al., 1993). Collagens may be classified into two main groups, fibrillar and non-fibrillar, and types I and III belong to the fibrillar group. These molecules provide the framework and strength needed for tissue structural stability. Collagen molecules are synthesised as propeptides (procollagens) that undergo further modifications both intra- and extracellularly. Large numbers of enzymatic steps are involved and deficiencies in some of these enzymes cause collagen disorders of the ECM (Uitto et al., 1986). Gingival fibroblast culture studies have been utilised to investigate the effects of both PHT and NIF on collagen gene expression (Salo et al., 1990; Shikata et al., 1993). These studies found that both drugs altered differently the expression of type I and type IV collagen genes, decreasing the level of mRNA for type I but enhancing the expression of type IV collagen. Schincaglia et al. (1992) showed that CsA caused increased collagen synthesis, but not DNA synthesis in cultured fibroblasts. The CsA-induced changes were related to a rise in both type I collagen mRNA and protein levels.

1.5.2 Fibronectin

Fibronectin (FN) is a high molecular weight glycoprotein produced by several cell types and with wide tissue distribution. It is present in high
concentration in plasma and is a major component of the ECM (Ruoslahti, 1988; Hynes, 1990). FN plays a vital part in mediating cell-matrix interactions via β1 integrins of the family of very late activation (VLA) antigens expressed by many cells (Hynes, 1987; Wayner and Carter, 1987; Takada et al., 1988; Wayner et al., 1988). FN is expressed in gingival and the deeper periodontal tissues, in which it is considered to play a pivotal part in the cell-matrix interactions which take place during cell attachment and proliferation (Pitaru et al., 1995). FN is expressed strongly in the gingival propria (Romanos et al., 1993) and along the attachment sites of PDL collagen fibres to the cementum but not to alveolar bone (Matsuura et al., 1995). In a recent study Kuru et al. (1998) examined its expression in cultured cells derived from gingiva and PDL tissues by FCM and analysis of fluorescence profiles showed that, although the majority of cells expressed FN, the levels were substantially higher in PDL cells compared with gingival fibroblasts.

FN consists of two identical polypeptide chains joined by disulphide bonds (Fig. 5.1). Each chain is composed of multiple homologous amino acid repeating sequences, designated types I, II and III. It has at least two major domains that mediate cell adhesion. One domain contains the Arginine-Glycine-Aspartate (RGD) amino acid sequence which recognises a variety of cells by binding to the integrin α5β1 (Petersen et al., 1983; Owens and Baralle, 1986; Rousslahti and Pierschbacher, 1986) and is located in one of the type III repeats. The second domain located in the IIICS region was identified by its ability to support melanoma adhesion and has the sequence Leucine-Aspartate-Valine (LDV) (Guan and Hynes, 1990). The receptor for this sequence is the α6β1 integrin family. FN also has direct binding domains for collagen, heparin and fibrin (Skorstengard et al., 1986; Owens and Baralle, 1986). The importance of FN in embryonic development was conclusively demonstrated by the use of 'knockout experiments' whereby a null mutation in the mouse FN gene had a lethal effect on its development (George et al., 1993). FN thereby has an important role not only in cell adhesion (Rousslahti and Piersbacher, 1986; Akiyama et al., 1986) but also in migration (Ali and Hynes, 1978; Critchley et al., 1979), cytoskeletal organisation and
differentiation (Guan et al., 1990), processes which are critical in embryonic development, wound healing and tumourogenesis (Ffrench-Constant and Hynes, 1988; 1989; Ffrench-Constant et al., 1989). These functions may underlie its role in periodontal wound repair and in the regeneration of tissue lost as a result of CIPD.

1.5.3 Osteocalcin

Osteocalcin (OC) is a noncollagenous vitamin K-dependent, calcium ion-binding protein, also called γ-carboxy-glutamic acid protein (bone-Gla-protein) (Hauschka et al., 1989). It is produced by osteoblasts, odontoblasts as well as fibroblasts and is found in bone matrix, dentine and other mineralised tissues. Although its precise function is unknown, it has many properties that suggest it plays an important role in calcified tissue metabolism (Cole and Hanley, 1991). Serum OC has been used as a biochemical marker that reflects bone turnover when resorption and formation are coupled and it is a specific marker of bone formation whenever formation and resorption are uncoupled (Van Daele, et al., 1994). Although osteoblasts synthesise a low basal level of OC, its synthesis is regulated by a number of factors including steroid hormones, particularly vitamin D₃, and growth factors (Cole and Hanley, 1991).

1.5.4 Osteonectin

Osteonectin (ON) is an acidic phosphoprotein rich in aspartic and glutamic acid residues and is also known as SPARC (secreted protein, acid and rich in cysteine) (Tracy and Mann, 1991). It is a single polypeptide chain comprising four main domains: an acidic region in the amino terminal end, a second cysteine-rich domain which forms extensive disulphide bonds, a hydrophilic third domain and a carboxy terminus domain involved in calcium binding (Engel et al., 1987). Although it is ubiquitously distributed, expressed by a variety of cells including fibroblasts, chondrocytes, platelets, endothelial and epithelial cells, ON is more abundant in bone (Tracey and Mann, 1991). It is expressed in PDL, being prominent around Sharpey's fibres, at the attachment sites between the PDL and cementum, and the PDL and alveolar
bone (Matsura, 1995). Apart from binding to calcium and hydroxyapatite, ON also binds with type I collagen suggesting that it may be involved in mineralisation. Other proposed function of ON include interactions with other calcium binding proteins during cell proliferation. It has been suggested that interaction between ON and other ECM proteins including types III and IV collagen may promote cell attachment and spreading (Tracey and Mann, 1991).

1.5.5 Osteopontin

Osteopontin (OP) is glycosylated phosphoprotein and has an unusually high content of serine, aspartic and glutamic acid residues (Denhardt and Guo, 1993). Although one of the major noncollagenous proteins in calcified tissues, it is also found in nonskeletal tissues including the central nervous system, kidney and placenta (Nomura et al., 1988). The protein contains an RGD cell-binding sequence which has been shown to be capable of cell attachment via an integrin receptor present on many types of cell including osteoblasts and osteoclasts (Oldberg et al., 1986). The presence of acidic residues gives the protein an overall negative charge which is implicated in calcium ion binding. Although its precise function is not clear, it is considered to be important in the mineralisation and resorption of bone matrix. Its synthesis and secretion in bone appear to be under the control of both osteotropic hormones (vitamin D₃, corticosteroids, retinoic acids and parathyroid hormone) and growth factors including PDGF, EGF and TGF-β (Denhardt and Guo, 1993).

1.6 Statement of the problems

Several diseases affect the integrity of the periodontal structures. CIPDs are the major diseases that affect the periodontium caused by dental plaque leading to destruction of the connective tissue matrix, loss of periodontal attachment, resorption of alveolar bone and ultimately tooth loss. The goals of periodontal therapy include the prevention of the disease, slow the progression of disease once begun and finally regenerating the supporting structures that have been lost. However, CIPDs undergo alternate periods of tissue destruction and repair and hyperplasia may represent an exaggerated
phase of growth, which involves the enlargement of gingival connective tissue and excessive matrix accumulation. GH is also associated with the administration of drugs such as cyclosporin A, phenytoin and nifedipine. Moreover, GH resembles other hyperplastic lesions in which a number of growth factors and the anabolic androgen have been implicated. There is also evidence to suggest that the effects of anabolic steroids are mediated through certain growth factors and/or their receptors. These interactions are of relevance in the context of periodontal regeneration and overgrowth. The regenerative process is dependent on cellular activities such as migration, proliferation, differentiation and the synthesis of matrix proteins. Thus, as a biological approach the use of certain growth factors, androgens and other biochemical agents including the ECM proteins have the potential to initiate and mediate key cellular events associated with periodontal tissue repair, regeneration and overgrowth. The biological activities of growth factors are mediated through the cell surface receptors. The ability of cells to respond to these growth factors, therefore, depends on the presence of these receptors. Similarly, steroids act by forming complexes with intracellular receptors that function as transcription factors controlling the activity of many types of genes. However, the role of these receptors in periodontal tissues has not been well defined.

A number of studies have suggested that the cellular and molecular events associated with periodontal wound healing may be similar to those occurring during embryonic tissue development. In this context, the ECM FN serves many important cellular functions such as cell-cell interactions, migration and differentiation. FN exists as different isoforms i.e different molecular forms of the proteins are expressed from a single gene, as a result of alternative splicing at the messenger mRNA level. These isoforms generate proteins with differing functional activities in embryonic development and wound healing. However, FN isoform expression has not been investigated in periodontal tissues and therefore this information may be useful in the therapeutic approach to periodontal repair and regeneration.
Both GF and PDL cells have been extensively used in vitro for the investigation of cellular mechanisms involved in periodontal destruction and regeneration. However, these studies have been confined to short-term cultures which have a limited life-span and are phenotypically unstable and this precludes cloning and characterisation of individual cells to enable to develop in vitro models for the investigation of cellular and molecular mechanisms of periodontal disease and wound healing.

Thus, in order to understand both the periodontal disease pathogenesis and to evaluate therapies directed at the periodontal regeneration, certain basic information pertaining to the cellular and molecular mechanisms underlying these biological processes is required. The aims of this study are therefore:

a) to determine the expression of steroid hormone receptors in periodontal tissues and cells and their role in periodontal disease, growth and renewal.

b) to study the expression of certain growth factor receptors in periodontal cells and their role in periodontal regeneration and overgrowth.

c) to examine whether the presence of specific alternatively spliced variants of FN are associated with the periodontal pathogenic, regenerative and overgrowth processes and to investigate whether certain growth factors influence the expression of FN isoform patterns in periodontal cells.

d) to obtain long-term cell lines of both gingival and PDL cells by retroviral transduction with temperature-sensitive SV40 large T antigen construct to investigate periodontal pathogenesis and regeneration in vitro.
CHAPTER 2
MATERIALS AND METHODS

2.1 METHODS

2.1.1 Tissue samples

Normal gingival and periodontal ligament tissues were obtained from premolar teeth extracted for orthodontic reasons. The gingival tissue (GT) was obtained by removal of the soft tissue from the tooth collar and the periodontal ligament (PDL) was scrapped off from the middle third of the root surface. Precautions were taken to avoid cross-contamination between the periodontal and gingival samples. Inflamed gingiva and drug-induced gingival overgrowth tissues were obtained from patients undergoing periodontal surgery as part of their clinical management. The tissues were first placed into alpha Minimum Essential Medium (α-MEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml fungizone, 2 mM L-glutamine (GIBCO, Paisley, UK) and 10% foetal calf serum (FCS) (PAA Laboratories, Linz, Austria). The PDL scrapings and GT tissues (which were cut into 1-2 mm^2 pieces) were washed twice with full medium containing fungizone. The tissues were then either cultured as explants or used to extract total RNA.

2.1.2 Cell culture

Between 3 and 8 GT explant pieces were placed in 6-well culture dishes and covered with glass coverslips No. 0 (BDH, Poole, UK) prior to the addition of complete alpha-MEM without fungizone. The PDL scrapings were grown in 24-well plates in alpha-MEM supplemented with 10% FCS. The explants were incubated at 37°C in humidified air with 5% CO₂ for 2 to 4 weeks, changing their medium every 3 to 4 days. When the outgrowth of adherent cells reached confluency, the remaining tissue was removed and the cells detached from the monolayer by treatment with trypsin-EDTA for 10 min at 37°C, centrifuged for 10 min at 200 x g, washed with full medium and re-cultured in 25 cm^2 tissue culture flasks until confluent monolayers were obtained. By the third passage, all cells in both the GT and PDL cultures had the typical spindle-shaped
morphological appearance of fibroblasts. The human breast adenocarcinoma cell line MCF7 (Hall et al., 1990) was also grown in the same way.

2.2 Treatment of cells

2.2.1 Treatment with sex hormones

The fibroblasts were grown to approximately 80% confluency in complete medium, which was then replaced with phenol red-free RPMI 1640 medium supplemented with 1% charcoal-treated serum (Lapp et al., 1995). This medium avoided the possible oestrogenic effects of the pH indicator and removed the steroid hormones which are normally present in FCS. After 48h of culture in this medium, diethylstilbesterol (DES) or DHT were added to replicate cultures at concentrations of 0.2, 2.0 and 20 ng/ml (0.1, 1.0 and 10.0 nM, respectively). Untreated cells were used as controls.

2.2.2 Treatment with growth factors and cytokines

PDL cells were grown to approximately 80% confluence in medium consisting of Dulbecco’s Minimum Essential Medium (DMEM) which was then replaced with medium containing only 1% FCS. After 48 h in this culture medium, the following growth factors and cytokines were added to the flasks at the concentrations indicated for a further period of 24 h: platelet derived growth factor (PDGF-BB) at 1, 4 and 20 ng/ml; insulin like growth factor (IGF-1) at 1, 10, and 100 ng/ml; transforming growth factor-beta (TGF-β) at 1, 5, and 10 ng/ml; interleukin-1β (IL-1β) at 1, 5, and 10 ng/ml. The concentrations of growth factors used were based on findings from previous studies (Blom et al., 1994; Dennison et al., 1994). At the end of this period the cells were harvested and the total RNA extracted.

2.2.3 Drug treatment

Fibroblasts from normal and hyperplastic gingiva were grown to about 80% confluency in complete DMEM medium. The cells were then treated with 0.1, 1, 10, 100 and 1000 ng/ml of CsA, 20 μg/ml PHT and 10^{-6}, 10^{-7} and 10^{-8} M NIF for 5 days. The concentrations of drugs used were based on findings from
previous studies (Bartold, 1989; Modeer and Andersson, 1990; Henderson et al., 1997). Following the treatment the cells were harvested for FCM analysis.

2.3 RNA isolation, RT-PCR and Northern blotting

2.3.1 RNA extraction

Total RNA was extracted from both tissues and cells according to the method of Chomczynski and Sacchi (1987). Tissue samples were snap-frozen in liquid nitrogen and the homogenised with denaturing solution (4M guanidinium thiocynate, 25 mM sodium citrate, pH 7.5, 0.5 % sarcosyl and 0.1 M 2-mercaptoethanol) whereas the cells were grown as confluent layers and trypsinised. After washing the cells in PBS the cells were treated with denaturing solution until all the cells had been lysed. The homogenates/lysates were then transferred to eppendorf tubes and phenol/chloroform solution added. The samples were vortexed vigorously for 1 min, left on ice for 10 min and the centrifuged at 13,000 x g for 10 min. The upper aqueous phase was removed and transferred to a fresh tube. An equal volume of isopropanol was added and the solution was left at -70°C for 30 min and then centrifuged at 13,000 x g for 15 min. The supernatant was removed and the RNA pellet washed with 70% ethanol, re-centrifuged and then re-suspended in diethyl pyrocarbonate (DEPC) treated water. RNA was quantified by measuring absorbance at 260 nm and the concentration calculated by multiplying the optical density (O.D.) by 40 to obtain the amount of RNA as µg/ml.

2.3.2 Reverse-transcribed polymerase chain reaction (RT-PCR)

Polymerase chain reaction is a powerful technique used to amplify small amounts of DNA and for the analysis of gene expression since messenger RNA (mRNA) reverse transcribed into complementary DNA (cDNA) may be subjected to PCR amplification (Fig. 2.1). PCR is based on the enzymatic amplification of a DNA fragment and it is a cyclic reaction carried out at three different temperatures. Double stranded DNA template is first denatured to give two single stranded DNA templates (denaturation). Oligonucleotide
primers direct the synthesis of the new strands by hybridising to opposite strands (i.e. the primers are oriented with their 3' ends pointing towards each other) of the target sequence (annealing). Synthesis of new strands is initiated by the extension of the annealed primers using Taq DNA polymerase (extension). The polyA-mRNA in total cellular RNA can be converted into cDNA by annealing the RNA to oligo-dT primers and synthesising cDNA using an enzyme reverse transcriptase. The RT reaction was performed using 5 μg of RNA in buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol (DTT), 3 mM MgCl₂, 500 μM of each dNTP (dATP, dCTP, dTTP, dGTP), 1 μg of oligo (dT₁₈) and 50U of reverse transcriptase in total volume of 50 μl. The reaction was incubated at 37°C for 1h. Following reverse transcription, each PCR reaction was performed in 50 μl volume containing 5 μl of cDNA in buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 500 μM of each dNTPs (dATP, dCTP, dTTP, dGTP), 50 pmol of each of the primers and 1U of taq polymerase (Stratagene, UK). Sequences of the oligonucleotide primers used for PCR amplification and the size of the predicted PCR products are shown in Table 2.1. The PCR amplification programme consisted of initial denaturation at 94°C for 3 min followed by variable number of cycles each consisting of denaturation at 94°C for 45 sec, annealing temperature depending on the primers (shown in table 2.2), extension at 72°C for 90 sec and final extension at 72°C for 10 min. DNA fragments were analysed by 2% agarose gel electrophoresis in 1x TBE buffer (10x TBE: 0.9 M Tris-borate, 2 mM EDTA (pH 8.3) and stained with ethidium bromide (0.5 μg/ml). A 1 Kb DNA ladder (BRL) was used as a size marker. Generally 35 cycles were chosen for the PCR amplification. However, for semi-quantitative mRNA analysis the number of cycles employed was chosen such that the amplification was in the exponential range for each molecule being studied. To monitor the amplification rate, 5 μl of cDNA products were amplified simultaneously for 15, 20, 25, 30, 35 and 40 cycles of PCR with each primer pair. After electrophoresis the gel was stained with ethidium bromide and the intensity of bands were measured using the NIH Image program and the
relative levels of expression were calculated by comparison with the amplified internal control (GAPDH).

2.3.3 Northern blot analysis

For the Northern analysis (Sambrook et al., 1989) RNA samples (20 µg) were heat denatured at 65°C for 5 min in a solution containing 6.5% formaldehyde, 50% de-ionised formamide and 1.0x gel running buffer (10x gel buffer: 0.2 M MOPS[3-(N-Morpholino) propanesulfonicacid], pH 7.0. 50 mM sodium acetate, 10 mM EDTA) and separated electrophoretically on 1% agarose formaldehyde gel. 18S and 28S ribosomal RNAs were stained with ethidium bromide and used as a control for RNA quantification. RNAs were transferred to Hybond-N+ (Amersham, UK) blotting membrane in 20x SSC (3 M sodium chloride, 0.3 M sodium citrate) and fixed by UV cross-linking. Membranes were hybridised overnight at 42°C with labelled probes in a solution containing 50% formamide, 2x SSPE (20x SSPE: 3 M NaCl, 0.2 M NaH₂PO₄, 0.2 M EDTA, pH 7.4), 1% SDS and 5x Denhardt's (100x Denhardt's: 2% each of bovine serum albumin, ficoll and polyvinylpyrrolidone). Following hybridisation, membranes were washed in 2x SSC/0.1% SDS at room temperature for 30 min and then for 30-60 min at 50°C in 0.2x SSC/0.1% SDS. Autoradiography was performed at -70°C using intensifying screens. Membranes were subsequently re-hybridised with different probes after stripping in 0.1x SSC/0.5% SDS at 95°C. The radioactive probes were prepared using Amersham’s Megaprime DNA labelling System based on the random priming of Feinberg and Vogelstein (1983).

2.4 Determination of IL-6 secretion by ELISA

Cells were seeded into 24-well plates at 1x10⁴ cells in 2 ml of culture medium. DHT was added at concentrations of 0.2, 2.0 and 20 ng/ml on the following day. Control cultures were incubated in the absence of DHT. In some experiments, an aliquot of the medium was removed at daily intervals for a period of 5 days. IL-6 released into the medium was measured by an ELISA, as described below. In other experiments, to investigate the role of the
androgen pathway in IL-6 expression, the cells were treated with the anti-androgens cyproterone acetate (CPA) and flutamide (FT) at a concentration of $10^{-6}$ M each, in the absence and presence of 20 ng/ml DHT. The media were collected on the fifth day only.

The ELISA assay to measure IL-6 was performed as follows. Microtitre plates (Immuron 4, Dynatech UK) were coated with 1 µg/ml of immuno-affinity purified goat polyclonal anti-IL-6 antibody G150/BM (National Institute of Biological Standards, Herts, UK), diluted in bicarbonate buffer (pH 9.6). The plates were incubated at room temperature for 2 h and then left overnight at 4°C. The antibody was decanted and the wells washed three times with PBS containing 0.1% Tween (PBS/T). 0.1 ml of the culture medium supernatants to be tested and known amounts of an IL-6 standard were added to each well and incubated for 2 h at room temperature. The wells were washed as described previously and then biotinylated, affinity-purified anti-IL-6 antibody was added to each well and incubated for a further 1 h at room temperature. The plates were washed three times and the binding of the biotinylated antibody was detected by incubation with 100 µl of 1:5000 dilution of avidin-HRP (Dako Ltd UK), followed by 100 µl of 0.2 mg/ml orthophenylenediamine (OPD, Sigma UK) in 0.1M citric acid-phosphate buffer pH 5.0 containing 0.03% hydrogen peroxide. The reaction was terminated by the addition of 2 M H$_2$SO$_4$ and the absorbance measured at 492 nm using a Titertek ELISA plate reader. The level of IL-6 secreted into the culture media was determined by comparison with the absorbance of the IL-6 standard.

2.5 Flow cytometry (FCM)

Flow cytometry is a process in which certain physical and chemical characteristics of cells are measured as they flow in a moving fluid stream past a fixed laser beam (Shapiro, 1988). Light scattered by cells at low angle, called forward scatter (FSC), is related to cell size, whereas light scattered by cells at an angle of 90°, called side scatter (SSC), is related to the cytoplasmic granularity and intracellular complexity of the cells (Fig. 2.2). If the cells are pretreated with a fluorescent-labelled antibody or other fluorochrome ‘tag’, the
technique gives a quantitative measurement about the expression of both cell surface and intracellular components, including antigens. 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 which is measured by an optical detector. The intensity of the fluorescence is considered to be proportional to the quantity of the bound fluochrome. Confluent cell cultures grown were prepared as described previously (Bou-Gharois et al. 1994). Briefly, the cells were washed in PBS and detached by 20 mM EDTA (pH 7.2) in PBS for 10 min at 37°C. They were then centrifuged and fixed with 1% paraformaldehyde (BDH) in PBS for 30 min, followed by permeabilisation (Bou-Gharios et al., 1994; Sumner et al., 1991) with 0.1% saponin for 10 min. This allows the antibodies to enter the cells and therefore enabled the intracellular as well as surface-associated antigens to be detected. The cells were washed in buffer containing PBS, and 2% FCS (PBS/FCS) and centrifuged at 400 x g for 10 min after each step mentioned below. Aliquots of the cell suspension containing 10^5 cells were placed into separate tubes and used for measuring the level of each antigen. Mouse monoclonal antibodies (mAbs) against osteocalcin (Dako), procollagen type I (Developmental Studies Hybridoma Bank, Iowa city, USA), rabbit antisera against osteopontin, osteonectin, and the androgen receptor (Chemicon, California, USA) diluted 1:500 in PBS/FCS containing 0.1% saponin were applied for 60 min at room temperature, (ON, OP) provided by L Fisher, NIH, USA). For growth factor receptor expression following antibodies were used: PDGF-R (α, β) (Biogenesis), TGF-βR (Biogenesis), IGF-IR (Biogenesis), FGF-R and EGF-R (Chemicon), diluted 1:100 in PBS/FCS. As a negative control, mouse IgG, was used for mAbs while PBS/FCS served as a negative control for rabbit polyclonal antibodies. Fluorescein isothiocynate (FITC)-labelled rabbit anti-mouse or goat anti-rabbit IgG, diluted 1:50 PBS/FCS containing saponin was then added for 30 min at room temperature. The cells were suspended in 400 μl of PBS/FCS and the size (FCS), granularity (SSC) and fluorescence intensity of 10,000 individual cells were measured by FCM using a FACScan flow cytometer (Becton Dickinson, UK). The results were analysed using CELLQuest software and are
presented as arbitrary units of scattered light and the level fluorescence expressed as the average fluorescence intensity (AFI).

2.6 Immortalisation of gingival and PDL cells

2.6.1 Retroviral transfection

Cultures of gingival and PDL cells were infected with retroviral vector pZipNeoSV(X)1 containing sequences encoding a temperature sensitive SV40 large T antigen (tsA58) and a neomycin (G418) resistance gene. This was done by incubating the cells with conditioned filtered (0.45 μm) medium from a packaging line (PA/tsA58-U19/8) producing an amphotropic retrovirus in the presence of 8 μg/ml polybrene for 4 h at 37°C. The infection protocol was repeated for three consecutive days. Three days after final infection the cells were selected for resistance to G418 (500 μg/ml, geneticin, GIBCO) for approximately 3 weeks at permissive temperature of 34°C. The resultant G418 resistance cells were maintained in culture by continuous passage at the permissive temperature.

2.6.2 Cloning procedure

Transduced gingival and PDL cells after drug selection were trypsinised and single cell suspensions prepared in complete medium containing 15% FCS. Cells were plated in 96 well plates by limiting dilution to 1-3/cells per ml. The plates were incubated undisturbed for two weeks after which wells with single colonies were scored for determining the cloning efficiency. The colonies were grown to confluency and subcultured to 25 cm² flasks.

2.6.3 Growth characteristics of immortalised cells

The growth of transduced cells was measured by seeding 1 x 10⁵ cells into 25-cm² culture flasks in complete medium. After 24 h incubation at 34°C, half of the cultures were transferred to 39°C. At 2 day intervals, replicate cultures were subsequently harvested by detaching with trypsin and single cells counted using haemocytometer, after staining with trypan blue.
2.6.4 Immunocytochemical staining of T antigen

Cells were grown on coverslips and washed in PBS, then fixed in ice-cold acetone-methanol (1:1). The cells were then treated with 10% normal goat serum (NGS) (GIBCO) and 0.1% saponin for 30 min at room temperature to block non-specific binding and to render cells permeable, respectively. Mouse monoclonal antibodies 419 and 423 (kindly provided by Prof D Lane, University of Dundee), diluted in TBS containing 10% NGS and 0.1% saponin, which react with amino- and carboxy-terminal epitopes, respectively were incubated for 1 h at room temperature. Non-specific mouse IgG1 antibody (Dako) was used as a negative control. After washing in TBS, the cells were incubated with biotin-conjugated goat anti-mouse IgG (Sigma) for 1 h, the avidin-biotin complex (ABC) (Dako) was applied and the antigen visualised using 3,3'-diaminobenzidine (DAB) (Sigma). The cells were counter stained with Mayers haematoxylin. The expression of the antigen was examined by light microscopy.

2.6.5 Alkaline phosphatase (AP) assay

The AP activity of the cells was measured colourimetrically using paranitrophenylphosphate disodium (Sigma) as the substrate. This substrate is hydrolysed by the enzyme AP to para-nitro phenol which under alkaline conditions is converted to a yellow product and its absorbance is subsequently measured at 405 nm (A405). The cells were seeded into 96-well plates at a density of 10^4 cells/well and incubated at 34°C and 39°C in the absence and presence of 10^-8 M dexamethasone (DEX) and vitamin D3 (D3). For each of the samples, 6 replicate wells were used in this experiment. After 7 days of incubation, the cells were washed with PBS and solubilised by the addition of 50 μl/well of 1% Triton-X (BDH) for 20 min. AP was determined using a kit purchased from Sigma (AP kit 104-LL). 50 μl of the assay mixture, consisting of 1.5 mM 2-amino-2-methyl-1-propanol (pH 10.3) (Sigma) and 4 mM paranitrophenylphosphate disodium (Sigma) was added to each well. The mixture was incubated for 30 min at 37°C and the reaction was terminated by the addition of 150 μl of 1 M NaOH and the relative amount of ALP activity
determined by measuring the absorbance at 405 nm ($A_{405}$) using spectrophotometer (Titrek, Helsinki, Finland). The International Units (IU/L) of the experimental samples were calculated from the standard curve of the Sigma Units and the $A_{405}$. 
Fig. 2.1 Schematic representation of reverse-transcriptase polymerase chain reaction (RT-PCR). RT is a process in which DNA copy (cDNA) of polyA-mRNA is synthesised by annealing oligo-dT primer to the mRNA and using the enzyme reverse transcriptase. PCR begins with the denaturation of DNA to form two single strands. This is followed by annealing specific primers to the denatured strands. Once bound, the enzyme Taq polymerase extends the primers to synthesise a double stranded DNA. This process is repeated many times.
The laser light applied to a cell in the flow cytometry is diffracted at low angles (Forward scatter, FSC) is proportional to cell size whereas light reflected at right angles (Side scatter, SSC) is proportional to cell complexity and granularity.
Table 2.1. PCR oligonucleotide primers and predicted sizes of PCR products using each primer pair

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCE 5-3</th>
<th>PREDICTED SIZES (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>gctgcaaggtcttcttcaaa</td>
<td>463</td>
</tr>
<tr>
<td>ANTISENSE</td>
<td>tgcgacaagtgtaaggtgcttcaaa</td>
<td></td>
</tr>
<tr>
<td>AP</td>
<td>acgtggctagaatgtcatc</td>
<td>475</td>
</tr>
<tr>
<td>ANTISENSE</td>
<td>ctggtagggtgcgttaagtctt</td>
<td></td>
</tr>
<tr>
<td>COL I</td>
<td>tgacgagacaaagagacgt</td>
<td>599</td>
</tr>
<tr>
<td>ANTISENSE</td>
<td>ccacgagacaaagagacgt</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>cataagcactataagtgtccagc</td>
<td>659</td>
</tr>
<tr>
<td>ANTISENSE</td>
<td>aacgagagacaaaagacgt</td>
<td></td>
</tr>
<tr>
<td>FN ED-A</td>
<td>gactattggaagcgtgtccagt</td>
<td>420, 151</td>
</tr>
<tr>
<td>ANTISENSE</td>
<td>cttgtgcgtgtgcgttcgagt</td>
<td></td>
</tr>
<tr>
<td>FN ED-B</td>
<td>ggtcctagcgtgctacagcgttcgagc</td>
<td>458, 185</td>
</tr>
<tr>
<td>ANTISENSE</td>
<td>caggtgacacgcactgtgtctg</td>
<td></td>
</tr>
<tr>
<td>FN IIICS</td>
<td>ggctactattactggcctgg</td>
<td>484, 409, 391, 316, 124</td>
</tr>
<tr>
<td>ANTISENSE</td>
<td>ctgagagacactgtcttgtcctgcc</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>ccacccatggcattcctggcata</td>
<td>600</td>
</tr>
<tr>
<td>ANTISENSE</td>
<td>ctagacggccaggtgctggccatcctgg</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>atgaactctctctccacagcgc</td>
<td>628</td>
</tr>
<tr>
<td>ANTISENSE</td>
<td>gaagagccctcagggctgcattg</td>
<td></td>
</tr>
<tr>
<td>OP</td>
<td>ccaagtaagtccaagcagaag</td>
<td>347</td>
</tr>
<tr>
<td>ANTISENSE</td>
<td>ggtgatgtctcgtgctgta</td>
<td></td>
</tr>
<tr>
<td>OC</td>
<td>catgagacacacctcaca</td>
<td>310</td>
</tr>
<tr>
<td>ANTISENSE</td>
<td>agagcgacacctagac</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2   PCR amplification annealing conditions

<table>
<thead>
<tr>
<th>PRIMER PAIRS</th>
<th>TEMP (°C)</th>
<th>TIME (SECS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH, AR, ER</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>FN</td>
<td>55</td>
<td>35</td>
</tr>
<tr>
<td>IL-6</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>OC, OP, COL I, AP</td>
<td>55</td>
<td>120</td>
</tr>
</tbody>
</table>
CHAPTER 3
THE ROLE OF SEX HORMONE RECEPTORS IN PERIODONTAL DISEASE AND GROWTH

3.1 RT-PCR analysis of oestrogen and androgen receptor expression in periodontal tissues and cells

3.1.1 Introduction

The role of sex steroids in the function of sex-associated tissues has been widely studied. However, a number of studies have suggested that sex steroids may play an important part in CIPD, and gingival inflammation and hyperplasia are closely associated with hormonal changes (Löe, 1965; Hugoson, 1970; Nyman, 1971; Sutcliffe; Mombelli et al., 1989). In addition, previous investigations have demonstrated that gingival tissues actively convert testosterone to DHT and, furthermore, this activity is enhanced in inflammation and GH. Oestrogens and androgens exert their influence by binding to specific intracellular receptors which belong to a family of ligand activated transcription factors that control a wide range of biological processes (Evans, 1988; Beato, 1989; Brinkman, 1994; Zhou et al., 1994). These include modulation of adhesion molecules, cytokine and the ECM genes, the hormones thereby playing an important part in cell interactions, inflammation and connective tissue turnover (Cavailles et al., 1988; Weisz et al., 1990; Freeman et al., 1991; Fox et al., 1991). These processes are fundamental to the pathogenesis of periodontal diseases and regeneration. However, the role of the sex hormone receptors and the precise effects of the hormones on periodontal tissues and cells have not been well defined.

Therefore, the aim of this part of the study was to examine the presence of messenger RNA (mRNA) transcripts for the oestrogen and androgen receptors in periodontal tissue and in gingival and PDL cells cultured in vitro using the specific and highly sensitive RT-PCR technique.
3.1.2 Materials and Methods

The methods were as described in chapter 2. Gingival and PDL cells were cultured as described in section 2.1.2. In order to determine the effects of steroid hormones, the cells were treated as described in section 2.2.1. Total RNA was extracted from both tissues and cultured cells as described in 2.3.1 and the RT-PCR was performed as described in 2.3.2 to detect the GAPDH, AR and ER genes using primer pairs shown in table 2.1. The PCR amplification annealing temperature for these primers are shown in Table 2.2.

3.1.3 Results

Initially, in order to assess the integrity of the extracted RNA and the subsequent fidelity of first strand cDNA synthesis, all the cDNA preparations from both tissues and the cultured cells were analysed by PCR using the primers for GAPDH, a standard housekeeping gene. Only those which yielded the predicted 600 bp DNA fragment were then amplified with ER and AR-specific primers. As a positive control in this experiment cDNA obtained from the MCF7 cell line was used. As shown in Figs 3.1a and 3.1b the expected amplification products of 659 and 463 bp were detected with ER and AR primers, respectively, in the MCF cDNA. However, analysis of 8 GT and 16 PDL tissue samples, and also of 5 gingival and 6 PDL cultured cells, showed that none of these samples expressed transcripts for the oestrogen receptor (Fig 3.1a). In marked contrast to the expression of the oestrogen receptor, 6 of the GT and 8 of the PDL, and all cultured fibroblast samples, were positive for the androgen receptor (Fig. 3.1b). Again, in each PCR reaction the positive control, MCF cDNA, was amplified appropriately. Negative control, containing no template, was appropriately negative and all samples contained the GAPDH (Fig 3.1c).

A number of reports have suggested that both oestrogen and testosterone modulate their own receptors (Quarnby et al., 1990; Simerly R B and Young B J, 1991; Liberato et al., 1993). Therefore, the effects of DES and DHT on the expression of sex steroid receptors in both gingiva- and periodontal ligament-derived cells were examined. As described in the Materials and
Fig. 3.1  Analysis of RT-PCR products on agarose gel stained with ethidium bromide and visualised under UV light. Total RNA was extracted, and subjected to RT-PCR using GAPDH, ER and AR-specific primers. Lanes: M, 1-Kb DNA ladder; 1, Negative control (without cDNA); 2, MCF7 cell line; 3, gingival tissue; 4, PDL; 5, GF; 6, PDL cells.
methods, the gingival and periodontal fibroblasts were first cultured with phenol red-free and hormone-free media for 2 days, then incubated with different concentrations of hormones for 6 days. Cells grown in the absence of the hormones served as controls. cDNAs were prepared from these cells and then amplified with the ER and AR primers. None of the cells treated with either DES or DHT resulted in levels of ER which could be detected by the RT-PCR procedure, whereas the AR transcripts, which were expressed constitutively in all the cells grown in the absence of the hormones, appeared to be unaffected by either DES or DHT (data not shown).

3.2 The effects of androgens on gingival and PDL cells

3.2.1 Introduction

The results reported in the previous section showed unequivocally that mRNA transcripts corresponding to androgen receptor are expressed in gingival and periodontal tissues and in fibroblasts derived from these tissues. Both GF and PDL cells are therefore potentially responsive to the molecular effects of testosterone and, moreover, previous studies have shown that periodontal tissues are capable of metabolising testosterone to form its potent metabolite, DHT (Vittek et al., 1979; Ojanotko et al., 1980). This metabolite is particularly active as an anabolic agent which may have implications in periodontal regenerative processes. There is also evidence to suggest that testosterone may be involved in GH which is induced in vivo by cyclosporin, phenytoin and nifedipine (Nyska et al., 1994; Nishikawa et al., 1996). Therefore the aim in this section was to examine 1) the effects of DHT on gingival cell proliferation, 2) the levels of AR mRNA in normal and hyperplastic gingival tissues (by semi-quantitative RT-PCR analysis), 3) the effects of the drugs on the levels of AR protein in gingival cells cultured in vitro by FCM.

As mentioned earlier, periodontal diseases are associated with the local invasion of inflammatory cells and the production of soluble mediators that ultimately damage periodontal tissues (Page, 1991). A number of cytokines including IL-6 are produced by infiltrating leukocytes and activated GF (Bartold et al., 1991; Takahashi et al., 1994). The cytokine IL-6 is a major mediator of
inflammation, tissue injury and bone resorption. This cytokine has been shown to be produced by GF, the main cellular component of gingival connective tissue, and IL-6 levels in GCF have been correlated with adult periodontitis (Gemmell and Seymour, 1993; Reinhardt et al., 1993; Gievelis et al., 1993). Moreover, IL-6 has been proposed to play a pathogenic role in fibrotic diseases including GH (Williamson et al., 1994). The expression of IL-6 is in turn regulated by a number of cytokines and mitogens. In addition, other studies have shown that androgen deficiency in male hypogonadism and in castrated males is associated with bone loss, a process in which IL-6 has been reported to play a central role (Greenspan et al., 1986; Stepan et al., 1989). In this section, therefore, the effects of DHT were also examined with regard to the expression and regulation of IL-6 in gingival and PDL cells.

3.2.2 Materials and methods

The effects of DHT and growth factors on cell proliferation were evaluated by the measurement of DNA synthesis in the replicating cells. This technique is based on the incorporation of the pyrimidine analogue bromodeoxyuridine (BrdU) into the DNA of proliferating cells and detection by immunoassay using Boehringer Manheim ELISA kit (Manheim, Germany). GF and PDL cells were plated in six replicate 96-well plates at a density of 1x 10⁴ cells per well in medium containing 10% FCS. The cells were incubated for 24 hours and then the medium decanted and the cells incubated for a further 24 h in medium supplemented with 0.5% FCS to growth arrest the cells. Initially, the cells were treated with 0.2, 2.0 and 20 ng/ml DHT for 24 h and the untreated cells served as controls. In subsequent experiments the cells were pre-treated with 20 ng/ml DHT for the same period and then with a variety of growth factors. For the final 2 hours of incubation BrdU was added to each well. After labelling, the cells were fixed, DNA denatured and the incorporated DNA measured by ELISA as described in the manufacturer’s protocol. The reaction product was quantified by measuring absorbance at 450nm using an ELISA plate reader.
Confluent cultures of normal gingiva- and hyperplastic-derived fibroblasts, and MCF7 cells were prepared for FCM as described in 2.5. The antibodies used to detect the AR were mouse mAbs (Novacostra, Newcastle-upon-tyne, UK), Biogenesis (Poole, UK), Biogenex (San Remo, USA) and the rabbit polyclonal (Chemicon, Harrow, UK), diluted 1:100 in PBS/FCS.

The size (FSC) and granularity (SSC) and fluorescent intensity of 10 000 individual cells were measured by FCM. The data were processed by CELLQuest software which provides statistical profiles of each of the samples. The arithmetic means of the FSC and SSC parameters and geometric means of the fluorescence intensity (average fluorescence intensity, AFI) were used for statistical analysis. The parameters are presented as arbitrary units of scattered light and fluorescence. In order to determine the effects of the drugs (CsA, PHT and NIF) on the AR expression, cultures of GF were incubated with these drugs as described in 2.2.3 and then immunostained with the rabbit polyclonal antibody against the AR. The effects of DHT on the levels of IL-6 secretion was measured by ELISA as described in 2.4.

3.2.3 Results

3.2.3.1 Cell proliferation

In preliminary experiments, the effects of various concentrations of DHT on both GF and PDL cell proliferation was measured by the incorporation of BrdU into the cellular DNA. Since the results for both GF and PDL cells were similar, the data (Fig. 3.2) are representative of GF and show that at all concentrations tested, DHT did not cause cell proliferation. However, the addition of TGF-β (10 ng/ml), PDGF (20 ng/ml) or IGF-I (100 ng/ml) resulted in response which were 165%, 160% and 205% of control respectively (Fig 3.3). In order to determine the regulatory influences of DHT on the response to growth factors by GF, the cells were pre-treated with DHT at 20 ng/ml for 24h followed by incubation with a variety of growth factors for the same time period, and the cell DNA synthesis measured. Pre-incubation with DHT produced significant enhancement of DNA synthesis when the cells were subsequently treated with PDGF and TGF-β. PDGF response in cells pre-treated with DHT
Fig. 3.2  Effects of increasing concentrations of DHT on DNA synthesis were determined by BrdU incorporation into GF. The cells were treated with DHT for 24 h and BrdU added in the final 4 h of incubation. Untreated cells served as a control. BrdU incorporation was measured by ELISA. The results shown represent the mean and standard deviation (SD) of 6 replicate wells from one experiment and represent three independent experiments. Note there was no difference in response between control and DHT-treated cells.
Fig. 3.3  Effects of DHT pre-incubation on DNA synthesis in GF as determined by BrdU incorporation. The cells were pre-incubated with DHT (20 ng/ml) for 24 h then with PDGF-BB (10 ng/ml), TGF-β (20 ng/ml) and IGF-1 (100 ng/ml) for a further 24 h and compared with the control (untreated cells), DHT-treated and the growth factors alone. The results are shown as mean ± SD of six replicate wells and represent three independent experiments. Note that all the growth factors alone significantly increased DNA synthesis, while pre-incubation with DHT further enhanced DNA synthesis when subsequently treated with PDGF-BB and TGF-β but not with IGF-1. * indicates significantly different from growth factor alone (P < 0.05).
3.2.3.2 Measurement of AR mRNA level in normal and GH tissue

Total RNA extracted and cDNA prepared from 7 normal gingiva and 10 CsA-induced GH tissue samples were subjected to PCR, performed as described in Materials and methods. To determine the linear range of amplification, aliquots of cDNA products were amplified simultaneously with 15, 20, 25, 30, 35 and 40 cycles of PCR for each primer pair, electrophoresed in agarose gels and bands visualised by ethidium bromide staining. Photographs of the gels were taken and band intensities quantified by using NIH Image program. The gel photographs are shown in Fig 3.4 and 3.5 for GAPDH and AR primers, respectively. The results of this analysis (Fig. 3.6) identified appropriate cycles within the exponential range. Based on densitometric analysis of GAPDH production, 20 to 30 cycles of amplification showed linear increase in density. Although a band was visible at cycle number 15 with GAPDH primers, the products amplified with the AR primers did not yield detectable bands after cycles 15 and 20 but a band appeared on the 25 cycle. The linear increase in density for the AR was between 25 and 35 cycles. Therefore in subsequent experiments 25 cycles were employed for GAPDH as internal control and 30 cycles for the AR. The relative levels of the AR expression were quantitated by comparison with the amplified internal control (GAPDH). The ratio of AR:GAPDH transcript in normal gingiva (Fig. 3.7) was 0.67 ± 0.096 (n=7) whereas in GH tissue it was 0.66 ± 0.11 (n=10). This showed that there was no difference in the level of the AR mRNA in CsA-induced GH tissues compared with normal gingiva.

3.2.3.3 Measurement of AR protein level in GF by FCM

Initially, MCF7 cell line was immunostained for the AR since this cell line has been shown to express the receptor at a high level. Three different monoclonal (Biogenesis, Poole, UK), (Novacastra, Newcastle-upon Tyne, UK) and Biogenex, (San Ramon, USA) and one rabbit polyclonal (Chemicon, Harrow, UK) antibodies were used to test their efficacy. Fig 3.8 shows a representative scatter plot of a GF culture, each cell being represented by one dot.
**Fig 3.4** Optimal amplification cycle for GAPDH primers. Total RNA samples were reverse transcribed and amplified with GAPDH primers and products electrophoresed in agarose gel and stained with ethidium bromide. Band intensities quantified by densitometry.

**Fig 3.5** Optimal amplification cycle for AR primers. Total RNA samples were reverse transcribed and amplified with AR primers and products electrophoresed in agarose gel and stained with ethidium bromide. Band intensities quantified by densitometry.
Fig. 3.6   Kinetic analysis of GAPDH and AR mRNA levels. Band intensities shown in Figs. 3.4 and 3.5 were determined using an NIH Image programme and plotted against the number of amplification cycles. Note the linear range for GAPDH is between 20 and 30 cycles and for AR is between 25 and 35 cycles.
Fig. 3.7 Relative levels of AR mRNA with respect to GAPDH mRNA in normal gingival (NG) tissue (n=7) compared with gingival hyperplasia (GH) tissue (n=10). mRNA subjected to RT-PCR and the amplified cDNA band intensities quantified by densitometry. The result is represented as the ratio of AR and GAPDH intensities.
The solid line indicates the gating region selected so as to eliminate cell debris and cell aggregates to measure antigen expression by fluorescence. The fluorescence profiles of these experiments are presented as histogram plots (Fig. 3.9), in which the intensity of the fluorescence represents the level of antigen expression. MCF cells treated with the rabbit polyclonal antibody stained positively for the AR (Fig. 3.9b) but when the cells were treated with the mouse monoclonal antibodies, they did not stain positively for the antigen (Fig. 3.9a).

In subsequent experiments rabbit polyclonal antibody was used to measure the level of the AR protein in fibroblasts obtained from normal gingiva (n=5) and GH (n=5) tissues from CsA-treated patients. Initially the parameters of FSC and SSC of the cultured cells were measured by FCM to determine the size and granularity of all the individual cells in the population. All the cell strains analysed produced narrow, bell-shaped peaks of fluorescence and all cells in the population were positively stained. The level of the AR protein in normal fibroblasts (AFI of 17 ± 2) was 24% of that in MCF7 cells (Fig. 3.10). Similar levels of AR protein (AFI of 16 ±1.5) were detected in fibroblasts from GH tissues (Fig. 3.10). In a further series of experiments to examine whether the drugs modulated the expression of the AR protein, the cells were treated with CsA at concentrations of 0.1, 1.0, 10, 100 and 1000 µg/ml, PHT at 20 µg/ml and NIF at 10⁻⁶, 10⁻⁷, and 10⁻⁸ M for 5 days and the level of the AR protein measured. Untreated cells served as control. Figs. 3.9c and d show histogram plots of the AR expression in normal and hyperplastic gingival fibroblasts, respectively. Three cell strains derived from normal healthy gingiva and 3 from CsA-induced GH patients were used to examine the effects of CsA and the results (Table 3.1) show the means of FSC and SSC profiles from replicate experiments, in each of which 10,000 individual cells were examined. Both the average size and average granularity of the drug-treated cells were found to be similar compared with those of the untreated cells. The fluorescence profiles were obtained from these experiments and the results are shown in Figs 3.11 and 3.12 as mean ± standard deviation and were analysed using a one-way analysis of variance.
Fig. 3.8  Representative FCM dot-plot profile of $10^4$ individual cells showing the forward scatter (FSC), representing the relative size, and the side scatter (SSC), representing the relative granularity and are presented in arbitrary units. The solid line indicates the gating region.
Fig. 3.9  Representative histogram plots showing fluorescence intensities of MCF7 cells stained with mouse (monoclonal) antibody (a) stained with rabbit (polyclonal) antibody (b). The green line represents the negative controls. (c) and (d) represent histogram plots of normal gingiva and hyperplasia-derived fibroblasts, respectively stained with rabbit antibody. Fluorescence intensity units are arbitrary.
Fig. 3.10  Comparison of AR expression by fibroblasts derived from normal gingiva (NG), gingival hyperplasia (GH) and MCF7 cell line cultured under drug-free conditions, the vertical lines showing the standard deviation of replicate experiments. The values are arbitrary. The data represent the mean ± SD of duplicate experiments of 5 strains of fibroblasts from NG and GH each, and MCF7 data represent the mean ± SD of triplicate experiments.
Table 3.1: Average size and granularity of gingival fibroblasts measured by FCM and are shown as arbitrary units of FSC and SSC, respectively. The numbers are mean ± SD. The results were analysed by ANOVA and found not to be significant.

<table>
<thead>
<tr>
<th>Condition</th>
<th>FSC</th>
<th>Range</th>
<th>SSC</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (untreated)</td>
<td>469 ± 84</td>
<td>346-529</td>
<td>361 ±24</td>
<td>331-393</td>
</tr>
<tr>
<td>CsA 1000 ng/ml</td>
<td>510 ± 39</td>
<td>472-551</td>
<td>374 ±63</td>
<td>311-439</td>
</tr>
<tr>
<td>CsA 100 ng/ml</td>
<td>468 ± 77</td>
<td>356-510</td>
<td>366 ±19</td>
<td>339-382</td>
</tr>
<tr>
<td>CsA 10 ng/ml</td>
<td>464 ± 76</td>
<td>350-513</td>
<td>365 ±20</td>
<td>337-383</td>
</tr>
<tr>
<td>CsA 0.1 ng/ml</td>
<td>468 ± 77</td>
<td>355-525</td>
<td>359 ±24</td>
<td>323-379</td>
</tr>
<tr>
<td>PHT 20 μg/ml</td>
<td>499 ± 20</td>
<td>476-516</td>
<td>362 ±24</td>
<td>335-380</td>
</tr>
<tr>
<td>NIF 10⁻⁶ M</td>
<td>505 ± 16</td>
<td>484-526</td>
<td>353 ±32</td>
<td>316-377</td>
</tr>
<tr>
<td>NIF 10⁻⁷ M</td>
<td>503 ± 20</td>
<td>484-508</td>
<td>353 ±28</td>
<td>331-374</td>
</tr>
<tr>
<td>NIF 10⁻⁸ M</td>
<td>489 ± 59</td>
<td>385-563</td>
<td>364 ±59</td>
<td>291-436</td>
</tr>
</tbody>
</table>
Fig. 3.11  Effects of CsA on AR expression by normal gingival fibroblasts compared with untreated cells. Note that the differences in AFI between CsA-treated and control were not significant. The results represent means ± SD of duplicate experiments of 3 strains of fibroblasts.
Fig. 3.12  Effects of CsA on AR expression by fibroblasts derived from GH tissue. Note that the differences between CsA-treated and control cultures were not significant. The results represent means ± SD of duplicate experiments of 3 strains of fibroblasts.
Fig. 3.13  Effects of PHT and NIF on AR expression by normal gingival-derived fibroblasts. Note that the differences between the drug-treated and control cultures were not significant. The results represent means ± SD of duplicate experiments of 3 strains of fibroblasts.
(ANOVA). When normal fibroblasts were treated with CsA, the AFI values were similar to those of the untreated cultures except that the AFI was higher at a CsA concentration of 0.1 ng/ml but this was not significant. Similarly, when cells derived from hyperplastic tissues were incubated with the drug, the AFI values were higher at a CsA concentration of 1 and 10 ng/ml compared with the untreated cells, and again these results were not significant. This experiment was repeated using 3 normal gingival fibroblast samples treated with PHT and NIF, and the AR expression examined. Although the AR antigen level was higher in PHT treated cultures (Fig. 3.13), this was not significant. The antigen levels in NIF-treated cells were similar to those of the untreated cells. The size and shape of the peak remained similar to those observed using control cultures.

3.2.3.4 Regulation of IL-6 expression by androgen in gingival and PDL cells

In initial experiments, the effects of increasing concentrations of DHT on IL-6 secretion by normal GF were measured. DHT was added at 0.2, 2.0 and 20.0 ng/ml and the culture supernatants collected daily for 5 days and analysed by ELISA. Culture media from untreated cells were used as controls. The results (Fig. 3.14) show that IL-6 secretion in control cultures was similar on days 3 and 4 and increased by day 5. However, the presence of DHT in the culture medium was found to inhibit IL-6 secretion, and this inhibition was both dose- and time-dependent. Thus, by day 3, DHT concentrations of 0.2, 2.0 and 20 ng/ml progressively decreased IL-6 secretion to approximately 80, 60 and 40% respectively of the level present in the control culture medium (no added DHT). By day 4, inhibition of IL-6 secretion was even more pronounced, IL-6 levels decreasing to 40, 40 and 35% respectively of that present in control cultures. On the fifth day of culture, all concentrations of DHT decreased IL-6 secretion by approximately 80%.

In further studies the effects of DHT on IL-6 secretion by fibroblasts derived from inflamed gingival tissue, GH tissue and normal periodontal
Fig. 3.14  Effects of varying concentrations of DHT on IL-6 production by normal gingival-derived fibroblasts. Control cultures were incubated in the absence of DHT. IL-6 levels in culture supernatants measured by ELISA. The values shown represent the means ± SD of three replicate experiments.
Fig. 3.15 Effects of DHT on IL-6 production by diseased gingival and normal PDL fibroblasts. Gingival cells from patients with gingivitis, GH and normal PDL fibroblasts were incubated with varying concentrations of DHT for 5 days. Control cultures were incubated in the absence of DHT. IL-6 levels measured by ELISA. The values shown represent the means ± SD of three replicate experiments.
ligament cells were examined. The same concentrations of DHT were used as above but the culture media were collected on day 5 only. The results (Fig. 3.15) show that, in the absence of DHT, the inflamed and overgrowth gingival cells (G119 and GH6, respectively) produced a similar level of IL-6 (approximately 3700 pg/ml), which was lower than the level secreted by normal GF (approximately 7000 pg/ml) on day 5 (Fig. 3.13). Nevertheless, as with the normal GF, the presence of increasing concentrations of DHT progressively down-regulated IL-6 in the fibroblasts derived from both the inflamed and drug-induced gingival overgrowth tissues. However, whereas 0.2 and 2.0 ng/ml of DHT reduced IL-6 production by the inflamed gingival cells by 32% and 43%, respectively, the same concentrations of DHT had far less inhibitory effect on the gingival overgrowth fibroblasts, down-regulating IL-6 secretion by these cells by only 12 and 27%, respectively. At the highest concentration of DHT (20 ng/ml), IL-6 production by both types of cell was inhibited by approximately 50%. Unexpectedly, neither cell line of PDL cells produced detectable levels of IL-6.

To explore the regulation of IL-6 synthesis and the apparent inhibitory role of DHT, the cells were treated with two drugs generally considered to be androgen antagonists, CPA and FT, and measured the effects of these anti-androgens on IL-6 production. Since androgens appeared to down-regulate IL-6, it was anticipated that the presence of either of these antagonists would block the inhibitory effect of the hormone. Thus, the cells were treated with the drugs in the absence and presence of DHT and the culture supernatants analysed by ELISA, as described in the Materials and methods. Each of the drugs had a unique effect on IL-6 secretion (Fig. 3.16). In the presence of CPA alone, IL-6 production was inhibited by approximately 85%, and similar down-regulation of IL-6 was obtained when DHT as well as CPA were added to the cultures. In marked contrast, FT had no inhibitory effect on IL-6 when added alone, IL-6 levels in the culture medium actually increasing slightly. However, when DHT was added as well as FT, the hormone down-regulated IL-6 synthesis to the same low level as that produced by DHT in the absence of FT.
Fig. 3.16  Effects of anti-androgenic drugs on IL-6 production. Normal GF were incubated with CPA or FT alone and in the presence of 20 ng/ml of DHT. Untreated cells were used as controls, and the supernatants of each culture analysed for IL-6 by ELISA. The values shown represent the means ± SD of three replicate experiments.
Fig. 3.17  RT-PCR analysis of the effects of DHT on IL-6 and GAPDH mRNA levels. GF were incubated in the absence (lane 1) and presence of DHT (lane 2). The figure shows the ethidium bromide staining of the cDNA amplification product after 25 cycles, visualised under uv illumination. Note the difference in relative band intensities of IL-6 compared with GAPDH in the control and DHT-treated culture.
(approximately 30% of control levels). Thus, FT had no effect on IL-6 levels in the absence or presence of DHT.

In order to establish whether the inhibitory effect of DHT on IL-6 secretion occurred at the level of mRNA transcription in the cells, gingival fibroblasts were grown and incubated with 20 ng/ml of DHT for 5 days, as described in the Materials and methods. Untreated cells were used as controls.

Total RNA was then extracted and the level of mRNA determined by semi-quantitative RT-PCR. The number of cycles was chosen so that the amplification reaction was in a linear range and was determined as described previously for the AR. Both GAPDH and IL-6 were amplified at 25 cycles. Fig. 3.17 shows a representative ethidium bromide-stained agarose gel of the IL-6 and GAPDH PCR products. Whereas the ratio of the IL-6:GAPDH transcripts in the untreated gingival cultures was 0.74, in cultures incubated for 5 days with 20 ng/ml of DHT the ratio decreased to 0.36. Thus, comparison of the relative effects of DHT on the activity of these two genes showed that IL-6 transcription was selectively reduced in cells treated with the androgen, to approximately 50% of the level of IL-6 mRNA in control, untreated cells.

3.3 Discussion

While a number of studies have shown that periodontal tissues are capable of metabolising sex hormones and thereby able to form their respective potently active metabolites (Vittek et al., 1979; Ojanotko et al., 1980; Soory and Gower, 1990), thus far neither ER nor AR have been conclusively demonstrated in these tissues. In the present study the RT-PCR procedure was used, this allows rapid detection of mRNA from a very small amount of sample, enabling examination of the expression of ER and AR in periodontal tissues and in fibroblasts derived from these tissues. It is possible to obtain false-positive results with this technique and therefore several forms of control were included to ensure that there was no contamination of genomic DNA or cDNA. These controls included designing a specific PCR primer pair such that they
spanned intron-exon boundaries and blank controls during each PCR reaction. The samples were also examined for the presence of GAPDH message. The presence of this message is often used as a control to indicate the amount and integrity of mRNA and as an internal control in quantitative PCR because it is constitutively expressed at high levels in nearly all tissues and cells. The data obtained from these experiments showed that the expression of the AR is readily detected in a high proportion of the gingival and PDL tissue samples and in all of the fibroblasts cultured from these tissues. However, ER transcripts was not detected in any of the samples tested, in agreement with a previous immunological study of oral mucosa (Ojanotko-Harri et al., 1992). The reason why earlier studies concluded that ER were expressed in gingival tissue and PDL cells grown in vitro (Vittek et al., 1982; Lewko and Anderson, 1986; Forabosco et al., 1992) is not clear, but may be due to the lack of specificity of the techniques used in the earlier experiments.

The results in this study strongly suggest that the AR but not the ER is expressed in gingival and deeper periodontal tissues. The AR binds two biologically active androgens, testosterone and dihydrotestosterone, and acts as a transcriptional regulatory protein that plays a major role in male differentiation and development (Xhou et al., 1994; Lindzey et al., 1994). However, the binding kinetics of these two androgens are different in that testosterone has faster association and dissociation than DHT (Grino et al., 1990). In addition to binding these androgens, the receptor also binds a variety of other synthetic steroid hormones including androgen analogues such as hydroxyflutamide and cyproterone acetate (McLeod, 1993; Kallio et al., 1994). Since the AR is expressed in many other tissues, androgens are therefore considered to be the targets for a wide variety of tissues.

While there is some evidence to suggest that oestrogens have a role in CIPD, the present observations indicate that any pharmacological effects exerted by oestrogen on the tissues are indirect but that at least some gingival and periodontal cells are potentially directly responsive to testosterone. Therefore, gingival inflammation seen during sex hormone imbalance in vivo could be due to secondary effects of oestrogen, perhaps on the leukocytic
infiltrate which is present in inflamed periodontal tissue. ER have been detected in peripheral blood cells including T cells and macrophages (Stimpson, 1988; Gulshan et al., 1990). Furthermore, oestrogens have been shown to regulate macrophage phagocytic activity (Boorman et al., 1980) and the production of IL-1 (Hu et al., 1988). In marked contrast, testosterone and DHT, acting directly on the periodontium, may have an important anabolic role in connective tissue and bone repair processes. Thus, in CIPD, when anabolic activity in affected tissue is considerably reduced, the effects of testosterone would be of maximal importance. Elevated levels of hormone-receptor complexes may thereby contribute to the repair of the wound, for example, by increased ECM synthesis by fibroblasts. Androgens are known to be the primary regulator of growth and differentiation of male sex tissues and although gingival tissues may be responsive to androgens their role in gingival function is not understood. Many studies have indicated that DHT has a proliferative effect on cell lines derived from prostate (Sato et al., 1993; Ashton et al., 1995). The present study, in contrast, showed that DHT had no effect on gingival and periodontal cell growth in agreement with the work of Bebo et al (1999), however, they demonstrated that androgens altered the synthesis of specific cytokines in AR-positive mouse T cells without affecting proliferation. Similarly, oestrogen has been shown to have no effect on the growth of ER-positive osteoblast-like cells but enhances collagen synthesis in these cells (Komm et al., 1987). In the present study, it has been demonstrated that DNA synthesis was increased significantly in the presence of a variety of growth factors compared with the control cultures. Furthermore, pre-treatment with DHT resulted in synergistic enhancement of DNA synthesis in response to PDGF and TGF-β compared with growth factors alone. In contrast, IGF-I had no significant effect on DNA synthesis following pre-treatment with DHT. Numerous studies have shown that healthy gingival tissue has the ability to form DHT from testosterone and that this activity is increased, particularly, during inflammation and in GH (Soory et al., 1988, 1990; Soory and Gower, 1989). The metabolism of testosterone has been studied extensively in gingival fibroblast cultures and has been shown to be further enhanced in
response to a number of growth factors and cytokines including PDGF, TGF-β, IGF-I and IL-1 when used singly but there was variable response when used in various combinations (Kasasa and Soory, 1996a; 1996b, 1998; Soory and kasasa 1997). Thus, DHT synthesis was less when a PDGF/IGF combination was used compared with the individual factors alone, whereas PDGF/TGF-β resulted in elevated DHT synthesis when compared with each of the growth factors alone. Similarly, DHT levels were elevated in gingival fibroblasts when treated with IL-1 and EGF in combination with PHT. These findings together suggest that the growth factors, drugs and cytokines investigated act together to influence the androgen metabolic pathway, which in turn can stimulate matrix synthesis and thus contribute to periodontal repair processes. In addition, it has been reported that the mitogenic action of androgen in prostate-derived cells may involve up-regulation of growth factor/growth factor receptors (Fiorelli et al, 1991; Eaton et al, 1991; Ashton et al, 1995). Taken together, these results suggest that the actions of androgens may be mediated by an autocrine loop involving the growth factors/growth factor receptors. The role of growth factor receptors in gingival and PDL cells will be discussed in a later chapter (Chapter 4).

Although androgen metabolism has been studied extensively in gingival tissues and cells, very few studies have examined AR expression in these tissues. Since the androgens exert their effects by interacting with intracellular receptors, therefore, it was pertinent to investigate their expression in GH. In the present study, tissue levels of AR mRNA were measured using the RT-PCR technique. In order to compare the levels of mRNA by RT-PCR, it was first necessary to determine the exponential phase of amplification. This was done by measuring the band intensities of PCR products after different numbers of cycles. The results obtained showed that the drugs did not appear to up-regulate the level of expression of AR in GH. In a series of experiments, the effects of the study drugs on AR protein levels were determined in normal and hyperplastic gingiva-derived fibroblasts by FCM. This procedure enables certain physical and chemical characteristics of individual cells to be measured simultaneously as they pass through a laser beam and when cells have been
immunostained using fluorochrome-conjugated antibodies, the emitted fluorescence light measures the relative levels of antigens. The FCM analysis showed that the antigen was undetectable with the (mouse) monoclonal antibody but positive staining was obtained with (rabbit) polyclonal antibody to a MCF-7 cell line, which is known to express the AR. The reason for this could be that AR in the absence of hormones interact with a number of proteins including ‘Heat shock proteins’ (Hsp) and cyclophilins (cyclosporine binding proteins) (Zhou et al., 1994) which could mask the epitopes recognised by the monoclonal antibodies. However, monoclonal antibodies have been used for immunohistochemical staining of tissue sections following antigen retrieval using a variety of fixation techniques (Williams et al., 1997) but such techniques are not feasible for FCM analysis. In this study, GF used were explanted from both healthy and overgrowth gingiva because studies have suggested variability of individual cell lines to susceptibility to the study drugs (Tipton and Dabbous, 1989). These authors examined gingival fibroblasts exposed to CsA and suggested that the effects of the drug on fibroblast metabolism was dependent on the presence of CsA-responsive cell subpopulations. It has also been shown that the primary cells derived from these tissues, at least in early passages, maintain their characteristics which can be correlated with their behaviour in vivo (Wakabayashi et al., 1997). Jarvis and Hassell (1984) using GF from healthy and inflamed gingiva, evaluated cell response to PHT and showed that matrix synthesis was greater in gingival cells from inflamed compared with healthy tissue. Similarly, Bartold (1989) compared response of fibroblast from healthy and overgrowth gingiva to CsA and concluded that CsA could stimulate both cell groups to synthesise DNA but the fibroblasts from healthy gingiva responded to lesser extent. NIF has also been shown to differentially affect gingival fibroblasts from responders and non-responders in the same way (McKevitt and Irwin, 1995). Since the effect of the study drugs was evaluated by treating each cell culture with drug alone, the pathogenic mechanism in vivo may be expected to involve cytokines and/or growth factors and their receptors.
A number of studies have suggested that sex hormones may also have an important role in periodontal diseases, since gingival inflammation is frequently seen during puberty, pregnancy and menstruation (Loe, 1965; Hugoson, 1970; Nyman, 1971; Sutcliffe, 1972; Mombelli et al., 1989). Gingival inflammation also often occurs in women taking oral contraceptives (Kalkwarf, 1978; Pankhurst et al., 1981). While it is clear that sex steroids are closely associated with periodontal physiology and pathology, the underlying mechanisms of androgens have not yet been delineated. However, studies of other tissues and cells have suggested that male sex hormones and IL-6 are closely associated at the molecular level. This is evidenced by the down-regulation of the cytokine by androgen in bone marrow-derived cells (Girasole et al., 1992) and by the bone loss caused by sex steroid deficiency, which has been shown to be mediated by IL-6 (Greenspan et al., 1986; Stepan et al., 1989). In order to examine the relationship between androgens and IL-6 in periodontal diseases, the present study therefore examined the role of DHT-AR complex on IL-6 production by GF in tissue culture.

This study has demonstrated that increasing concentrations of DHT progressively down-regulated IL-6 secretion by normal gingival cells, and that this inhibition of IL-6 production progressively increased during the period of cell culture. This was not due to failure of the cells to secrete the cytokine in the presence of the androgen, since the results of semi-quantitative RT-PCR confirmed that the hormone was acting at the transcriptional level to down-regulate IL-6 mRNA. Although the IL-6 gene promoter itself does not have a DNA consensus sequence for this complex, it has a glucocorticoid response element (GRE) which is also able to bind the androgen-activated AR (Roche et al., 1992).

The AR has affinity for other steroids in addition to testosterone and DHT including synthetic anti-androgenic drugs (Zhou et al., 1994). To determine whether the inhibitory effect of DHT on IL-6 synthesis by gingival cells was mediated by the AR, the effects of steroidal (CPA) and non-steroidal (FT) drugs, which have been reported to compete with androgens for the AR binding site (Matsuda et al., 1993), were analysed. However, it was found that
CPA acted as an androgen analogue and, like androgens, potently inhibited IL-6 synthesis in both the absence and presence of DHT. In contrast, FT in the absence of DHT had no effect on IL-6 expression and failed to abrogate DHT inhibition of IL-6 synthesis. Thus, the action of FT differed notably from that of CPA and also FT did not act as a conventional antagonist, as it has been shown to do in other cells (Matsuda et al., 1993; Zhou et al., 1994). The reason for these unexpected effects of the drugs is not known, but it appears likely that CPA and DHT both interact with the AR and both down-regulate IL-6 synthesis by binding to the GRE present in the IL-6 promoter, as discussed above. In contrast, it appears from the results that the FT binding may have caused conformational modification of the AR in these cells which would have rendered the FT-AR complex unable to bind to the consensus sequence of the IL-6 promoter. Similar agonist- and antagonist- induced changes in conformation have previously been shown to have a marked influence on the functional activity of the progesterone receptor (Zhou et al., 1994). The results of this study also show that androgen effectively reduced the levels of IL-6 secreted by inflamed and hyperplastic gingival fibroblasts as well as markedly down-regulating IL-6 production by normal gingival cells. It was striking, however, that PDL cells secreted only barely detectable levels of this cytokine, in the absence or presence of the hormone. Despite the failure of the PDL cells to produce IL-6 directly, however, the cytokine is nevertheless likely to be involved in the initiation and progression of periodontal disease. This may occur via up-regulation of IL-6 production by gingival connective tissue cells exposed to oral bacteria, viruses and bacterial products such as lipopolysaccharides (Geivelis et al., 1993; Irwin and Myrillas, 1998). Elevated levels of IL-6 in periodontal sites is not matched by increase in serum level indicating local production of the cytokine. The work of Williamson et al. (1994) showed increased levels of IL-6 in GH compare with normal gingival tissue and the authors suggested GF as the main source of the cytokine. Moreover, recent study by Myrillas et al. (1999) confirmed the work of Williamson et al. (1994) but the study disagreed on the cellular source of IL-6 and postulated that the infiltrating mononuclear cells may be the source of this cytokine. IL-6
may also play a role in pathological conditions involving bone loss (Greenspan et al., 1986; Stepan et al., 1989). Therefore, the pathogenic effects of IL-6 on osteoclast activation, loss of bone and damage to the periodontium may be most strikingly manifest when androgen deficiency or dysfunction fails to down-regulate the IL-6 gene (Bellido et al., 1995). In a recent study, Gornstein et al. (1999) came to the same conclusion as the present study regarding the androgen-mediated inhibition of IL-6 production by GF. In addition, the authors showed that flutamide did not reverse the DHT-induced down-regulation of the cytokine.

In conclusion, the results of this study established that the androgen receptor but not the oestrogen receptor is expressed in periodontal tissues. This demonstrated that the periodontium does not respond to oestrogen but may be under direct influence of androgens which are likely to be of major importance in periodontal repair process. The results showed that no substantial differences in the level of expression of AR were detected in normal and hyperplastic gingival tissue. In addition, the drugs that cause GH in vivo did not appear to up-regulate the expression of AR in vitro. Thus, there was no difference in the amount of AR in normal and hyperplastic gingival tissue and in cells treated with the drugs. In contrast, the activity of the AR was found to be important in the pathogenesis of periodontal diseases, since androgens were found to down-regulate IL-6, a cytokine involved in connective tissue and bone turnover.
CHAPTER 4
FLOW CYTOMETRIC ANALYSIS OF GROWTH FACTOR RECEPTOR EXPRESSION BY GINGIVAL AND PDL CELLS

4.1 Introduction

Growth factors are naturally occurring biological mediators which play a fundamental part in the complex wound healing events that control tissue repair and remodelling (Clark and Henson, 1988). Such processes have a major role in the normal turnover of periodontal tissues and in periodontal repair and regeneration following the loss of tissue as a result of CIPD (Ramney, 1993; Loe, 1993). Studies in vivo and in vitro have suggested that growth factors such as PDGF, TFG-β, FGF, EGF and IGF may play an important part in periodontal wound healing. Several recent studies have also shown an association of GH with growth factors including PDGF, TGF-β, EGF and FGF in vitro and in vivo (Modeer et al., 1990; Dill, et al., 1993; Nares, et al., 1996; Plemons, et al., 1996; Iacopino, et al., 1997) and GH resembles other hyperplastic lesions in which growth factors have been implicated (Annoni et al., 1992; Logan et al., 1994; Becks et al., 1994; Finkelstein et al., 1994). Furthermore, a number of studies have implicated sex steroids, particularly androgens, in the development and progression of hyperplasias and their effects have been shown to be mediated through the modulation of growth factors and/or their receptors (Fiorelli et al., 1991; Polimeno et al., 1994; Sciarrà, 1995). The response of target cells to growth factors depend on the expression of their specific receptors. These receptors are transmembrane antigens which, on binding of the respective growth factors, produce a cascade of intracellular signals that stimulate chemotaxis, cell growth, differentiation and the production of ECM (Matsuda, et al., 1992; Graves and Cochran, 1994). The growth factor receptors are therefore also likely to be of fundamental importance not only in the growth and regeneration of the periodontium but also in the pathogenesis of GH. However it remains unclear as to how these various parameters are interacting. The aim of this study, therefore, was to compare growth factor receptor expression by normal GF and PDL cells in vitro
and examine the effects of DHT and CsA on the growth factor receptor expression by GF.

4.2 Materials and methods

The cells for growth factor receptor expression were grown as described in section 2.1.2. To examine the effects of growth factors present in serum, confluent cells grown in complete medium was replaced with serum free medium for three days. To examine the effects of DHT and CsA on the growth factor receptor expression, the cells were treated with these reagents as described in 2.2.1 and 2.2.3, respectively. The cells were then analysed by FCM as described in section 2.5.

4.3 Results

4.3.1 Expression of growth factor receptors by GF and PDL cells

The relative levels of the growth factor receptors in the cultured GF and PDL cells were measured by FCM. Cells were either grown in complete medium or in medium depleted of serum for three days in order to examine the possible effects of factors which are likely to be present in the FCS. Fig. 4.1 shows representative histograms of the fluorescence profiles of the receptor expression in the GF and PDL cells cultured in complete medium. Similar profiles were obtained when cells were grown in the absence of serum (data not shown). Neither the GF nor PDL cells expressed the PDGF-Rα or EGF-R (Fig. 4.1 a and e, respectively) but both types of cell appeared to express the PDGF-Rβ, TGF-RβI, -RβII and IGF-R, showing a narrow, bell-shaped peaks of fluorescence. However, while the positive fluorescence profiles of PDGF-Rβ in the GF and PDL cells were exhibited by the entire cell population in the cell culture as demonstrated by the shift of the peak to higher fluorescence intensity (to the right compared with the control) (Fig. 4.1b), the shift of the peaks of TGF-βRI, -βRII and IGF-R (Fig. 4.1 c, d and g respectively) were considerably lower, respectively, and involved only a proportion of the total cells.
Fig. 4.1  Representative histograms of growth factor receptor expression by GF and PDL cells shown as red lines (a) PDGF-Rα (b) PDGF-Rβ (c) TGF-βRI (d) TGF-βRII (e) EGFR (f) FGFR (g) IGFR. The corresponding negative control profiles are presented as green lines. Note that there is no expression of PDGF-Rα and EGF. In contrast, PDGF-Rβ is expressed by all the cells whereas TGF-βRI, TGF-βRII, FGF-R and IGF-R is expressed differentially. Fluorescence intensity units are arbitrary.
Fig. 4.2 Comparison of growth factor receptor expression by GF and PDL cells. The results show (a) the receptor density (b) % of positive cells cultured in the presence of serum. 1, PDGF-Rβ; 2, TGF-βRI; 3, TGF-βRII; 4, FGF-R; 5, IGF-R. Data are represented as mean ± SD of four replicate experiments. * indicates significantly different from GF at P < 0.05.
Unlike PDGF-Rβ, TGF-βRI, -βRII and IGF-R, the FGF-R fluorescence profile was not uniform but showed a broader and more extended distribution. These experiments were repeated twice for each individual cell culture and both the antigen levels (AFI) and the percentage positive cells calculated by gating the negative control fluorescence profile. Fig 4.2 is a summary of 4 individual experiments in which the receptor levels and the proportion of antigen-expressing cells are compared in cultures of GF and the PDL cells. The receptor density of PDGF-Rβ was found to be lower in the GF compared with PDL cells (AFIs of 37 and 50, respectively), although this difference was not statistically significant. The percentage of positive cells was also higher in the GF compared with the PDL cells (90% and 75% of the total cells, respectively). TGF-βRI expression was higher in the GF compared with PDL cells (AFIs of 28 and 21, respectively) but this was not significant, although approximately 50% of both cell types expressed this receptor. In contrast, although TGF-βRII was expressed at a lower level in GF compared with the PDL cells (AFIs of 19 and 24, respectively), only 18% of the latter cells were positive for this receptor compared with 50% of the GF cells. As with PDGF-Rβ, the AFI values for FGF-R were lower in GF than in the PDL cells (AFIs of 25 and 32, respectively), although the proportion of receptor-positive GF cells were significantly higher (28%) than the PDL cells (12%).

The same experiments were also carried out using replicate cell cultures incubated for 3 days in the absence of FCS, in order to examine the possible effects of a range of unknown quantities of many mediators, including growth factors, which are likely to be present in the FCS. It was notable, however, that in cells incubated in the absence of FCS there was no evidence of a statistically significant changes in the receptor density or the proportion of receptor-positive cells. The results of these experiments are summarised in table 4.1. For negative controls for the above experiment, the cells were treated with non-specific mouse IgG primary antibody or with the FITC conjugated secondary antibody. The AFI values were low with mean of 6 and the profiles for these cells were narrow bell-shaped and are represented as green lines.
Fig. 4.3 Growth factor receptor expression by GF in non-treated (dashed line) and DHT-treated (solid line) cells. Background staining of IgG control antibody has been subtracted for each value. Note that there was no difference between the non-treated and DHT-treated cells.
Fig. 4.4  Representative histogram showing the effect of DHT on IGF-R expression in GF. Untreated cells (blue, shaded), DHT treated cells (red line) and corresponding negative control is shown as green line. Note the shift of DHT treated histogram to lower fluorescence intensity.
Fig. 4.5  Effects of DHT on IGF-R expression by normal GF. Note the dose-dependent decrease of receptor-positive cells.
Fig. 4.6 Growth factor receptor expression by GF in non-treated (dashed line) and CsA-treated (solid line) cells. For clarity only half the drug-treated cells are shown. Background staining of IgG control antibody has been subtracted for each value. Note that there was no difference between the non-treated and CsA-treated cells except for FGF-R. After incubation with CsA, FGF-R histogram shifted marginally to a higher fluorescence level with no apparent change in antigen distribution.
Table 4.1 Growth factor receptor density (AFI) on GF and PDL cells as measured by FCM

<table>
<thead>
<tr>
<th>Receptor type</th>
<th>GF (+serum)</th>
<th>GF (-serum)</th>
<th>PDL cells (+serum)</th>
<th>PDL cells (-serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGFR-β</td>
<td>37 ± 14</td>
<td>44 ± 8</td>
<td>50 ± 19</td>
<td>56 ± 23</td>
</tr>
<tr>
<td>TGFβ-RI</td>
<td>28 ± 10</td>
<td>30 ± 11</td>
<td>21 ± 3</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>TGFβ-RII</td>
<td>19 ± 2</td>
<td>26 ± 6</td>
<td>24 ± 3</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>FGF-R</td>
<td>25 ± 6</td>
<td>36 ± 6</td>
<td>32 ± 12</td>
<td>36 ± 13</td>
</tr>
<tr>
<td>IGF-R</td>
<td>23 ± 6</td>
<td>26 ± 5</td>
<td>59 ± 9</td>
<td>60 ± 10</td>
</tr>
</tbody>
</table>

4.3.2 The effects of DHT and CsA on growth factor receptor expression by GF

In order to examine the effects of DHT, gingival cells were incubated with DHT for 7 d in the presence of 0.2, 2.0 and 20 ng/ml DHT and the growth factor receptor expression analysed by FCM. Untreated cells served as controls. DHT had no effect on the levels of PDGF-Rβ, TGF, FGF and EGF receptor expression and the shapes of the peaks also remained unchanged (Fig 4.3). No expression of PDGF-Rα and EGF-R was seen after DHT treatment (data not shown), however, DHT did affect IGF-R expression markedly. A representative fluorescence profile of IGF-R expression in GF obtained is shown in Fig. 4.4. In the non-treated cells, which had a relatively uniform distribution, high proportion of cells were positive for the receptor. However, on incubating the cells with DHT there was dose-dependent decrease in the proportion of receptor-positive cells. These experiments were repeated three times and the results summarised in Fig. 4.5. The receptor densities of treated cells compared with untreated cells were similar. Untreated cells had AFI values of 42, whereas, DHT treated cells at concentration of 0.2, 2.0 and 20 ng/ml had values of 40, 40 and 44, respectively.
Similarly, gingival cells were treated with CsA as described in section 2.2 and the growth factor receptor expression analysed. Fig. 4.6 shows a representative histogram of three experiments. There was no appreciable effect of the drug on the growth factor receptor expression in these cells except for FGF-R, where there was slight shift in histogram to the right compared with the untreated cells with no apparent change in antigen distribution, but this was not significant.

4.4 Discussion

Growth factors receptors are of fundamental importance in mediating the cellular response to the corresponding factors, but their expression in human gingival and PDL cells is not well defined. The demonstration of growth factor receptor expression by such cells is likely to identify the specific growth factors which could significantly affect periodontal growth and renewal.

Growth factors on binding to their respective cell receptors stimulate chemotaxis, proliferation, differentiation and synthesis of components of the ECM through a complex signal transduction cascade which results in modulation of genes involved in growth controlling activities. Therefore the interaction of growth factors to their receptors on PDL cells play an important role in wound healing and regeneration. Furthermore, in vitro studies have demonstrated the mitogenic effect of different growth factors on gingival fibroblasts suggesting that stimulation of fibroblasts by growth factors could enhance new periodontal attachment formation. Since fibroblasts are the main cell type of gingival connective tissue, their stimulation by growth factors could result in overproduction of ECM components that ultimately cause GH. In this study, a number of growth factor receptor expression including PDGF-Rα and -Rβ, TGFR-β1 and -βII, EGF-R, FGF-R and IGF-R were analysed by FCM.

The ligand for PDGF-R, PDGF, is composed of two structurally related but distinct polypeptide chains designated A and B. Three isoforms of PDGF (AA, AB, BB) with difference in their biological activities and potencies have been identified and their effects are mediated through two distinct receptors PDGF-Rα and -Rβ. The α receptor binds all PDGF isoforms, while β receptor
bind PDGF-BB with high affinity and PDGF-AB with low affinity but does not bind PDGF-AA. Thus, the type of PDGF receptors present on cells will likely to determine the response to various PDGF isoforms. On PDL cells, BB has been shown to be the most potent stimulator of mitogenesis followed by AA and AB. Previous studies have demonstrated that the PDL cells responded to all forms of PDGF in dose-dependent manner including AA, however, the BB was the most potent (Matsuda et al., 1992; Boyan et al., 1994) suggesting that the α-receptor as well as the β-receptor is likely to be expressed by the PDL cells, as also reported by Oates et al. (1995), although at a lower level. In contrast, the results of the present study clearly show that in both the GF and PDL cells, β receptor was present in a high proportion of these cells whereas α receptor could not be detected by FCM. This discrepancy may have arisen because the functional studies are carried out in cultured cells and could be partly explained by different techniques used and receptor expression may be altered under different culture conditions, particularly by other growth factors (Ishikawa et al., 1990; Oates et al., 1998). Moreover, the degree of confluency of the cultured cells also seems to affect the level of receptor expression (Tiesman and Rizzino, 1989). Nevertheless, the present study suggests that both the GF and PDL cells should be responsive to the corresponding ligands, PDGF-AB and -BB. Furthermore, a previous study has shown that these factors co-localise with the receptor in a gingival wound healing model (Green et al., 1997). Moreover, this same study also demonstrated the absence of the α-receptor in both healthy and healing gingival wound sites. Similarly, in a recent study Kuru (1998) examined normal gingival tissue and the PDL by immunocytochemistry and showed that there was a moderate expression of the β receptor in both the tissues but the α receptor was not detected in any of the tissues. Taken together, these results suggest that the PDGF-Rα and its corresponding ligand may have a restricted role in periodontal growth and regeneration. A number of studies have demonstrated that the PDGF-BB growth factor is up-regulated in GH and may be a factor in the development of this lesion (Dill, et al., 1993; Nares, et al., 1996; Plemons, et al., 1996; lacopino, et al., 1997). Since the present study has shown that GF expressed PDGF-Rβ ubiquitously, this
implies that all the cells are responsive to the growth factor. However, this conflicts with the idea that specific sub-populations of cells may be sensitive to GH.

Six types of TGF-βR have previously been described in a number of cells but only the types RI to RIII have been extensively investigated thus far. Types I and II but not the type III are involved in intracellular signal transduction processes, although the latter receptor may aid in enhancing the binding of the growth factors to type I and II receptor complex (Massaguè, 1994). Five different isoforms of TGF-β have been identified and only TGF-β1 to -β3 are expressed in mammalian tissues but the affinity of these isoforms for the different TGF-β receptors has not yet been clearly delineated. The present study demonstrated that both the gingival and PDL cells expressed TGF-βRI and -RII receptors but to a different extent. Whilst similar proportion of gingival cells were found to express both types of receptors, greater proportion of PDL cells expressed type I compared with the type II. In an immunohistochemical analysis of normal and GH tissue, Saito et al. (1996) were able to detect type II TGF-βR in normal tissue but elevated level of the receptor was observed in hyperplastic tissue. Similarly, Kuru (1998) examined TGF-β type I and II receptor expression in normal gingival connective tissue, PDL and regenerated tissue (RT) by immunohistochemistry. Although this work showed weak expression of the receptor in normal gingival and PDL tissues, the receptor expression was shown to be up-regulated in the RT.

Previous studies on the expression of the EGF-R have shown that although this receptor was not expressed in normal gingival tissue, positive staining has been observed in stromal cells of inflamed tissue (Irwin et al., 1991). However, Cho et al. (1988; 1991) showed that the EGF-R was expressed by both the precursor and mature PDL cells in vivo. GF in vitro also expressed low levels of this receptor, which were found to be elevated in cells from gingival hyperplasia patients (Modeer et al., 1990). However, despite the presence of this receptor on PDL cells, EGF did not have a mitogenic or chemotactic effect on the cells (Matsuda et al., 1993). Similarly, the growth factor has been shown to have only a limited and variable mitogenic and
chemotactic effects on normal GF (Matsuda et al., 1992; Blom et al., 1994). The expression of EGF-R observed in PDL precursor cells in vivo (Cho et al., 1988; 1991) could be due to the presence of undifferentiated cells which on differentiation decrease their receptor level, as also suggested by the down-regulation of EGF-R in differentiated PDL cells in vitro (Matsuda et al., 1992). In a recent study, Matsuda et al. (1998) investigated the role of EFG-R in PDL cells under mechanical stress conditions in vitro. These workers used ‘western’ blotting technique to examine the level of EGF-R protein and demonstrated that the receptor level decreased in stress induced differentiated PDL cells. In contrast, this study using FCM has shown that both the gingival and PDL cells lack the receptor and this was confirmed by RT-PCR analysis.

FGFs are members of the heparin-binding growth factor family which have diverse functions including mitogenesis and ECM formation and have potent angiogenic activity in vivo. Two of the best characterised forms are aFGF and bFGF which bind to the same receptor. A number of in vitro studies have examined the effects of FGF on gingival and periodontal cells (Terranova et al., 1989; Blom et al., 1994; Takayama et al., 1998) and two classes of receptors have been identified in PDL cells, with low and high affinity (Takayama et al., 1998). The present FCM analysis of both the gingival and PDL cells demonstrated that only a proportion of the total cells were positive for the FGF-R (which was higher in GF compared with the PDL cells), the broad distribution of the fluorescence profile suggesting that the two classes of receptors are expressed at different levels. Immunohistochemical staining of normal and drug-induced hyperplastic tissue was examined by Saito et al. (1996) and these authors were able to detect the receptor in normal gingival connective tissue but observed higher expression in hyperplastic tissue. In contrast, the work of Kuru (1998) showed negative staining for this receptor in both the normal gingival and PDL tissues whereas RT tissue showed very weak staining.

IGF-I has been shown to have a mitogenic effect on mesenchymal cells, including periodontal cells (Blom et al., 1992; Matsuda et al., 1992) and ligand-binding analysis has also demonstrated the presence of IGF-R on normal PDL
cells *in vitro* (Blom *et al.*, 1992). Although *in vivo* use of IGF-1 alone has been shown to have some effect on periodontal regeneration (Lynch *et al.*, 1991; Rutherford *et al.*, 1992), studies using this growth factor in combination with PDGF-BB has been shown to accelerate bone regeneration (Howell *et al.*, 1997). In the present study, the expression of IGF-R was confirmed in GF and PDL cells. Furthermore, this study also demonstrated that a high proportion of GF expressed the receptor compared with the PDL cells, but this was not significant. This study also examined the effects of both DHT and CsA on growth factor receptor expression by GF. Although CsA had no effect on the expression of any of the growth factors, DHT reduced the proportion of IGF-receptor positive cells in dose-dependent manner similar to its inhibition of IL-6 synthesis. It is interesting to note that when the effects of growth factors on GF proliferation were examined (section 3.2.3.1), there was significant increase in DNA synthesis when PDGF and TGF-β was added to DHT pre-treated cells. In contrast, there was no significant increase in proliferation when the cells were treated with IGF-1 further confirming the results obtained with the DHT mediated down-regulation of the IGF-R receptor expression.

In conclusion, FCM analysis demonstrated that although the PDGF-Rβ and IGF-R were present in a high proportion of both the GF and PDL cells, these same cells did not seem to express either the PDGF-Rα or EGF-R. Moreover, this study also demonstrated that the TGFβ-RI, -RII and FGF-RI were expressed by a sub-populations of cells showing that only this subset of cells is likely to be responsive to the corresponding growth factors. Furthermore, DHT had no effect on growth factor receptor expression with the exception of IGF-R. The hormone down-regulated the expression of the receptor in dose-dependent manner. However, CsA increased the expression FGF-RI but this was not significant and it did not affect the expression of the other growth factor receptors. Thus, the FCM technique has highlighted the presence of diverse sub-populations of cells which are likely to have functionally distinct roles in periodontal growth and renewal.
CHAPTER 5
EXPRESSION AND SPlicing OF THE FIBRONECTIN GENE IN
PERIODONTAL DISEASE AND RENEWAL

5.1 Introduction

FN is cell adhesion protein that mediates a variety of cellular functions including phagocytosis, cell migration, cell adhesion and cell differentiation (Hynes, 1990). These biological activities result from its ability to interact with both cell surface and ECM components (Wayner et al., 1988; Takada et al., 1988). Although FN is coded by a single gene, there is nevertheless considerable heterogeneity between the molecular forms of the protein expressed in different tissues. This diversity is due to alternative splicing of the primary transcript. Most mammalian genes contain regions called exons that are separated by non-coding regions called introns. Both of these regions are transcribed and the RNA is processed by intron excision to form mRNA. However, the presence of different splice sites within the RNA molecule leads to the inclusion and/or exclusion of exons. This mechanism is called alternative splicing and gives rise to structurally related but functionally different proteins. In FN, this occurs in each of the three distinct regions, referred to as extra domain A (ED-A), extra domain B (ED-B) and type-III connecting sequences IIICS (Kornblitht et al., 1985; Norton and Hynes, 1987) (Fig. 5.1). Single splice sites in the former two regions give rise to mature transcripts in which ED-A and ED-B are either included or exon-skipped, whereas the type IIICS segment exists in five different forms because of multiple splice sites within this exon (Schwarzbauer et al., 1983; Sekiguchi et al., 1986; Zardi et al., 1987). These variants are named IIICS-0, IIICS-64, IIICS-89, IIICS-95 and IIICS-120 according to the number of amino acid residues generated in the IIICS region of the resulting FN polypeptides.

Although the functional roles of these different molecular forms are not yet known, their expression has been shown to be markedly affected in a number of situations. For example, changes in FN splicing profiles have
Fig 5.1 The generalised structure of the FN protein, showing the amino-terminus on the left and carboxy-terminus on the right. Types I, II, III amino acid repeating sequences are indicated by the open rectangles, triangles and ovals, respectively. The regions encompassing the various functional binding sites are also shown. The diagram also shows the corresponding regions of the FN gene which are subject to alternative splicing. In exons ED-A and ED-B, these regions are indicated by the solid ovals and show that only the exon-included and exon-skipped isoforms are generated. The 5 variants produced in the IIICS region, indicated by the large open and striped rectangles, include 3 which arise from partially-skipped exons. bp : base pairs.
been demonstrated in regenerating rat liver (Caputi et al., 1995), during cardiac ontogeny (Farhadian et al., 1994) and in hypertensive disease (Tasaki et al., 1992), in neoplastic human breast tissue (Kaczmarek et al., 1994) and in cutaneous wound healing (Brown et al., 1993). Changes in FN splicing have also been found during ageing processes, both in vitro and in vivo (Magnusson et al., 1991).

However, despite the importance of FN in the integrity and pathology of periodontal tissues, the expression of the different molecular isoforms has not yet been investigated. In this study, therefore, it was examined, firstly, whether the normal GT, CIPD and GH tissues are associated with the presence of specific spliced variants and, secondly, whether growth factors influence the expression of FN isoform patterns in PDL cells in vitro.

5.2 Materials and methods

Total RNA was isolated and the RT-PCR performed as described in section 2.3.1 and 2.3.2, respectively. The cells were treated with growth factors as described in 2.2.2. The primer pairs and the annealing temperature used are shown in tables 2.1 and 2.2, respectively.

5.3 Results

5.3.1 FN splicing in gingival and PDL tissues

In this study, the alternative splicing of FN transcripts was examined in 5 each of, GT, GH and PDL samples and in 11 gingiva from patients with CIPD. Fig. 5.1 shows the general structure of the FN molecule and also illustrates the possible FN variants that can be generated by alternative splicing of the primary transcript. The exons corresponding to the domains designated ED-A and ED-B can either be retained in the mature FN mRNA, giving fragment sizes of 420 and 458 bp, respectively, or alternatively can be skipped, producing 151 and 185 bp fragments, respectively. Multiple splice sites in exon IIICS can lead to five isoforms, ranging from one where the entire exon is included (484 bp) to one in which the exon is fully-skipped (124 bp).
Fig. 5.2 RT-PCR analysis of alternative splicing of FN mRNA in healthy PDL tissue (lane 1) and in tissue from patients with CIPD (lane 2). The line shows the sizes of the low molecular weight, exon-skipped isoforms derived from the ED-A, ED-B and IIICS exons. The arrows indicate the fully- and partially-spliced FN variants.
Fig. 5.3  RT-PCR analysis of alternative splicing of FN mRNA in GH tissue. The arrows indicate the over-expression of the low molecular weight, exon-skipped isoforms derived from the ED-A and ED-B exons.
The primers used for PCR are located in the exons flanking these alternatively spliced exons, as noted above. The sequence of each of the primers and the predicted product sizes are shown in Table 2.1. Initially, all the cDNA preparations were examined by PCR using the primers for GAPDH, a standard housekeeping gene. This was done to assure the integrity of the extracted RNA and the subsequent fidelity of first strand cDNA synthesis. Only those samples which yielded the predicted 600 bp fragment were subsequently amplified with the FN primers. As a negative control, PCR amplification was performed without any template. No signal was produced under these conditions.

All alternatively spliced FN isoforms were found to be expressed in the extracts of healthy PDL (Fig. 5.2 Lane 1, indicated by arrows). Thus, two PCR products each were obtained for exons ED-A (151, 420 bp) and ED-B (185, 458 bp), the smaller fragments arising from exon-skipping while the larger products retained their respective exons. However, in exon IIICS, five PCR products were obtained from the normal tissue (124, 316, 391, 409 and 484 bp). The smallest is again due to the skipped exon, the largest includes the whole exon and the other products result from partial exon-skipping due to additional splicing sequences within the exon (Fig. 5.1). An identical pattern of expression was seen in all the GT and although GH samples showed similar pattern of expression, the isoforms corresponding to exons ED-A, ED-B in these tissues showed up-regulation (Fig. 5.3, indicated by arrows). In marked contrast to these normal and GH tissues, the CIPD tissues (Fig. 5.2, lane 2) generated only the low molecular weight exon-skipped FN transcripts, i.e., single PCR products of 151, 185 and 124 bp corresponding to exons ED-A, ED-B and IIICS, respectively. The above results were obtained consistently for each of the types of tissue examined, the figures shown being representative of the samples tested. The additional bands occasionally observed (for example, the ones just above the 420 and 458 bp bands in ED-A and ED-B, Fig 5.2) are due to PCR amplification artifacts. These also appear in Figs 5.3, 5.4 and 5.5 and arise during PCR amplification due to primer mis-annealing.
Fig. 5.4 The influence of growth factors on FN splicing in cultures of PDL cells. Lane 1, cells grown in 10% FCS; lane 2, cells grown in 1% FCS; lanes 3, 4 and 5 shows the effects of PDGF-BB (1, 4 and 20 ng/ml, respectively; lanes 6, 7 and 8 shows the effect of IGF-I (1, 10 and 100 ng/ml, respectively).
Fig. 5.5 Changes in FN splicing resulting from incubation of the PDL cells in the presence of 1, 5 and 10 ng/ml of the inflammatory cytokine IL-1β (lanes 1, 2 and 3, respectively). The arrows indicate the FN isoforms which have been relatively down-regulated by the highest cytokine concentration (lane 3).
5.3.2 Effects of cytokines and growth factors on FN splicing

To determine the effects of the cytokines and growth factors on FN splicing, cultures of PDL cells were incubated with only very low levels of FCS (0.5%) in order to reduce the possible effects of unknown amounts of the multiple factors which are present in serum. The RNAs were isolated from the cells after the cultures were incubated with various concentrations of the factors and the cDNAs prepared as described in the Materials and methods. PCR analysis revealed that the exon-skipped, low molecular size FN isoforms (ie. 151, 185 and 124 bp for exons ED-A, ED-B and IIICS, respectively) were expressed by all the cells under all conditions. However, the experiment in Fig. 5.4 shows that there were marked changes in the alternatively spliced isoforms which were produced when the cells were grown under varying conditions. Thus, although all isoforms were generated when the cells were grown in complete media containing 10% FCS (Fig. 5.4, lane 1), no full-length or partially-skipped FN transcripts were produced when the cells were grown under conditions of serum starvation (lane 2). Moreover, the addition of each of the growth factors induced the expression of specific FN profiles. Fig. 5.4 shows that cells treated with PDGF-BB at a concentration of 1 ng/ml produced only a relatively small amount of the larger isoform of ED-A and ED-B (lane 3), whereas incubation with 4 and 20 ng/ml of the growth factor resulted in elevated levels of these non-spliced transcripts (lanes 4 and 5). However, PDGF failed to elicit expression of the FN isoform retaining the entire IIICS exon until its concentration was raised to 4 ng/ml (lane 4). Similarly, treatment with IGF-1 at concentrations of 1, 10 and 100 ng/ml also enhanced the relative expression of the intact, exon-containing FN isoforms of ED-A and ED-B (lanes 6, 7 and 8), but even the lowest level of this particular factor was sufficient to induce multiple splicing in the IIICS region (lane 6). Identical results were obtained using the same concentrations of TGF-β.

In marked contrast to the dose-related effects of the growth factors, progressively increasing concentrations of the pro-inflammatory cytokine IL-1β caused a pronounced decrease in the relative expression of the intact and partially exon-skipped FN isoforms. Fig 5.5 shows changes in alternative
splicing in the ED-A, ED-B and IIICS resulting from incubation of the PDL cells in the presence of 1, 5 and 10 ng/ml of the cytokine IL-1β (Lanes 1, 2 and 3 respectively). Thus, whereas the highest levels of PDGF (Fig. 5.4, lane 5), IGF-1 (Fig. 5.4, lane 8) and TGF- β had up-regulated the expression of all the FN isoforms, the highest concentration of IL-1β (10 ng/ml, Fig. 5.5, lane 3) resulted in a notable decrease in the relative proportion of FN transcripts containing the intact ED-A and ED-B domains and also the loss of the non- and partially-skipped IIICS transcripts.

5.4 Discussion

ECM components play an important role in embryonic development as well as in remodelling and regeneration of damaged tissues. One of the major matrix molecules involved is FN, which has number of ligand binding domains that mediate the processes of cell adhesion and cell migration via its ability to bind both cell surface integrin receptors and other ECM molecules. Another important feature of FN is that, although it is transcribed from a single gene, it exists in several different molecular isoforms, which are temporally and spatially regulated. It is this diversity that gives the molecule its multifunctional role. For example, while normal adult tissue has been shown to express only the exon-skipped isoforms of the ED-A, ED-B and IIICS regions, the higher molecular weight transcripts in which the exons are included are considered to be characteristic of developmentally immature tissue where cell migration is active (Ffrench-Constant and Hynes, 1988; Ffrench-Constant et al. 1989). Although the reasons for the inclusion of spliced domains during embryonic development and wound healing are not clear, it has been suggested that the presence of these domains may facilitate the migration of cells by modulating the adhesiveness of the substrata (Chen and Culp, 1996). While fibroblasts normally bind to the RGD sequence of FN via the classical integrin receptor α5β1, in wound healing situation these cells express the integrin α4β1 which then bind to the IIICS domain of FN and thereby facilitating cell migration. Once cell migration into wounds is completed, fibroblasts increase the expression of the classical α6β1 integrin (Chan et al., 1992). The alternative splicing of FN RNA
has been analysed by a number of techniques including immunohistochemical methods, nuclease protection assays and primer extensions (Norton and Hynes, 1987; Schwarzbauer et al., 1987; Borsi et al., 1987; Ffrench-Constant et al., 1989).

To determine the patterns of FN gene expression and splicing in periodontal tissues, RT-PCR was used in this study. The results from this study showed that healthy GT, PDL and GH tissues expressed all alternatively spliced embryonic isoforms, although the low molecular weight isoforms of A and B showed up-regulation in GH, whereas only the exon-skipped transcripts were generated in tissue from CIPD patients. This indicates extensive and ongoing rebuilding processes in normal tissues and therefore it is not surprising to find these ‘embryonic’ isoforms to be produced in periodontal tissues, since a number of studies have suggested that the PDL contains progenitor cells which are capable of repopulating and re-forming damaged tissue (Gould et al., 1980; Iglhaut et al., 1988). However, the same FN profiles were also found in healthy gingival tissue which, unlike the PDL, is not considered to be a stem cell compartment. The expression of the embryonic isoforms in gingiva therefore most probably reflects continuing wound healing processes aimed at maintaining homeostasis by repairing tissue lost as result of normal, on-going gingival damage in response to dental plaque. It is notable that wound healing in foetal tissue occurs with minimal or no scarring, whereas adult tissues usually results in scar formation due to the inflammatory response to injury resulting in the release of cytokines. No such response takes place in foetal wound healing situation suggesting that this difference may be related to the profile of growth factors and cytokines released at foetal and adult wound sites (Brocker and Reiter, 1994; Olutoye et al., 1996). Furthermore, a number of studies suggest that phenotypic differences between foetal and adult fibroblasts may also be a key factor (Chen et al., 1989; Alaish et al., 1994; Ellis and Schor, 1996). The studies of Irwin et al. (1994) and Schor et al. (1996) showed that gingival fibroblasts had many of the characteristics of foetal derived cells, including responses to mitogens, proliferative potential, migratory activities and synthesis of ECM. These cells were derived from the papillary
tips of gingiva and have been called PAP fibroblasts. In contrast, the cells which exhibited the adult-like phenotypes were obtained from the deeper reticular tissue (RET fibroblasts). The papillary fibroblasts proliferated faster than those from reticular layer. In addition the PAP fibroblasts were smaller and had spindle shaped appearance compared with RET fibroblasts, which were larger and had much more spread morphology. Similar subsets of fibroblasts have been identified in other tissues and, furthermore, the foetal-like characteristics of PAP fibroblasts have been shown to undergo transition to adult-like phenotypes during cell growth in vitro. It has been postulated that mesenchymal cells in foetal associated tissue produce growth factors that allow faster, scar free wound healing (Adzick and Lorenz, 1994). In gingiva, Irwin et al. (1994) demonstrated that PAP fibroblasts produce a factor termed 'migration stimulating factor' (MSF) which is not produced by adult fibroblasts and, in addition, showed that during the transition of PAP to RET phenotype in vitro the cells ceased production of MSF.

In marked contrast to the healthy tissues, it was found that only the low molecular weight, exon-skipped FN transcripts characteristic of adult tissue were expressed in CIPD. Although the reason for the apparent down-regulation of the embryonic isoforms is not yet known, it may be one of the molecular consequences of the over-production of certain cytokines which accompanies the disease process. Chronic periodontitis is considered to involve the local invasion of inflammatory cells, resulting in the production of soluble mediators that cause damage to periodontal soft and hard connective tissues. Several studies have shown the presence of higher numbers of macrophages and plasma cells in CIPD than in normal gingiva and the simultaneous elevated presence of cytokines in inflammatory periodontal disease. Analysis of gingival crevicular fluid from patients with periodontitis has shown that a high level of IL-1β and also IL-1β mRNA are present in inflamed gingival tissues (Matsuki et al., 1993), and experiments using a quantitative ribonuclease protection assay also demonstrated that IL-1β was the major cytokine expressed in chronic periodontitis (Tokoro et al., 1996). Similarly, Gemmell and Seymour (1998) analysed individual cytokine-producing
cells by FCM and showed that the percentage of IL-1\(\beta\) producing macrophages and B-cells were both higher in CIPD compared with normal gingival tissue. IL-1 has specific catabolic activity with regard to both soft connective tissue and mineralised tissue and thus it is a key mediator of periodontal destruction (Birkedal-Hansen, 1993). IL-1 induces major changes in matrix composition mainly through the secretion and activity of MMPs and TIMPs (Birkedal-Hansen, 1993). One of the effects of MMPs is the proteolysis of intact FN molecule to generate fragments and, although these fragments interact with PDL cells, these show less response to chemotaxis than the intact FN molecule. Furthermore, the PDL cells exhibit greater proliferative response to intact FN compared to FN fragments. Similarly it has been shown that IL-1 is also involved in stimulating bone resorption. It is therefore notable that, in the present study, it was found that a high concentration of this cytokine down-regulated the expression of the high molecular weight isoforms of FN in PDL cells in vitro but had little, if any, effect on the exon-skipped transcripts. Thus, the pathogenesis of CIPD, which appears to be accompanied by the excess production of inflammatory mediators such as IL-1\(\beta\), is also associated with reduced synthesis of the embryonic forms of FN.

The production of partially-and non-spliced FN variants is characteristic of regenerative processes, as discussed previously, and it is notable that growth factors such as PDGF, IGF-1 and TGF-\(\beta\) were all capable of inducing the expression of these transcripts. Previous studies have shown that such growth factors can up-regulate the levels of FN as well as induce mitogenic and proliferative responses by PDL cells in vitro (Oates et al., 1993; Matsuda et al., 1992). Moreover, the growth factors have also been reported to specifically promote the repair of the periodontal ligament which sometimes follows tissue damage in CIPD in vivo (Lynch et al., 1991; Graves et al., 1994; Cho et al., 1995). Thus, although the functional activities of the differentially spliced isoforms of FN are not yet established, the findings of this study highlight the important relationship between wound healing processes and growth factors which up-regulate the expression of the embryonic FN transcripts. All the growth factors tested had a similar effects on the alternative splicing of FN.
RNA, although the concentration of growth factor required was variable, suggesting that the mechanism involved in splicing may be common to all the factors. A number of studies have implicated growth factors in GH and, therefore, this may explain the presence of ‘embryonic’ isoform profiles in these tissues despite the presence of inflammatory cytokines in this lesion. Thus, the presence of elevated amount of growth factors may override some of the effects of cytokines present in GH. Although previous studies have shown increased ECM production in GH, the data from this study indicated that only the low molecular weight exon-skipped isoforms A and B of FN are up-regulated in GH. These results also suggest the potential clinical value of suppressing inflammatory cytokines that act to generate the low molecular weight exon-skipped isoforms of FN. Conversely, it has been suggested that the development of CIPD could be the consequence of imbalance between foetal-like and adult-like subsets of fibroblasts present in gingiva where the adult-like phenotype predominate during the course of the disease (Schor et al., 1996). Thus, the use of growth factors that would promote subsets of foetal-like fibroblast proliferation may also have a potential therapeutic benefit.
6.1 Introduction

A number of studies suggest that cells in the PDL and gingiva are different from each other and also not homogenous, having distinct phenotypes and functions (Irwin et al., 1994; Fries et al., 1994; Schor et al., 1996; Phipps et al., 1997; Kuru et al., 1998). The PDL is responsible for attaching the tooth to the alveolar bone and its integrity is maintained by PDL cells (Berkovitz et al., 1995). Gingival cells play a major part in the turnover and pathology of the soft connective tissue which surrounds the alveolar bone (McCulloch and Bordin, 1991). The PDL is now generally considered to contain precursor cells which have the capacity to differentiate into osteoblasts and cementoblasts as well as fibroblasts (Nojima et al., 1990; Pitaru et al., 1994). As with PDL cells, recent studies have described the heterogeneity of gingival fibroblasts with respect to morphology, proliferative potential, migratory properties, metabolic activity and ECM production (Rose et al., 1987; Piche et al., 1989; Mariotti and Cochran, 1990; Hakkinen and Larjava, 1992; Hou and Yaeger, 1993). Moreover, subsets of gingival fibroblasts have been suggested as the reason for the variable sensitivity to drug-induced gingival hyperplasia (Seymour et al., 1996). Although both PDL and gingival cells have been extensively examined, these studies have been confined to short-term cultures which have a limited life-span and are phenotypically unstable, often showing changes in functional activity on prolonged culture in vitro (Adams et al., 1993; Kuru et al., 1998). This precludes the cloning of individual cells and limits the characterisation of the diverse cell populations which are present in primary cultures. Although long-term cultures of several types of human cells, including PDL cells, have been prepared following conversion to an 'immortal' phenotype using the SV40 large T antigen (Stacey et al., 1990; White et al., 1992; Stamps et al., 1994; Shiwen et al., 1995; Simon et al., 1996; Hoang et al., 1997), many characteristics as well as functional activities of cells are not manifest until after they have undergone differentiation from proliferating precursor cells (Simon et
al., 1996). Transduction with a viral construct encoding a temperature-sensitive T antigen (tsT) has therefore been used to obtain long-term cultures in which cells continue to divide at a low (34°C, permissive) temperature but become arrested at a high (39°C non-permissive) temperature at which the T antigen is inactivated. In this study a recombinant amphotropic retroviral vector was used to introduce this tsT construct into primary PDL and gingival cells. This procedure has enabled to examine cell growth and the expression and regulation of certain antigens which have a fundamental role in the physiology and pathology of these tissues.

6.2 Materials and methods

Early passage cells were transduced with a retroviral vector containing ts SV40 large T antigen as described in section 2.6.1. Transduced cells were cloned as described in 2.6.2. Growth characteristics of these cells were analysed as described in 2.6.3. The detection of the T antigen in transduced cells analysed by immunocytochemical staining as described in 2.6.4. The expression of ECM mRNA was analysed ‘Northern’ blotting (2.3.3) and RT-PCR (2.3.2) and the protein levels measured by FCM (2.5). The AP activity of the cells was assayed colourimetrically as described in 2.6.5.

6.3 Results

6.3.1 Characterisation of transduced cells

6.3.1.1 Morphology of transduced cells

Using a retroviral construct encoding the tsT antigen, long-term cell cultures were obtained from the PDL and gingival tissues, as described in the Material and methods. After the period of 3 weeks during which the transduced cells were selected in G418, and for more than 40 passages at a 1:5 split ratio over a period of more than two years, there was little evidence of any extensive cell death. The cells in these cultures were observed to be morphologically similar to their respective non-transduced, parent cultures. Phase-contrast micrographs of the PDL cultures grown at 34°C showed that the cells had a spindle-shaped appearance characteristic of fibroblasts (Figs. 6.1a),
Fig. 6.1 Phase contrast micrographs of transduced PDL cells cultured at (a) 34°C and (b) 39°C. The cells were plated in duplicate and cultured until semi-confluent. One of the duplicates was then transferred to 39°C and incubated for a further 7 days. All the cells retained the appearance of spindle-shaped fibroblasts at both low and high temperatures, but the cells incubated at 39°C appear to become more flattened and granular, as indicated by the arrows.
Fig. 6.2    Expression of the tsT antigen in transduced PDL cultures. The transduced PDL cells and untransduced parental counterpart were grown on a glass coverslips at 34°C and then stained for the T antigen. The micrographs show that the antigen is expressed in the nuclei of most of the cells, but some cells stained with less intensity (b). Note the absence of the T antigen in untransduced cells (a) (magnification x 200).
whereas the cells grown for 7 days at 39°C appeared to be much more granular (Figs. 6.1b). Very similar micrographs were obtained for the gingival cultures incubated at 34°C and 39°C.

6.3.1.2 Expression of the tsT antigen

The transduced PDL and gingival cultures were grown on coverslips at 34°C and immunostaining was carried out to examine the expression of the tsT antigen, as described in the Material and methods. The results in Fig. 6.2 shows that, at the third passage after selection for G418 resistance, the antigen was detected in the nuclei of most of the cells in PDL culture, although some cells in the culture appeared to express lower levels of the antigen. The same pattern of antigen expression was found with the transformed gingival cultures and there was no evidence that the antigen was expressed by any of the cells in the normal, non-transduced parent PDL and gingival cultures grown at 37°C.

6.3.1.3 Growth and cloning of transduced cells

Cell growth experiments were performed in order to examine the effect of the tsT antigen on the proliferation of the cells at 34°C and 39°C. Figs. 6.7a, b show that the PDL and gingival cells continued to divide at 34°C, although the rate of proliferation was somewhat higher in the latter cells, which reached a density twice that of the PDL cells at day 10 and three times at day 14. Thus, after 2 weeks of growth at 34°C the PDL reached a density of 10 times their original number and the number of gingival cells increased nearly 30 times. However, when transferred to the non-permissive temperature of 39°C, the cells in both of the transduced cultures stopped dividing within 4 days.

Cloned transduced cells were obtained by dilution plating method. Single cell suspensions were prepared from monolayer cultures and 0.1 ml of medium containing 1-3 cells per ml dispensed into 96 well plates. When colonies were observed after 3-4 weeks in some of the wells, each consisted of approximately 500-600 cells per well.
Fig. 6.3 Growth of tsT-transduced (a) PDL and (b) gingival cultures. Replicate cultures were seeded at initial density of $1 \times 10^5$ per flask and incubated at 34°C (open square; solid lines) and 39°C (open circles; dotted lines). The cells were harvested and counted using a haemocytometer.
The plating efficiency of PDL cells was approximately 0.5% whereas GF had cloning efficiency of 1% to 3%.

6.3.1.4 Expression of ECM mRNA transcripts in transduced cells

A number of markers associated with connective tissue and osteoblast cell phenotypes were examined in order to delineate the characteristics of the transduced cells. The expression of the mRNAs corresponding to OC, OP, COL I and AP was therefore measured in the PDL and gingival cells by Northern blotting, as described in the Materials and methods. The membranes were hybridised first with the GAPDH cDNA probe, a standard housekeeping gene whose expression remains constant. Fig. 6.4 shows an autoradiograph of a Northern blot of the RNA obtained from the PDL cells grown at 34°C and at 39°C (lanes 1 and 2, respectively) and then hybridised with GAPDH cDNA probe. A similar intensity and size (1.2 Kb) of GAPDH band was obtained when 20 µg of each RNA sample was placed on the gel. The membrane was subsequently hybridised with the cDNA gene probes for OC, OP, COL I and AP. However, no mRNA signals were detected for any of these genes. Northern blotting carried out with extracts of the transduced gingival cells also did not reveal the presence of any mRNA transcripts corresponding to these antigens although, like the PDL cells, they had intact and readily detectable levels of GAPDH. Moreover, the PDL cells (and the gingival cells) failed to express these transcripts even after they had been cultured for 7 days at 39°C in the presence of DEX and D3, although they expressed GAPDH (Fig. 6.4, lanes 3 and 4). Because the mRNAs corresponding to these antigens could not be detected by Northern blotting, a more sensitive technique was used to examine the expression of the transcripts. RT-PCR analysis was therefore carried out, as described in the Materials and methods, and demonstrated that mRNA corresponding to OC, OP, COL I and AP were all present in extracts of the transduced PDL cells (Fig. 6.4 b, c, d, and e, respectively) grown at 34°C (lane 1) and 39°C (lane 2) and at 39°C in the presence of DEX (lane 3) and D3 (lane 4).
Fig 6.4 The expression of mRNA in transduced cells. The Northern blot analysis of GAPDH in the transduced PDL cell cultures at 34°C (lane 1) and 39°C (lane 2) is shown in (a). Lane 3 and 4 show GAPDH band in cells cultured at 39°C in the presence of DEX and D3, respectively. RT-PCR analysis of OC, OP, COL I and AP mRNA expression in the transduced cells is shown by the arrows in (b)-(e), respectively.
Fig. 6.5  Representative histogram of the forward scatter of PDL cells grown at 34°C (green) and 39°C (pink), as measured by FCM. Note that there is no difference in the histogram pattern.
Fig. 6.6  Representative histogram of the side scatter of PDL cells grown at 34°C (green) and 39°C (pink), as measured by FCM. Note the shift of the histogram to the right of cells grown at 39°C indicating increased granularity.
Identical results were obtained for gingival cells cultured under the same conditions (data not shown). Cells cultured at 39°C in the presence of DEX and D3 also expressed these transcripts, as shown in Fig. 6.4 b-e, lanes 3 and 4.

6.3.2 FCM analysis of transduced cells

6.3.2.1 Size and granularity of transduced cells

FCM analysis of the tsT-transduced cells grown at 34°C showed that they were the same size as their non-transduced counterparts grown at 37°C (Table 6.1). Growth at 39°C in the presence or absence of DEX and D3 also had no effect on cell size (Fig. 6.5). However, the transduced PDL and gingival cells grown at 39°C were both far more granular (Fig. 6.6) (SSC of 572 and 578, respectively) than the same transduced cells grown at 34°C (SSC of 471 and 379) and the non-transduced cells grown at 37°C (SSC of 425 and 364). These differences were found to be statistically significant (p < 0.05), but there was no additional effect on their increased granularity when they were grown at 39°C in the presence of DEX and D3, as shown in Table 6.1.

6.3.2.2 FCM analysis of ECM components

Fluorescent-labelled antibodies were used to measure the levels of OC, ON, OP and COL I in the transduced cells. The PDL cells grown at 34°C were found to express readily detectable levels of all these antigens. Fig. 6.7 shows the average antigen levels in the PDL cells cultured at 39°C for 7 days relative to their expression in replicate cultures of the same cells incubated at 34°C. While OC showed a statistically significant increase after growth at the higher temperature (approximately 160% of the control 34°C cells; p<0.05), in contrast, ON was significantly down-regulated, to only 55% of the control cultures (p < 0.05). Both OP and COL I increased to some extent, but these differences were not statistically significant. Notably, incubation at 39°C in the presence of DEX and D3 had no further effect except on COL I expression,
Table 6.1. Size and granularity of PDL and gingival cells measured by FCM

The size and granularity of PDL and gingival cells are shown as arbitrary units of FSC and SSC, respectively, as described in the Materials and Methods. The numbers are the means ± SD of three replicate experiments. The results were analysed using the Student’s t-test.

* indicates statistically significant from transduced cells grown at 34°C (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Size (FSC)</th>
<th>Granularity (SSC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDL</td>
<td>Gingival</td>
</tr>
<tr>
<td>non-transduced, 37°C</td>
<td>542 ± 27</td>
<td>489 ± 5</td>
</tr>
<tr>
<td>transduced, 34°C</td>
<td>575 ± 2</td>
<td>567 ± 29</td>
</tr>
<tr>
<td>transduced, 39°C</td>
<td>613 ± 36</td>
<td>548 ± 5</td>
</tr>
<tr>
<td>transduced, 39°C + DEX</td>
<td>603 ± 25</td>
<td>545 ± 6</td>
</tr>
<tr>
<td>transduced, 39°C + D3</td>
<td>615 ± 18</td>
<td>535 ± 4</td>
</tr>
</tbody>
</table>
Fig. 6.7 FCM analysis of antigen expression in transduced PDL cells grown at 34°C and 39°C. The PDL cells were grown at 34°C and 39°C in the absence (solid bars), presence of DEX (dashed bars) and D3 (dotted bars). The cells were immunostained with antibodies for OC, ON, OP and COL I. Note the up-regulation of OC and down-regulation of ON at 39°C, and COL I in the presence of DEX.
Table 6.2  Comparison of ALP levels (IU/L) in PDL and gingival cells grown at 34°C and 39°C in the absence and presence of either DEX or D3. ALP activity was measured after 7 days of culture (statistical significance is indicated by *; p<0.05 compared with cells grown at 34°C)

<table>
<thead>
<tr>
<th></th>
<th>PDL cells</th>
<th>Gingival cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>34°C</td>
<td>35 ± 2.0</td>
<td>35 ± 2.0</td>
</tr>
<tr>
<td>34°C + DEX</td>
<td>41 ± 3.0</td>
<td>37 ± 2.0</td>
</tr>
<tr>
<td>34°C + D3</td>
<td>36 ± 1.5</td>
<td>37 ± 3.0</td>
</tr>
<tr>
<td>39°C</td>
<td>36 ± 2.0</td>
<td>36 ± 3.0</td>
</tr>
<tr>
<td>39°C + DEX</td>
<td>62 ± 6.0 *</td>
<td>39 ± 3.6</td>
</tr>
<tr>
<td>39°C + D3</td>
<td>40 ± 1.5</td>
<td>40 ± 3.0</td>
</tr>
</tbody>
</table>
which declined by 60% in the presence of DEX but not D3. Although FCM analysis showed the presence of all the antigens in the gingival cells, however, the levels of ON, OP, OC and COL I in the gingival cells grown at 34°C were 50%, 45%, 40% and 30% of the levels in PDL cells, respectively and these were too low to establish whether there were any temperature-dependent changes.

6.3.3 AP expression in transduced cells

AP is frequently associated with osteoblast-like cells and the activity of this enzyme was therefore measured in the transduced cultures using a colorimetric assay, as described in the Materials and methods. In the transduced PDL cells, AP activity remained the same after 7 days of incubation at 39°C (36 ± 2.0 IU/L) as it was at 34°C (35 ± 2.0 IU/L). This relatively low level of AP activity was also present in cells incubated at 39°C in the presence of D3 (40 ± 1.5 IU/L), but was significantly increased in cells incubated with DEX (62 ± 6.0 IU/L; p<0.05). As with antigen expression, AP activity in the transduced gingival cells remained low under all culture conditions, even when incubated at 39°C with DEX (range 35-40 IU/L) (Table 6.2).

6.4 Discussion

The PDL is considered to contain sub-populations of cells responsible for the development, repair and regeneration of the periodontium (McCulloch and Bordin, 1991; Pitaru et al., 1994; McCulloch, 1995). Primary cultures of this tissue have been used as model systems in vitro in order to understand the complex cellular and molecular events underlying these processes (Mariotti and Cochran 1990; Hou and Yaeger 1993; Giannopoulou and Cimasoni, 1996). However, PDL and gingival cells are heterogenous and also have a limited life span in culture. Long term-cultures of these cells which could be cloned and characterised would therefore provide a valuable tool for not only dissecting the cellular, biochemical and molecular events of periodontal repair and
regeneration but also benefit in investigating the abnormal phenotypic characteristics of gingival pathogenesis.

The finite proliferative life-span of human cells can be overcome by either cellular mutations or introduction of exogenous immortalising genes (Jat and Sharp, 1986; Kelekar and Cole, 1987). Such cells can proliferate indefinitely but are dependent upon the presence of the immortalising gene. In the present study, primary PDL and gingival cells were transduced with retroviral vector containing the tsT antigen to generate conditionally 'immortalized' cell lines. The advantage of using this particular gene rather than the wild type is that the antigen expressed is thermolabile and is inactivated when the transduced cells are grown at the higher temperature (Jat and Sharp, 1989). Another important feature of the retroviral transduction is that the cell lines produce tend to be stable throughout extensive growth and cloning. This is in contrast to wild type SV40 which tends to undergo repeated amplification and chromosomal integration causing cell lines to be unstable (Stacey et al, 1990). Furthermore, the vector also contains the gene for neomycin which allows for selection of transduced cells. Following transduction and shortly after selection for G418-resistance, during which only little cell death was evident, the cultures were found to contain a majority of cells which expressed readily detectable levels of the T antigen. However, a substantial proportion of the cells appeared to express lower levels of the antigen on the basis of immunoperoxidase staining, possibly because of the presence of only low copy numbers of the corresponding gene construct in some of these initial cells. However, after approximately 30 additional passages, the T antigen was clearly present in the nuclei of all the cells which had been grown for this extended period. These cells have therefore have not appeared to been through the 'crisis' phase. The cellular crisis is thought to occur during the generation of immortal cell line in which cells stop growing, show karyotypic abnormalities and usually die. However from these cultures few cells survive and give rise to immortal cell line. The emergence of an immortal cell line without crisis is not unprecedented as has been observed previously (Bartek et al., 1991; Shiwen et al., 1995).
Both the PDL and gingival cells continued to proliferate at 34°C and appeared to have a fibroblast-like morphology similar to that of the non-infected parent cultures grown at 37°C. In contrast to previous work (Marioti and Cochran, 1990; Ogata et al., 1995), the proliferation rate of PDL cells, however, were much slower than the gingival cells. In addition, differences in cloning efficiency was also noted, with PDL cells having lower cloning efficiency than the gingival cells further confirming the slower growth rate of PDL cells compared with the gingival cells. However, the studies of Ohshima et al. (1988), Somerman et al. (1988) and Giannopoulou and Cimasoni (1996) showed that there was no difference in growth rate between PDL and gingival cells. Although previous studies all used primary cultures, this study used immortalised cell line but taken together the data supports the notion of fibroblast heterogeneity. However, when the transduced cells were transferred to 39°C, at which the SV40 large T antigen is inactivated (Jat and Sharp, 1989), there was rapid cessation of growth. Furthermore, although the cell size remained very similar to that of their non-transduced counterparts, as has been reported previously (Kuru, et al., 1998), the cells increased significantly in granularity. These observations suggested that the growth of the transduced cells was dependent on the function of the T antigen and, moreover, that the growth-arrested cells (ie, those incubated at 39°C), appeared to have undergone some differentiation, as previously shown for myogenic cells (Simon et al., 1996), osteoblasts (Harris et al., 1995) and chondrocytes (Lefebvre et al., 1995).

In order to assess whether the tsT-transduced cells retained phenotypic characteristics of the parental cells, the expression of several antigens associated with the specific functional activity of PDL and gingival cells were examined, including those phenotypic markers and characteristics associated with osteoblasts such as OC, OP, COL1 and AP. OC is a calcium binding protein whose expression is associated with bone formation and osteoblastic activity and is used as a marker of bone turnover. AP is a phospho-hydrolytic enzyme with optimum activity at alkaline pH and its activity is considered to be an important indicator of bone formation. It is distributed heterogeneously in PDL
tissue but is most noticeable in the vicinity of the alveolar bone (Ogata et al., 1995; Nohutcu et al., 1997; Carnes et al., 1997). Moreover, because the expression of these markers is modulated in osteoblasts and other cells during differentiation (Keeting et al., 1992; Rickard et al., 1996), the thermolabile property of the large T antigen was utilised to cause the transduced cells to cease proliferation and withdraw from the cell cycle by incubating them at a high temperature, thereby enabling them to display differentiated characteristics (Warburton et al., 1993; Andersson et al., 1994; 1994; Simon et al., 1996). However, it was not possible to detect any differentiation-associated functions at either 34°C or 39°C by Northern blotting, which failed to reveal the expression of any mRNA corresponding to OC, OP, COL I or AP, although hybridisation with the control GAPDH probe indicated that the mRNA in each of the extracted samples was intact. It is possible that transduction with the T antigen may have interfered with the expression of all of these transcripts but previous studies have shown that a number of cell types retain their phenotypic characteristics even after tsT transduction (Shiwen et al., 1995; Simon et al., 1996). It is therefore more likely that the mRNA levels were too low to be detected by Northern blotting, and subsequent RT-PCR analysis was sufficiently sensitive to demonstrate unequivocally that the gene transcripts corresponding to OC, OP, COL I and AP were expressed in the tsT-transduced cells at both 34°C and 39°C. Although the reason for the apparently low levels of mRNA expression, even at 39°C, is not known, it is possible that the corresponding genes are active in only a relatively small sub-population of PDL cells. In order to address this question a number of single cell clones were obtained from the transduced heterogenous population, however, due to laboratory accident these clones were lost.

Although used primarily for the analysis of lymphocyte sub-sets, the FCM technique has also been applied to the study of surface and intracellular antigens in many types of cells (Sumner et al., 1991; Bou-Gharios et al., 1995; Kuru et al., 1998). In the present study, this technique was used to measure the levels of OC, ON, OP and COL I expression by the tsT-transduced PDL and gingival cells grown at 34°C and 39°C. While it is generally accepted that PDL
cells express markers characteristics of the osteoblast phenotype (Arcea, et al., 1991; Nohutcu et al., 1996), recent studies have shown that these are also expressed by gingival cells, albeit at much lower levels (Somerman et al., 1990; Nojima et al., 1990; Ogata et al., 1995; Giannopoulou and Cimasoni, 1996) and this has been confirmed by the present study. Moreover, because the osteoblastic phenotype has been reported to be enhanced when conditionally immortalised osteoblastic cells are grown under non-permissive conditions (Harris et al., 1995; Bodine et al., 1996), this study also compared the expression of these antigens in the tsT-transduced PDL cells cultured at 34°C and 39°C. The results showed that OC levels became significantly elevated when the PDL cells were growth-arrested. Although OP and COL I also increased at 39°C, these changes were not statistically significant. In contrast, the level of ON was significantly reduced at this non-permissive temperature compared with 34°C. Moreover, despite other studies which have shown that DEX and D3 can regulate the expression of bone-related antigens (Subramaniam et al., 1992; Broes et al., 1995), the present study showed highly specific effects of these steroids on the tsT-transduced cells. Thus, while D3 had no effect on any of the antigens, DEX significantly down-regulated COL I but selectively up-regulated the activity of AP in the PDL cells incubated at 39°C. This enzyme has frequently been used as an index of early osteoblast differentiation (Kawase et al., 1988; Goseki et al., 1995), and the finding of the present study that DEX-induced increase in AP activity in the tsT-transduced PDL compared with gingival cells is consistent with previous reports of (Yamashita et al., 1987; Kawase et al., 1988;). Although the reason for the differential responses to DEX and D3 are not yet known, they are likely to be due to their complex effects on gene expression in different cells and under different experimental conditions (Darwish and Deluca, 1993; Delany et al., 1994), which can result in either gene activation or gene suppression (Subramaniam et al., 1992; Broes et al., 1995). Several models have been used for studying osteoblast differentiation and maturation in culture and these include rat and chick calvaria and human bone marrow. Using these approaches, a number of chronological events associated with osteoblast
differentiation have been defined and these are cell proliferation, maturation and mineralisation (Stein and Lian, 1993). During cell proliferation a number of proteins are expressed at a high level including cellular oncogenes, c-myc and c-fos, and also growth factors such as TGF-β. This phase is followed by maturation stage which involves the expression of genes associated with differentiation function including AP and OP. The final stage in the process is the mineralisation of matrix in which OC has been implicated. Induction of osteoblast phenotype depends on the co-ordinated action of steroid hormones, cytokines and growth factors. These agents act at different stages of osteoblast differentiation pathway and the effects are complex and can vary according to the duration of exposure and stages of cell maturation resulting in specific expression of bone-related genes. It is therefore clear from these studies that the expression of osteoblast phenotype in culture is very complicated. The present results suggest the presence of sub-population of cells at different stages of differentiation with respect to antigens investigated in this study. Furthermore, although the tsT-transduced cells induced overall up-regulation of these antigens, the failure of steroid hormones (D3 and DEX) to elevate their levels could be due short incubation (7 days) of the cells at the higher temperature. As noted above, some of these antigens may be expressed at the later stages of differentiation.

In conclusion, the tsT-transduced PDL and gingival cultures obtained in this study appear to retain certain phenotypic and functional features of their non-transduced parental counterparts. When PDL and gingival cultures were compared, the PDL cells were found to have relatively higher levels of antigens associated with osteoblast phenotype in agreement with previous studies. Moreover, the PDL cells also showed some differentiated characteristics following incubation at the non-permissive, high temperature. In addition, gingival cells should provide phenotypically stable source of cells as a valuable tool in the investigation of gingival pathogenesis.
CHAPTER 7

SUMMARY AND FUTURE STUDIES

The repair and regeneration of periodontal tissues are wound healing processes which occur following damage to the tissue as a result of periodontal diseases. In CIPDs, which are induced by cytokines and other inflammatory mediators in response to dental plaque, there is soft tissue degradation and bone breakdown. In contrast, GH involves the enlargement of the gingival connective tissue and excessive accumulation of ECM resulting primarily from the administration of drugs including immunosuppressants, anti-epileptic drugs and calcium channel-blocking agents. Moreover, regenerative process following CIPDs, which involve active cell growth and the production of new ECM, could cause GH and may represent an exaggerated phase of repair and regeneration. As in other hyperplastic lesions, GH has been associated with growth factors and anabolic steroid hormones. Moreover, sex hormones play an important role in periodontal diseases since gingival inflammation and hyperplasia are closely associated with the hormonal changes which accompany puberty, pregnancy, menopause and the use of the oral contraceptive pill. Furthermore, several studies have shown that the androgens are actively metabolised in both the inflamed and hyperplastic gingival tissues. The sex hormones exert their influence by binding to specific intracellular receptors which belong to a family of ligand-activated transcription factors that control a wide range of biological processes. These include modulation of a number of adhesion molecule, cytokine and extracellular matrix genes thereby playing an important part in cell interactions, inflammation and connective tissue turnover. Although these processes are fundamental to the pathogenesis of periodontal diseases, the role of the sex hormone receptors and the precise effects of the hormones on periodontal tissues and cells have not been delineated. Previous studies have not provided unequivocal evidence that steroid receptors are expressed in periodontal tissues and it was therefore not clear whether cells in these tissues were potentially sensitive to the effects of sex steroids. In order to determine whether periodontal tissues could respond to oestrogen and androgen, in this study specific and highly sensitive
RT-PCR technique was used to detect mRNA for these receptors. The results established unequivocally that the androgen but not the oestrogen receptor was expressed in these tissues. This demonstrated that the periodontium does not respond to oestrogen but may be under the direct influence of androgen which are likely to be of major importance in periodontal homeostasis.

Normal periodontal cells were cultured in the absence and presence of DHT and ELISA assay was used to measure DNA synthesis to evaluate the effects of the hormone on cell proliferation. The results showed that, despite the presence of the AR, there was very little difference in cell proliferation between the control and DHT treated cultures. However, a number of studies have suggested that androgens have their effect on cell growth via its ability to modulate growth factors and/or their receptors through an autocrine mechanism. It was notable, therefore, that when the cells were pre-treated with DHT prior to incubation with the growth factors such as TGF-β and PDGF, there was significant enhancement in cell proliferation. In contrast, IGF-1 had no effect under similar conditions.

It has been shown that androgen metabolism is elevated in GH. It was therefore pertinent to ask whether the AR could be one of the factors in drug-induced GH. In this study, semi-quantitative RT-PCR was used to examine the levels of AR mRNA in these tissues. In addition, normal and hyperplastic gingival derived fibroblasts were treated with the GH-inducing drugs in vitro and the AR protein levels measured by FCM. A major advantage of FCM lies in being able to measure the properties of each individual cell in a population rather than measuring the cell population as a whole and thus to be able to analyse distinct subsets of cells in a heterogeneous population. Another advantage being that the relative levels of intracellular antigens can be measured by permeabilising the cell by treatment with the non-ionic detergent saponin. Although the FCM values are arbitrary, they depend on the electronic input and output detection settings of the specific cytometer and thus the relative values have been shown to be proportional to the actual number of molecules present in and/or on the cell. The present studies showed that there was no significant difference in the expression of AR in normal and hyperplastic
gingival tissue. In addition, the drugs that cause GH in vivo did not appear to up-regulate the expression of AR in vitro. Thus, there was no difference in the amount of AR in normal and hyperplastic gingival tissue and in cells treated with the drugs. However, the drugs could still cause GH by increasing the biological activity of the AR in vitro and in vivo. The biological activity of the AR could be tested by transfecting gingival cells, in vitro, a DNA construct containing the AR promoter linked to a reporter gene (Lake and Owen, 1991; Gigure, 1991). The reporter genes usually code for an enzyme including chloromphenicol acetyl transferase (CAT) and luciferase. Reporter genes have become powerful tools to study regulation of gene expression in eukaryotes. Thus, the effects of the drugs on the activity of the AR can be assessed by measuring the enzyme activity.

CIPDs have been associated with the local invasion of inflammatory cells and the production of soluble mediators that ultimately damage periodontal tissues. These factors, including IL-1 and IL-6, act in an autocrine or paracrine fashion to regulate cell function. IL-6 is a multi-functional cytokine which is secreted by many types of cell including GF, and has been implicated in the pathogenesis of gingival inflammation and hyperplasia. Moreover, previous studies in other tissues have suggested that IL-6 is regulated by anabolic steroids and, therefore, in the present study it was investigated whether DHT also controlled IL-6 expression by gingival fibroblasts. It was found, using ELISA assay, that increasing concentrations of DHT progressively reduced IL-6 production by cells from normal gingiva and also from patients with gingival inflammation and GH. Analysis using the RT-PCR established that DHT caused a marked reduction in the levels of IL-6 mRNA. Studies using androgen antagonists showed that anti-androgen cyproterone acetate did not reverse DHT-mediated down regulation of the cytokine in GF, but instead acted as an androgen analogue, potently inhibiting IL-6 production. In contrast, while another anti-androgen, flutamide, also failed to abrogate DHT inhibition of IL-6 production, it had no effect on IL-6 production in the absence of DHT. In marked contrast to GF, PDL cells produced barely detectable levels of IL-6.
These findings suggest that anabolic steroids could be used for the treatment of periodontal diseases by down-regulating IL-6.

Studies *in vivo* and *in vitro* have suggested that certain growth factors including PDGF, TGF-β, EGF, FGF, and IGF may play an important part in periodontal growth and disease. In order to exert their activity, these factors must first bind to specific cell surface receptors. The expression of these receptors is thus of fundamental importance for the response of the cells to the factors. The analysis of growth factor receptor expression by FCM showed that, although PDGF-Rβ was expressed by all the cells, TGF-β RI and RII, FGF-R and IGF-R were differentially expressed by periodontal cells. In contrast, none of the cells expressed the PDGF receptor-α or the EGF-R. The results suggest the presence of diverse sub-populations of cells which are likely to have functionally distinct roles in the periodontal homeostasis. It was increasingly becoming clear that some relationship exist between androgen and growth factors and/or their receptors. Therefore, the possible effect of DHT and the GH-inducing drugs was investigated. DHT had no effect on growth factor receptor expression with the exception of IGF-R. The hormone down-regulated the level of the receptor in dose-dependent manner suggesting that the cells that expressed the IGF-R were under negative androgenic regulation. This confirmed the earlier observation that DHT pre-treated cells, unlike TGF-β and PDGF, did not proliferate in the presence of IGF. However, CsA increased the expression FGF-RI but this was not significant and the drug did not affect the expression of the other growth factor receptors.

ECM plays a significant role in embryonic development of tissues as well as wound healing processes. FN is a major component of the ECM and is likely to have an important role in periodontal growth and renewal. The FN gene product has been shown to be subject to alternative splicing in three regions, each generating different mRNA transcripts that have been associated with normal adult tissue, embryogenesis and wound healing. In this study, using the RT-PCR, splicing profiles of the primary FN transcript was examined and found that healthy periodontal tissues expressed all alternatively spliced embryonic isoforms, indicative of the extensive repair which occurs in these
tissues. In marked contrast, only the fully spliced-out transcripts were generated in tissue from CIPD patients. The loss of the non-spliced and partially-spliced isoforms in these lesional tissue may be due to the excess production of mediators in this disease, since it was observed that high concentration of the cytokine IL-1β caused down regulation of these transcripts in cultured periodontal cells. It was also demonstrated that growth factors likely to be involved in periodontal regeneration, such as PDGF-BB, IGF-I and TGF-β, elicited pronounced up-regulation of the FN embryonic isoforms in these cells, which may explain the isoform profile obtained in GH as this lesion has been correlated with up-regulation of PDGF-BB. Although the expression of FN during tooth development has been shown, however, the mRNA profiles of the different isoforms has not been demonstrated. Thus, it would be interesting to analyse FN isoform expression during the development of the periodontium but this approach will require animal studies and the use of both in situ hybridisation and RT-PCR techniques to detect the different isoforms. In mammalian system, a number of methods are used by which protein isoform diversity is generated including the use of gene families, the use of different promoters in a single gene and the use of alternative splicing. However, it is not clear why a particular mechanism is favoured. Although the functional activities of the alternatively spliced variants of FN proteins are not known, however, the results presented here suggests that they may have highly specific roles in both periodontal breakdown and regeneration.

Although the main cell types of the periodontium are fibroblasts, epithelial cells, cementoblasts and osteoblasts, the fibroblasts are the predominant cell type of the soft periodontal connective tissue, comprising both the gingiva and periodontal ligament and play fundamental roles in normal function and pathology. These cells have been studied in vitro but they have only a limited life span and are phenotypically unstable. Nevertheless, functional and morphological differences have been shown to exist not only between but within tissue types. It has been suggested by several studies that during periodontal disease progression, both inflammatory and GH, that positive selection occurs of certain subsets of GF as a result of the effect of
inflammatory cytokines and drugs associated with GH. Similarly, PDL is postulated to contain sub-populations of cells which have the capacity to differentiate into specific cell types including osteoblasts, cementoblasts and fibroblasts. In order to obtain long-term cultures of these cells which can be cloned and characterise, primary cultures of gingival and PDL cells were infected with amphoteric retroviral construct encoding a temperature-sensitive SV40 large T antigen. The transduced cells were readily grown at the permissive temperature of 34°C and they showed morphological characteristics similar to those of parental cultures and remained unchanged following prolonged growth but were growth arrested at the non-permissive temperature of 39°C. Furthermore, the presence of the T antigen increased the life span of these cells but did not ablate the expression of parental characteristic phenotypic and functional features. Although using limited dilution method single cell clones were obtained, these were lost due to laboratory accident. Nevertheless, these studies showed that conditionally immortalised periodontal cells could be obtained and cloned which would provide valuable tool in investigating phenotypic and functional characteristics of periodontal cells. As mentioned before, a number of diverse drugs cause gingival overgrowth, however, the specific cell and molecular mechanisms underlying drug-induced gingival are not known. Although cell proliferation and ECM production are fundamental in hyperplastic reactions and a number of studies have been carried out to investigate the expression of genes thought to be involved in these processes, a more general method is required to detect genes which are differentially expressed in GH compared with normal gingiva. To this end a novel method described by Liang and Pardee (1992), differential display, would be a useful tool to analyse differences in gene expression associated with GH. This technique involves reverse-transcription of mRNA prepared from cells treated with and without drugs using anchored oligo-dT primers and subsequent amplification of subsets of mRNA by PCR with arbitrary oligodeoxynucleotide primers. The amplified cDNA fragments are radioactively labelled and separated on high resolution polyacrylamide gels. This method will delineate drug-induced changes in the expression of particular genes relevant
to GH. It is also clear that wound healing is a complex process which involves a number of cell types responding to a range of signals. Therefore, this technique will also be useful in the understanding of molecular mechanisms of differential gene expression in response to these signals in wound healing processes.
REFERENCES


Gemmell, E. and Seymour, G.J. (1993) Interleukin-1, interleukin-6 and transforming growth factor-β production by human gingival mononuclear cells
following stimulation with *Porphyromas gingivalis* and *Fusobacterium nucleatum*. J Periodont Res 28, 122-129.


180


Teachers are prepared to help if you wish to answer other past questions.
Could the presence of endocrine FN synergy with GT be one of the reasons that the cell membrane generally heals without scar

Do you think that elevated FN profiles may be due to cell rupture and growth factor profile in these lesions. Pre-inflammatory

Do you think that elevation of the pro-inflammatory GT tissue is the main reason that it works. This was seen in the liver and other organs. Also mainly seen in CHPD tissue.

Why do you think Northern Blotting failed to detect mRNA for OC, OP, COL1 and AP in transformed cells p 137 even when

Although with DET or 03 while PCR also Rgs detected revealed their presence. By Abs & FCM.

Do you think cells grown at the non-permissive temperature mature

other cells mature at the permissive temps. p 141.

What was the laboratory accident that preceded cloning. Could this be repeated on the cells you are currently growing.
Questions:
1. How did the ideas for this project first come to you?
2. Did you experience any particular problems in developing these ideas?
3. Why do you describe certain cells as fibroblast-like cells, rather than osteoblasts? p.23
4. What is the evidence that osteoblasts are derived from progenitors in the vascular spaces of the perosteal layer or endosteal spaces of bone? p.23
5. What is the evidence for different types of fibroblast in general & PL and mutants within these groups? p.24
6. Do you think that PL cells include mesenchymal cells which can give rise to several cell types e.g. osteoblasts, osteoclasts, fibroblasts or that there are distinct forms of skin cell for each cell type? p.24-25
7. Are the differences between the synthetic products of GF & PD1 cells only qualitative (or not qualitative?) p.24-25
8. Are the differences in the responses to biomolecules between CF and TAL2 cells subtle or quantitatively or qualitative? p25
9. Are cloned cells derived fromniival cells likely to differ from those from unontolateln. p26
11. How do oncogenes act to prevent cell senescence and increase proliferation? p27
12. Normal cancer development is thought to involve several rather than one key mutations. Therefore, in immortalization by one gene only one step in this process. p27
13. How does the use of a recombinant SV40 T-Ag gene overcome the 10th loss of differentiation by these cells? p27
14. How close the lipoprotein receptor T-antigen function to tumor or end of these cells. p27
(13) What is the main evidence to suggest that the pro-inflammatory cytokines IL-1 and IL-6 are particularly involved in pericelal pathology? p.29

(16) Is the pro-inflammatory processing enzyme the same for IL-1β and IL-6? If so does it encode the common area for the two forms? p.30

(17) What is the large body of evidence implicating IL-1β production in the pathogenesis of RA? p.30

(18) How can IL-1 function in two opposing ways to one to increase ECF production x the other to lead to its breakdown? p.30

(19) Why are ECF levels of IL-6 higher in CP whilst serum levels are normal? p.31

(20) Is there any direct evidence for the blockade of Ca' influx by drugs causing the activation of the apoptotic programme as these increase number of ECFs? p.31

(21) Why should the systemic x local effects of CsA on IL-6 be different? p.36

(22) Since increased osteogenesis by proinflammatory cytokine may increase the inflammatory component of PD both these any evidence for receptor of these homone in GT for general epithelium or vascular endothelium? p.39-40.
23. Does the different receptor specificity for PDGF affect the form you choose in your experiments i.e. PDGF BB. pg 43-55.

24. What would be the effect of down-regulating EGF receptor in response fibroblasts 
and regulating it in non-responder fibroblasts - re effects of PHT on EGF rec.

25. What is the probable mechanism in chondrocytes, if PHT increased expression growth factors p 46.

Also? growth factors last line.

26. What is the implication of the strong expression of FN along the attachment sites of collagen fibers to cementum but not to bone. pg 49.

27. Why is there considerable confusion about the presence of ER + AR on various cell types. p 52.

28. What constituted the complete medium p 54.

29. Why are chondro cells killed serum 1% rather than a serum free medium pg 55.
30. p55 2.2.2.

Why 1% FCS medium rather than serum free medium for clone experiments. Would not 1% FCS contain serum derived growth factors.

31. How were the concentrations of CSA, PHT and NIF determined. Also cells cleaved in 5 days. How much variation in previous studies and what do they base their on. p55.

32. How much tissue/cells are required as a minimum for RNA extraction. p56

33. What is the basis of multiplying the OD by 40 to obtain RNA as pg/ml. p56.

34. How much less sensitive is Northern blotting of PCR (RNA 20ng of Smq).

35. What were your sources of oligonucleotide primers p56.

36. How is the SV40 large T antigen & the reverse gene inserted into the retrovirus. p61

37. If there are no receptors for antigens e.g. GT, GF, PDL, PDL cells then what are
To possible pathways for the down

estrogen related effects in the gingival tissues.

10. Since there are variable results on

receptor studies in relation to both E2 + AR

what are the problems associated with

relatively diluting these receptors. p. 70-71.

41. What is the probable pathway

for the enhancing effects of DHT on

all proliferation produced by PDGF, TGFβ.

p. 73, 75

42. Why were there no similar effects in IGF-1.

p. 75.

43. Would you have expected to find a difference

and protein level.

between the AR expression in hypertrophic and

normal GI p. 76-80

44. Were you surprised that PA, CS, NIF

did not effect AR expression p. 88.

45. What is the significance of DHT control

of 12-6 secretions in its role in GH. p. 88

46. Why do you think the estrogen

antagonists, CPA + FT failed to produce

effects on this process p. 91.
47. Why do you think that androgens alter the synthesis of some growth factors but do not affect proliferin whilst these same growth factors do p. 96.

48. Why do you think that with FCS the protein antigen for AR was detectable with a polyclonal Ab but not with a monoclonal Ab. p. 98.

49. Do you feel that your work supports the contention that GF are the main source of 16-6 in penile也会 tissues p. 100.

50. Does your work show that the penile tumour does not respond to antigens p. 101. Could not the effects be indirect (see Talcher et al. Scz 1999 JCm 26, 723-3).

51. What is the significance of different expression of growth factor receptors by different cell groups & GFs p. 103.

52. Why do androgens alter the synthesis of some growth factors (p. 98), do not alter the expression of PDGF-Rb, TGF PR1+II & FGF-R but do significantly decrease expression of TGF-R.

53. If PDV cell only express PDGF-Rb why should PDGF have any mitogenic actions on these cells p. 113 line 3-7.
32. p. 123 Why do cells grown in 1% FCS fail to show any FN bands whilst those grown in 10% FCS do. Fig 3-4.

33. p. 119 Does the presence of extra bands of FN in PDL and CF cells lessen with their absence from incubated detached tissue indicate their respective nature or lack of it respectively.

34. Does the fact that growth factors increased the presence of all forms of FN in a step wise manner does this mean that these effect the FN synthesis at a given concentrating level. p. 125

35. Does it also indicate that the lack of FN bands at low FCS supplemented low levels mean that this occurred because of the lack of these factors. p. 125.

36. Does the effect of IL-1β reduce the levels of FN bands indicate that the presence of these pro-inflammatory cytokines in detached tissue may be the reason for the low levels there. p. 126-7. also p. 129.
Great progress! Thank you.

For reference, the following PL is CN not ERP (or perhaps):

<table>
<thead>
<tr>
<th>ERM</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCF</td>
<td></td>
</tr>
<tr>
<td>FTG</td>
<td></td>
</tr>
</tbody>
</table>

For Pollock, consider all factors.

Factors:
- IL-
- Legal - manual CP & Pollock by day count

For notice at CP & Pollock, any CP & PL.