CHAPTER 5
DISTRIBUTION OF DIFFERENT MACROPHAGE PHENOTYPES IN PERIODONTITIS

5.1 INTRODUCTION

The presence or expansion of different phenotypic subsets of macrophages may ultimately determine the severity of certain infections (Sorg, 1991, Rutherford et al., 1993). Hence, the sequential appearance of distinct subpopulations of macrophages within the inflammatory lesion may provide the most appropriate response at a given stage of the immune process (Rutherford et al., 1993). The converse may also occur with the correlation of certain populations of macrophages in disease states indicating a poor prognosis. For example, in human liver, heart and kidney grafts, acute rejection is associated with a strong infiltration of 27E10- positive macrophages, whereas the presence of RM3/1-positive macrophages reflects an uncomplicated clinical outcome (Burkhardt et al., 1990, Sorg, 1991).

The topographical distribution of subsets of macrophages within specific tissues presumably reflects the physiological and defence requirements of the tissues, for example, alveolar and interstitial macrophages in the lung. The distribution and role of specific macrophage phenotypes in most tissues in physiological health and in pathological conditions, such as periodontitis, requires further elucidation.

Identification of macrophage phenotypes and their distribution in pathological lesions, such as in hypertensive vascular lesions and rheumatoid arthritis has been investigated by the use of antibody probes with improved specificity for macrophages. Abumiya et al., 1996, investigated the distribution of macrophage subsets in hypertensive vascular lesions of stroke prone rats. Their findings indicated that the diversity of the vascular lesions was related to differences in accumulation of macrophage subpopulations and their role in lesion formation. To elaborate, in fibrinoid necrosis, macrophages extravasated in the early phase of lesion formation and acted only as scavengers, whereas in fibrocellular proliferative lesions, MHC class II positive macrophages accumulated perivascularly in the early phase and appeared to induce
immunological reaction and to stimulate cell proliferation. In a prospective study of polyarticular destruction in rheumatoid arthritis, Mulherin et al., (1996) found that synovial macrophages correlated with the radiological course and the outcome of rheumatoid arthritis. Importantly, the distribution of synovial macrophages as detected by anti-CD68 and the macrophage subsets defined by Ber-MAC3, anti-CD14, and RFD7 (a mature tissue macrophage marker) displayed distinct layering in the synovial lining where chronic inflammatory changes manifest and tissue destruction occurs. The full significance of the role played by the different subsets has not been clarified.

There have been few reliable phenotypic markers for macrophages available until recently. This limitation has been addressed in part by the development of the high specificity monoclonal antibodies Ber-MAC3 (Backe et al., 1991), RM3/1 (Zwadlo et al., 1987), 27E10 (Zwadlo et al., 1986), and 25F9 (Zwadlo et al., 1985). These markers together with anti-CD68 have been described in Chapter 3 and they are employed in the following study.

5.2 MATERIALS AND METHODS

5.2.1 Tissue specimens

Gingival tissue samples were obtained with approval by the Human Ethics Committee of the Central Sydney Area Health Authority and informed consent, from 48 patients with no record of previous periodontal therapy or known relevant medical or drug histories including tobacco smoking, who were attending the United Dental Hospital, Sydney, for dental extractions.

The tissues collected were classified by clinical, radiological and histological criteria into 2 categories; either minimally inflamed (clinically healthy) or advanced adult-type periodontitis.** The minimally inflamed group comprised 26 biopsies from 14 males and 12 females, with a sulcus depth of 3mm or less, no loss of attachment and no clinical signs of inflammation, as determined by the Löe and Silness gingival index (1963), as well as by a lack of bleeding within 30 seconds of gentle probing (Meitner, 1979). Twenty-two specimens were obtained from 16 male and 6 female donors diagnosed with

** It is considered that the new classification recommended at the 1999 International Workshop for a Classification of Periodontal Diseases and Conditions does not impact on the study design or classification of the study groups.
advanced adult-type periodontitis, with suprabony pocket formation, resulting in probing
depths ranging from 7 to 10mm. All biopsies were buccal gingivae taken from teeth
anterior to the second molars before tooth extraction. The biopsies included as much of
the sulcus or suprabony pocket wall as possible. The donor age ranges for the advanced
periodontitis and minimally inflamed tissues were 37 to 90 years, mean age 56.9 years
and 23 to 82 years, mean 48 years, respectively.

Biopsies were snap frozen in isopentane, mounted with a buccolingual orientation,
that is, crest of gingiva to base of pocket/sulcus (Figure 6, 7) in OCT embedding
compound and 6μm sequential sections cut at a cryostat temperature of -12 to -16°C,
then stored at -70°C until required. A tissue reference library was compiled for each
biopsy comprising every 10th sequential slide being stained with haematoxylin and
cosin.

5.2.2 Immunohistochemical probes

The high specificity macrophage marker Ber-MAC3 (Backe et al., 1991) and the pan
marker KP1, used to detect CD68 antigen, were obtained from Dako Co., Carpintaria,
CA, USA. The monoclonal antibodies for the different macrophage phenotypes were
27E10, (Zwadlo et al., 1986) an acute inflammatory marker, 25F9, (Zwadlo et al., 1985)
for resident histiocytes, and RM3/1, (Zwadlo et al., 1987) for reparative macrophages,
all obtained from Dianova, Hamburg, Germany.

5.2.3 Procedures

Prior to immunolabelling, sections were fixed in ice cold methanol for 3 min or cold
acetone for 10 min (for the methanol sensitive antigen recognised by RM3/1), washed in
phosphate buffered saline (PBS) and then blocked for 1 h in 20 per cent horse serum in
PBS.

A two-step immunoperoxidase staining procedure was used. Briefly, tissues were
incubated in mouse monoclonal anti-human primary antibody in 10 per cent foetal calf
serum (FCS) at a dilution of 1:50 for 1 hour in a humid chamber. Negative controls were
obtained by replacing the primary antibody with an irrelevant antibody of the same
isotype (mouse IgG1 Dako, Code X931). The tissues were then washed in PBS for 3x10
min and incubated with the secondary antibody, peroxidase-conjugated, goat anti-mouse IgG (Dako, Code P447) in 10 per cent FCS at 1:50 concentration. Conjugates were incubated in a humid chamber for 1 h at 23°C then washed in PBS for 3x10 min, and subsequently detected and visualised with the chromogen diaminobenzidine (DAB, Dako). Endogenous peroxidase labelling was blocked by pre-incubating slides in 3 per cent H₂O₂ for 1 min. The DAB was developed for 5 min at 23°C. Slides were counterstained with Mayer’s haematoxylin and coverslipped.

Positive controls were frozen sections of human cervical lymph nodes, kindly provided by Dr L McDonald, (Dept. Oral Pathology and Medicine, Westmead Hospital) prepared and appropriately stained. Specificity of staining was confirmed by a pattern of reactivity confined to macrophage-like cells#. Negative controls were incubated with 10% FCS + PBS without the primary antibody application. Additional controls consisted of substitution of the primary antibody with an isotype matched irrelevant murine antibody (IgG1 Dako XC931).

5.2.4 Analysis

The sequential stained sections were coded and divided into fields (Figure 8). At semi-focus random sites within each field were selected at low magnification then focused at magnification ×312. Multiple sites, the size of the rectangular photographic grid, were examined using a Leitz Dialux 20 EB microscope and photographed using Konica Impressa 50 film. The size of the areas counted was standardised and macrophages were identified and counted using a grid frame overlayed on the photomicrographs. Reproducibility was 95% for the calibrated observer. The variable thickness of frozen sections made quantification of intensity of staining for positive cells as an indication of the degree of activity inappropriate and misleading. All probes were studied in at least one bracket of sequential tissue sections and, where possible, in two or more widely spaced brackets.

5.2.5 Statistical analysis

Mean and standard errors were calculated from the cell counts taken over the representative fields in each specimen for MAC3, 27E10, 25F9 and RM3/1. Where appropriate, significance was determined using a 2 tailed Student t-test for unpaired data.

# The expected numeracy could not be confirmed as there are no published findings.
Figure 8  Diagram of a typical biopsy section showing an inverse incision and Field 1 which represents the base of the pocket and deep pocket epithelium, Field 2 the superficial pocket epithelium or oral sulcular epithelium (minimally inflamed biopsy), Field 3 the connective tissue and Field 4 the oral epithelium. In the minimally inflamed biopsy section the depth of the sulcus was insufficient to accommodate Field 1.
5.3 RESULTS

5.3.1 Histological appearance of biopsies

The minimally inflamed biopsies (Figure 3) all demonstrated similar features. The keratinised oral epithelium displayed regular rete pegs and the sulcular epithelium showed some thickening with a limited inflammatory cellular infiltrate observed adjacent to the epithelium. The supporting connective tissue was dense and consisted of bundles of collagen fibres, fibroblasts and blood vessels.

The advanced periodontitis biopsies (Figure 7) displayed an attenuated pocket epithelium with long, thin, irregular processes extending into the adjacent connective tissue. In some areas the epithelium was only a few cells thick. The connective tissue adjacent to the pocket epithelium had been largely replaced by an infiltrate of chronic inflammatory cells, predominantly plasma cells in perivascular foci. The deep connective tissue displayed varying amounts of intact tracts of fibrous connective tissue between foci of accumulated inflammatory cells. The connective tissue adjacent to the oral epithelium was intact.

5.3.2 CD68

Anti-CD68 stained macrophages, some neutrophils, as well as interdigitating and dendritic-type cells.

Using morphometric analysis mean profile densities for CD68-positive and MAC3-positive cells were determined then compared for similar regions. The results showed that between 50 and 70 per cent of the CD68-positive cell population also expressed MAC3 (Figure 9A and B). There was also evidence of anti-CD68 staining consistently non-macrophage positive cells across all fields. Therefore, CD68 was not included in further analyses.

5.3.3 MAC3, 27E10, 25F9 and RM3/1

The results of the mean positive cell counts for the markers MAC3, 27E10, 25F9 and RM3/1 for each field are presented in Figure 10.
Figure 9A and B  Photomicrographs of serial sections of advanced diseased tissue stained for (A) CD68 and (B) MAC3. Macrophages staining for both CD68 and MAC3 (arrows).
Figure 10 Positive immunoreactive cells in advanced periodontitis (■) and minimally inflamed gingival tissue (□) in Fields 4, 3, 2, 1 are presented for MAC3 (pan-macrophage marker), 27E10 (acute/inflammatory marker), 25F9 (resident histiocyte) and RM3/1 (reparative macrophage). Y-axis shows the number of positive cells per mm². The data are mean values ± SEM. For each marker t-tests between fields were * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. There was no statistically significant difference between the advanced and minimally inflamed groups for each marker.
Frequently, within a given field in both biopsy groups, wide variations in patterns of distribution and numbers of positive cells were noted. This was overcome by counting four randomly selected sites within a field to produce a mean count.

Advanced and minimally inflamed tissues displayed similar distribution patterns for all of the macrophage phenotypic markers (p>0.05).

The MAC3+ cell was the most highly represented phenotype across all fields in both advanced and minimally inflamed tissues. There were no significant differences between fields except for Fields 4 and 1, where there were fewer cells (p<0.05) at the base of the pocket in biopsies of advanced disease.

The RM3/1 phenotypic marker was the second most prevalent reaction displayed in the two tissue groups. Although there appeared to be fewer cells in Field 1, no statistically significant differences occurred across the fields.

Less numerous were the 27E10+ and 25F9+ phenotypes. In advanced and minimally inflamed tissues 27E10+ cells displayed a similar distribution pattern. Positive cells were most plentiful adjacent to the oral epithelium in Field 4 and decreased towards the sulcus and the base of the pocket (Field 1) (p<0.05). A similar pattern occurred for the 25F9 marker, with most cells in Field 4, fewer in Field 2 (p<0.05) and the least in Field 1 (p<0.01). These findings were supported by additional analyses comparing the different markers for the same field.

The combined cell count of 27E10, 25F9 and RM3/1 was greater than the pan MAC3 marker, possibly in relation to co-expression of 25F9 and RM3/1 by some cells.

5.3.4 Staining patterns and distributions

The antibody MAC3 stained the cytoplasm of macrophages detailing well defined ovoid, dendritic or irregular shaped cells (Figure 11), which were well represented throughout all fields in both specimen groups. Most positive cells showed discrete staining, however, some were clustered, occasionally producing a reticular pattern in both advanced and minimally inflamed tissues across all fields. Distribution of the labelled cells also displayed a strong association with blood vessels, presenting in ribbon-like bands.
Cells reacting with 27E10 displayed a dense surface and intracellular staining pattern as well as a soft local extracellular staining observed under high magnification (Figure 12). Positive cells were well defined, discrete and scattered, and strongly represented in the connective tissue adjacent to the oral epithelium. A stained extracellular connective tissue was observed in some areas adjacent to the pocket wall.

The antibody 25F9 stained both the cell membrane and intracellularly as well as producing a limited, localised extracellular stain (Figure 13). Also stained were the cell membranes of the basal cell layer of the oral epithelium and an extracellular reaction was noted in the sulcular epithelium and at the base of the pocket. The immunolabelled cells displayed an ovoid or irregular appearance and were scattered through all fields with clusters sometimes observed adjacent to the oral epithelium and along the advanced tissue pocket wall, that is, Fields 1 and 2.

Antibody RM3/1 produced a surface staining on irregular or dendritic-shaped macrophages. A reticular pattern was frequently observed particularly in the connective tissue adjacent to the oral epithelium (Figure 14) and in the deep connective tissue of both groups.
Figure 11  Tissue biopsy from a patient with advanced periodontitis showing the appearance of discrete, irregularly shaped MAC3+ cells in connective tissue adjacent to the oral sulcular epithelium (OSE).

Figure 12  Tissue biopsy from a patient with minimally inflamed gingival tissue stained for 27E10 showing large, intensely stained positive cells occurring in aggregates in the connective tissue. Note the localised extracellular staining.
Figure 13  Tissue biopsy from a patient with advanced periodontitis stained for 25F9 showing positive cells with intense membrane and intracellular staining as well as a limited localised extracellular stain, occurring in clusters in connective tissue adjacent to the oral epithelium.

Figure 14  Biopsy from minimally inflamed gingival tissue stained for RM3/1 and showing strong presentation of numerous positive cells with dendritic appearance beneath the oral sulcular epithelium (OSE) and oral epithelium (OE).
5.4 DISCUSSION

CD68 does not display high macrophage-specificity in gingival tissue. Cells expressing CD68 included dendritic-type cells, presumably Langerhans cells, in epithelium and interdigitating reticulum cells and neutrophils in the connective tissue. This range of cell types staining for CD68 is in agreement with other reports (Holness and Simmons, 1993). The results showed that between 50 and 70 per cent of the CD68-positive cell population also expressed MAC3 antigen (Figure 9A and B), with wide variations occurring between fields. Meaningful interpretation of the macrophage distribution using anti-CD68 was not possible and hence further comparisons with other macrophage phenotypes would have been inappropriate. This finding also has implications for other studies that have used CD68 antigen to identify and quantify macrophages in periodontitis.

In the advanced lesion, the marker for MAC3 antigen revealed a relatively even distribution of reactive cells throughout the tissues, with the exception that the density of macrophages found at the base of the pocket was significantly less than adjacent to the oral epithelium. The reparative phenotype identified by RM3/1 represented approximately 50 per cent of the total MAC3 macrophage count and displayed a similar distribution pattern to those identified by MAC3, again with the least number of reparative cells detected at the base of the pocket. The acute inflammatory (27E10) and resident histiocytic (25F9) macrophages each represented approximately 30 per cent of the total macrophage count. Cells identified by these markers displayed a similar distribution pattern manifested by a descending gradient of cell density from the oral epithelium to the base of the pocket. These findings did not support the accumulation of acute inflammatory macrophages in close proximity to the microbial insult.

This study also found that the macrophage populations in advanced periodontitis tissues displayed similar distribution patterns to minimally inflamed tissues. Macrophage densities were consistent between advanced and minimally inflamed tissues after adjusting for the additional field, the base of the pocket, studied in the advanced diseased tissues.
There were, however, regionally-specific differences in the populations identified by these phenotypic markers.

All of the macrophage phenotypes were strongly represented in the tissue adjacent to the oral epithelium. Others (Schlegel-Gomez et al., 1995) have also noted this finding. It has been suggested that bacterial, mechanical or chemical stimulation of the oral epithelium may elicit cell recruitment to this region; however, the function of these macrophages has not been determined.

Schlegel-Gomez et al., (1995) conducted a cross-sectional, semi-quantitative study of macrophages in gingival tissues using the 27E10, 25F9 and RM3/1 monoclonal antibodies to probe healthy gingivae, gingivitis lesions and pooled tissues obtained from treated early to advanced, periodontitis lesions. They found that the distribution trends for the phenotypic macrophage populations in gingivitis and periodontitis tissues were similar, a finding confirmed in the present study. They also found, consistent with the present findings, constant low numbers of acute inflammatory, histiocytic and reparative macrophages along the sulcus or pocket wall of the different biopsy groups. In the treated periodontitis lesions there was strong representation adjacent to the oral epithelium for 27E10, 25F9 and RM3/1.

Topoll et al., (1989) used an experimental gingivitis model and studied 8 young male adults (mean age 27.6 years), to follow cellular changes over a 33 day period. Quantitative changes in macrophage subpopulations were defined by the 27E10, 25F9 and RM3/1 antibodies. They found the acute and reparative macrophage populations to be highly reactive to changes in levels of inflammation. There was up to a six fold reciprocal change in 27E10 and RM3/1 cell numbers during the development and resolution of inflammation. As inflammation developed 27E10 cells increased and RM3/1 cells decreased, and as inflammation resolved 27E10 cell numbers decreased and RM3/1 increased. In contrast, the number of histiocytic cells identified by 25F9 remained stable. The additional finding of no significant change in the total macrophage population with the development of experimental gingivitis in humans was also noted by Brecx et al., (1987) and Seymour et al., (1988). Topoll et al., (1989) limited their study to the region adjacent to the gingival sulcus. The present study of the minimally inflamed lesion was extended to include representational sites throughout the biopsy.
The antigens recognised by the panel of antibodies could represent separate functional activities or an array of unknown functions or the maturation stage of the macrophage. This raises the issue of simultaneous co-expression of activation markers for different functions. Zwadlo et al., (1987) found co-expression of 25F9 and RM3/1 on macrophages in some chronic diseases, including rheumatoid arthritis; however, it is unknown whether these markers represent distinct and separate functions. Dual expression could explain the discrepancy in total cell count between MAC3 and the combined counts for 27E10, 25F9 and RM3/1 positive cells observed in the present study.

During chronic inflammation increased production of monocytes in the bone marrow results in the number of circulating monocytes increasing temporarily by two to three-fold relative to the number under normal steady-state conditions (van Furth, 1988). Labelling studies in mice have shown that at least 70% of circulating monocytes migrate to the site of inflammation, resulting in approximately a ten-fold increase in the numbers of macrophages in the inflammatory exudate (van Furth et al., 1973). In chronic inflammation, such as periodontal diseases, macrophages at the site of the lesion are primarily bone marrow-derived monocytes while the contribution by local production to the number of tissue macrophages is relatively small, on average 7% (van Furth, 1992).

A hallmark of chronic inflammation is the accumulation of macrophages at the site of inflammation. The findings of this study are contrary to documented evidence that chronic inflammation leads to an increase in macrophage numbers (van Furth, 1988). Others (Brecx et al., 1987, Seymour et al., 1988) who studied the developing gingivitis lesion found constant total macrophage numbers in periodontal inflammation.

The influx of monocyte-derived macrophages at a site of inflammation has been well documented, however, little is known about the mechanisms controlling persistence and clearance of macrophages from the tissues. The possibility of altered kinetics might account for the lack of observed differences in macrophage numbers in minimally inflamed gingival tissues and advanced periodontitis. An accelerated influx might be balanced by an increased clearance. For example, numbers may remain constant due to macrophage migration through the sulcular epithelium and into the pocket. If this were so then an increased density of macrophages should have been evident adjacent to the
sulcular epithelium which was not the case. A recent study investigated *in vivo*, the fate of inflammatory macrophages during the resolution of inflammation. It was found that inflammatory macrophages, after performing scavenging activities did not die locally, by apoptosis or phagocytosis, but emigrated to draining lymph nodes (Bellingan *et al.*, 1996). Enhanced removal of macrophages via the lymphatics might account for the observed constant macrophage numbers.

Another explanation for steady cell numbers could be that migration of newly recruited cells is impeded by pathologically altered blood vessels. Pinchback *et al.*, (1996a) found marked thickening of the basement membrane as a result of increased deposition of type IV collagen and laminin in advanced periodontitis tissues.

An animal model used extensively in the study of periodontal inflammation showed an association between ageing and decreased macrophage numbers (Berglundh and Lindhe, 1993). This is unlikely to explain the findings of the present study as the age range of the two groups showed considerable overlap.

The significance of the reduced density of different subpopulations of macrophages along the pocket wall can only be speculated upon. It has been demonstrated *in vitro* that *A. actinomycetemcomitans* produces a leukotoxin, a member of the RTX toxin family of pore forming cytotoxins (Lally *et al.*, 1989) that kills monocytes and PMNs (Taichman *et al.*, 1980, Rabie *et al.*, 1988). Leukocyte function-associate antigen-1 (LFA-1, CD11a/CD18) is the cell surface receptor for RTX toxins on target cells (Lally *et al.*, 1997) and is crucial in mediating reversible leukocyte-endothelial adherence. Further investigations are required to determine whether this potent leukotoxin is present in the tissues in an active form capable of suppressing macrophage numbers.

In conclusion, this study has shown that macrophages within the untreated lesion of periodontitis formed a heterogeneous cell population displaying different developmental and functional stages. The phenotypical heterogeneity and patterns of distribution of different macrophage subpopulations have been demonstrated by the presence of macrophages displaying antigens for CD163 (MAC3 and RM3/1), 27E10, and 25F9. This study found that advanced periodontitis and minimally inflamed tissues displayed similar distribution patterns and densities for the macrophage phenotypic markers. However, regionally-specific differences in the populations occurred. The full significance of the roles played by the different phenotypic subpopulations remains to be elucidated as does the mechanism for the recruitment of macrophages to specific sites within the lesion of periodontitis.
CHAPTER 6
MACROPHAGE ACTIVATION FOR DIGESTION AND ANTIGEN PRESENTATION IN PERIODONTITIS

6.1 INTRODUCTION

The distribution of different phenotypic macrophage populations in diseased and healthy gingival tissues was examined in the previous chapter. The presence of macrophages tells us little about their activation or function within periodontal tissues.

There have been few investigations into the functional status of macrophages in treated periodontitis lesions and only one in untreated tissue in which nonspecific esterase and acid phosphatase activities were examined (Charon et al., 1981). There is now a requirement to define the functional status of macrophages in relation to pathological changes in untreated periodontitis.

While there has been considerable success in developing antibody probes that distinguish specific macrophage populations, there are few reliable activation-related antigens described for macrophages (Sunderkotter et al., 1994). This issue is further complicated by the plethora of separate functional states, including phagocytosis and killing of opsonised microorganisms, antigen presentation to T cells, cytotoxicity and the secretion of over one hundred products including potent cytokines and growth factors that can be attained by macrophages (Sunderkotter et al., 1994). Therefore, in order to assess the activation of macrophages in periodontitis this chapter describes an investigation of three recognised specific functional activation markers for macrophages; HLA-DR, AP and TRAP, utilising immunohistochemistry and histochemistry procedures. MHC class II, HLA-DR represents the capacity of macrophages for antigen presentation to T-cells, while the presence of acid phosphatase (AP) and tartrate-resistant acid phosphatase (TRAP) indicate the digestive capacity of macrophages.

Based on previous observations of pathological features in the lesion of periodontitis, including accumulations of degenerate plasma cells (Freeman et al., 1968, Joachim et al., 1990), remnants of basement membrane material, and a restricted capacity to
develop reparative granulation tissue (Zoellner and Hunter, 1989, Pinchback et al., 1996a), it is postulated that macrophages are not appropriately activated in periodontitis.

6.1.1 Activation

Activation, the enhancement of important functions by macrophages has been discussed in Chapter 2. The concept of the requirement for activation prevails in almost all studies of macrophage function. The potent role of activated macrophages in angiogenesis is a good example of this (Sunderkotter, 1994).

When macrophages are activated they display increased size and surface ruffling, an increased number of mitochondria and free ribosomes, as well as an enlargement of the Golgi complex. Depending on the functional state of the activated macrophage there can be an increased content of hydrolytic enzymes, enhanced production of reactive oxygen intermediates, or increased surface expression of major histocompatibility complex class II molecules (Adams and Koerner, 1989).

6.1.2 Functional activation markers

Antigen presentation and digestion as part of scavenging activities by macrophages have been described in Chapter 2. Functional activation markers for these activities are described.

6.1.2.1 Major histocompatibility complex class II molecule HLA-DR

The major histocompatibility gene complex (MHC) class II gene consists of at least four subregions: HLA-DR, -DC, -DP and -DQ. The products of the HLA class II gene subregion display a similar biochemical structure, that is, alpha and beta polypeptide chains.

Anti–HLA-DR recognizes a human class II major histocompatibility complex (MHC) antigen. The antigen is a transmembrane glycoprotein composed of two non-covalent immunoglobulin-like polypeptide chains, α and β that have molecular weights of approximately 34 and 28 kDa, respectively. HLA-DR antigen is expressed on monocytes and macrophages, as well as on B lymphocytes, activated T lymphocytes,
activated natural killer (NK) lymphocytes, dendritic cells, interdigitating Langerhans cells, and some keratinocytes.

6.1.2.2 Hydrolases

A variety of hydrolytic and proteolytic enzymes are involved in degradation. Two hydrolase enzymes representing digestive capacity in activated macrophages are described.

6.1.2.2.1 Lysosomal acid phosphatase (AP)

Acid phosphatase activity is associated with lysosomal hydrolases (Weissmann, 1965). Activated macrophages displaying phagocytic activity and enhanced bactericidal capacity have increased numbers of lysosomes associated with increased synthesis and secretion of lysosomal hydrolases. It has been demonstrated in vitro that macrophages activated by endotoxin from dental plaque synthesise and release lysosomal enzymes (Page et al., 1973, Wahl et al., 1974).

6.1.2.2.2 Tartrate resistant acid phosphatase (TRAP)

TRAP, also referred to as human purple phosphatase, has emerged as an important marker of macrophage activation and differentiation (Janckila et al., 1996, Chun et al., 1999). TRAP, a metalloprotein, is expressed by activated macrophages (Yaziji et al., 1995) and by some specialized cells of macrophage lineage, including osteoclasts (Chun et al., 1999) as well as some activated lymphocytes (Janckila et al., 1996). Confocal fluorescence studies have confirmed that TRAP is localized to the lysosomal compartment of macrophages (Hayman et al., 2000) and is not secreted (Drexler and Gignac, 1994).

Studies in vitro suggest that TRAP may have a similar physiological activity in both osteoclasts and macrophages (Halleen et al., 1998). Although the biological role in relation to bone resorption and phagocytosis has not been fully elucidated, it has been postulated that TRAP can generate oxygen-derived free radicals that participate in microbial killing (Hayman and Cox, 1994) and bone resorption (Drexler and Gignac, 1994). In TRAP knock-out mice disruption of endochondral ossification and mild osteopetrosis occurs. From these observations and studies in vitro Halleen and others postulated that TRAP destroys endocytosed matrix degradation products. This
mechanism is presumably important in connective tissue matrix degradation and remodelling (Halleen et al., 1999).

6.2 MATERIALS AND METHODS

6.2.1 Tissue specimens

The gingival tissue samples obtained for the previously described study were used in this study. Briefly, gingival tissues were collected from 48 consenting donors who had no record of previous periodontal therapy, significant associated diseases, including diabetes, or a history of relevant drug usage. The tissues collected were classified using clinical and histological criteria as either minimally inflamed or advanced periodontitis. Twenty-six biopsies, collected from teeth with no loss of gingival attachment, probing depth ≤ 3mm and with no clinical signs of inflammation, were classified as minimally inflamed. Twenty-two gingival specimens were obtained from patients diagnosed with advanced adult type periodontitis, with bone loss and pocket formation, resulting in probing depths ranging from 7 to 10mm. All biopsies were buccal gingivae taken from teeth anterior to the second molars before extraction. The donor age ranges for the advanced periodontitis and minimally inflamed tissues were 37 to 90 years and 23 to 82 years, respectively.

Biopsies were frozen and processed as described in the previous chapter.

6.2.2 Immunohistochemical and histochemical probes

The monoclonal antibodies against HLA-DR for the expression of MHC class II, and Ber-MAC3 for the identification of macrophages were purchased from Dako Co., Carpintaria, CA, USA. DAKO-HLA-DR, CR3/43 reacts with the β-chain of all products of the gene subregions DP, DQ and DR and is suitable for labelling acetone-fixed cryostat sections. MAC3 specificity was described in the previous chapter.

6.2.3 Procedures

6.2.3.1 Dual immunohistochemistry

Dual staining was performed to determine coincidence of markers HLA-DR and MAC3 and to enhance identification of the macrophage phenotype, using a peroxidase
reaction followed by an alkaline phosphatase staining technique as described by Pinchback et al., 1996a.

Briefly, sections were fixed in acetone at room temperature for 10 min, then blocked for 1 h with 20% HS in PBS. Incubation with the primary antibodies against MAC3 and HLA-DR was for 1 h at room temperature. Slides were washed after each step for 30 min in PBS for the peroxidase reaction and in TRIS buffer for the immunophosphatase reaction.

Secondary antibodies were goat anti-mouse Ig conjugated with horseradish peroxidase and rabbit anti-mouse Ig conjugated with alkaline phosphatase. Secondary antibodies were incubated for 45 min at room temperature.

The chromogen used for the first stage was DAB, developed for 5 min and for the second stage Fast-Blue BB salt (Sigma) was developed for 20 min.

To allow for quenching, incubation with MAC3 was carried out initially. All antibodies were diluted in 10% FCS to the appropriate concentrations which were determined on the basis of preliminary experiments.

Positive controls were single stained gingival tissues. Single anti-MAC3 and anti-HLA-DR antibody staining for gingival tissue elements displayed typical patterns as previously reported. Negative controls were incorporated to test each stage of the double staining process. Negative controls were incubated without the primary antibody application. Additional controls consisted of substitution of the specific antibody with an isotype matched irrelevant antibody. These controls were negative in all cases.

6.2.3.2 Histochemistry

The detection of acid phosphatase (AP) was carried out by Burstone’s technique (Danenberg et al., 1981) utilising naphthol AS-B1 phosphate as substrate and fast Red Violet LB salt (Sigma Chemical Co., St Louis, MO, USA) at pH 5.2. The addition of inhibitor sodium tartrate to 0.01M was used to detect the presence of tartrate-resistant acid phosphatase (TRAP). Tissues were fixed in ice cold methanol then rinsed in acetate buffer before being incubated at 23°C with the stain reagents for 1 h. The slides were washed in distilled water, dried, counterstained with Mayer’s haematoxylin for 2 min, then coverslipped.

For a positive control for TRAP, sections of periapical granuloma were used.
6.3 RESULTS

Cells reacting for HLA-DR were closely related to blood vessels and strongly represented in the reactive pocket epithelium of periodontitis. The typical presentation was of a reticular appearance in all fields for both minimally inflamed and advanced disease specimens. Dual staining with MAC3 (Figure 15) showed that very few cells displayed both markers. To verify this finding labelled cells were counted in consecutive sections with each labelled antibody and then the sum of these counts were compared with that for the dual immunolabelling on one section.

In advanced periodontitis the acid phosphatase (AP) stain reaction typical of macrophages was strongly represented immediately adjacent to the pocket wall (Figure 16A, 17A) and for some specimens, beneath the oral epithelium. The distribution of reactive cells was highly variable in the deep connective tissue. In minimally inflamed tissues AP positive cells were predominantly adjacent to the sulcular epithelium and scattered beneath the oral epithelium.

Tartrate-resistant acid phosphatase was not found (Figure 16B) with the exception of an occasional positive cell located near bone fragments (Figure 18) or in the connective tissue adjacent to the pocket wall in eight (8/16) advanced specimens (Figure 17B). Macrophage-like TRAP+ cells were located at the base of the OSE in eighteen (18/26) minimally inflamed tissues probed.
Figure 15  Tissue biopsy from a patient with advanced periodontitis showing dual staining for MAC3+ cells (brown staining) and HLA-DR+ cells (blue staining) along the pocket wall comprising pocket epithelium (PE) and connective tissue (CT). There is no evidence for co-expressing cells.
Figure 16A  Tissue from a patient with advanced periodontitis stained for acid phosphatase. High power magnification of the pocket wall showed numerous, strongly reactive cells typical of macrophages between the projections of attenuated epithelium (PE). In contrast, Figure 16B showed no staining for TRAP along the pocket wall in the tissue biopsy from the same patient.
Figure 17A Tissue biopsy from a patient with advanced periodontitis stained for acid phosphatase. The pocket wall showed numerous, strongly reactive cells typical of macrophages between the projections of attenuated epithelium (PE). B. Tissue from the same patient stained for TRAP showed occasional, positive stained cells typical of macrophages.
Figure 18  Tissue biopsy from a patient with advanced periodontitis stained for TRAP, showing positive stained cells associated with alveolar bone fragment.
6.4 DISCUSSION

Examination for antigen presentation capacity revealed that very few macrophages expressed detectable HLA-DR. Although HLA-DR was strongly represented in the reactive pocket epithelium of periodontitis and in vascular structures, in both advanced disease and minimally inflamed specimens, there were few HLA-DR+ macrophages in any region, as determined by dual staining with MAC3. Not all macrophages have the capacity for antigen expression as MHC class II molecules on macrophages are transitory and are eventually lost. The small subset of MAC3+/HLA-DR+ macrophages requires further study to define the precise phenotype.

In the process of chronic inflammation concurrent tissue destruction and ongoing repair occurs, with the immunosuppressive effect of TGF-β being an essential regulator of wound healing and repair (Roberts et al., 1986). An immunosuppressive effect of TGF-β1 is the down-regulation of MHC class II expression on macrophages (Czarniecki et al., 1988) while expression on B-cells is modestly up-regulated (Cross and Cambier, 1990). Recently, TGF-β1 has been found to be expressed by many cell types, including macrophages, T and B-cells, neutrophils, mast cells, endothelial cells, and fibroblast-like cells in periodontitis tissue (Steinsvoll et al., 1999, writer’s unpublished data).

There have been few studies undertaken to examine the antigen presentation capacity of macrophages in healthy and in diseased gingival tissues. Macrophages were identified by morphology and staining for AP as part of a series of studies to determine the pattern of MHC class II antigen expression in treated periodontitis tissues (Walsh et al., 1985) and in developing experimental gingivitis (Seymour et al., 1988). In treated tissue beneath the oral epithelium the dominant macrophage-like cells were HLA-DR+ and AP−, whereas cells within the deeper connective tissues were HLA-DR+, and AP+ suggesting phagocytic function (Walsh et al., 1985). In the developing experimental gingivitis model studied by Seymour and others, the dominant macrophage population was HLA class II negative but AP+ indicating a phagocytic population. It was also noted that interdigitating cells subjacent to the oral epithelium expressed HLA class II.
molecules. Walsh et al., (1986) had found earlier that there was an increase in the number of HLA-DR expressing Langerhans cells in experimental gingivitis compared with healthy gingiva (Walsh et al., 1986). In the present study interdigitating HLA-DR+ cells were dominant subjacent to epithelium and in perivascular spaces. Further, an investigation of HLA-DR expression in gingival tissues using monoclonal antibodies found that Langerhans cells (LC), dendritic cells (DC) and keratinocytes were all HLA-DR+. HLA-class II antigen was distributed along both oral epithelium (OE) and pocket epithelium (PE) and notably, HLA-DR+ keratinocytes were detected along the whole extension of both OE and PE (Nunes et al., 1994). In this context, it has been postulated that “non-professional” APCs, such as keratinocytes, because of their strategic tissue localisation, can amplify and sustain local immune reactions during normal and pathological responses (Tykocinski et al., 1996).

Dendritic cells such as Langerhans cells and interdigitating dendritic cells, are the most efficient antigen-presenting cell type to activate T cells. As APCs they are 100-fold more potent than macrophages (Di Nicola and Lemoli, 2000) and their expression of HLA-class II molecules is more stable (Bjercke and Gaudernack, 1985). They are however, characterised by a low phagocytic capacity (Dijkstra and Damoiseaux, 1993). Macrophage depletion studies in mice have demonstrated that these cells are important in enhancing the antigen presenting function of DCs (Nair et al., 1995). A dual accessory cell concept has been proposed whereby macrophages (pre)process particulate antigens then transfer the fragmented antigens to better equipped APCs, such as dendritic cells (van Rooijen et al., 1992, 1996, Nair et al., 1995). Accordingly, there is a need to elucidate this potential role of the macrophage in periodontitis.

Based on the findings of the dual labelled HLA-DR/MAC3 study and the earlier studies of MHC class II expression in gingival tissues, it is speculated that dendritic cells are the dominant APCs in gingival tissues, in health and in periodontitis.

Acid phosphatase, important in the digestive activity of macrophages, was detected in sites proximal to the sulcus/ pocket wall and the oral epithelium in minimally inflamed and advanced diseased tissues. In the connective tissue of the advanced lesion
the distribution pattern of this hydrolase was highly variable. Charon et al., (1981) used morphological features to identify macrophages in untreated periodontitis tissues and noted the detection of acid phosphatase associated with macrophage activation. Other cells also produce AP; however, in inflamed gingiva the macrophage is readily identified by its strong staining pattern. The findings of the present study confirm those of Charon et al., in relation to distribution, with the additional observation that some macrophages were detected at the periphery of clusters of plasma cells but not within the plasma cell foci even when the majority of cells were clearly degenerate. This finding indicates a lack of appropriate macrophage activation for digestive activity. Without the removal of effete cells and debris by macrophages, tissue turnover and repair is compromised.

This study found that TRAP was limited to occasional positive cells, presumably osteoclasts, located near bone fragments observed in some biopsies of advanced disease tissue. This is consistent with other observations of TRAP+ cells associated with bone resorption (Shibutani et al., 1997). Interestingly, more minimally inflamed tissues than advanced specimens displayed the occasional macrophage-like TRAP+ cell in connective tissue adjacent to the base of oral sulcular epithelium (OSE). This could be an appropriate response for successful defence and maintenance of minimally inflamed gingival tissues. In contrast, the persistent microbial challenge and protracted immune response in advanced disease, results in disruption of tissue maintenance and repair.

The striking reduction of macrophage activation and numbers adjacent to the pocket wall is notably different from the observations in other epithelial-connective tissue inflammatory processes. Pathogens and tumour cells have been shown to deactivate macrophages by the production of macrophage-deactivating agents. It has been demonstrated in vitro that LPS from different strains of P. gingivalis can inhibit inflammatory macrophage activation (Shapira et al., 1998). There may also be a need for host cells to deactivate macrophages under certain circumstances, to restrict the tissue damage caused by cytotoxic products produced by macrophages. Further research is needed to determine whether these mechanisms operate in vivo in periodontitis.

From this study it is concluded that the majority of macrophages of the MAC3 phenotype are not activated for antigen presentation although there is abundant MHC
class II molecule expression in periodontitis. Further, the lack of AP expression in macrophages in close proximity to foci of plasma cells and the restricted expression of TRAP indicate a lack of appropriate macrophage activation in advanced periodontitis. These findings support the hypothesis that macrophages are not appropriately activated in periodontitis. The unique environment of the gingival tissues and the associated conditions resulting in periodontal inflammation together with the unusual pathological features of the untreated lesion may explain in part, the apparent failure of recruitment and activation of macrophages. This is further considered in Chapter 9, the general discussion.

The next chapter focuses on features of vascular remodelling and disruption associated with periodontitis.
CHAPTER 7
VASCULAR REMODELLING IN PERIODONTITIS

7.1 INTRODUCTION

Periodontitis is a chronic inflammatory disease of the highly vascularised supporting tissues of the teeth. In this chapter a series of investigations are described that define vascular changes in periodontitis and in the following chapter the potential role of the macrophage is examined in relation to these changes.

There has been little research on vascular changes in the untreated lesion of periodontitis. Studies using animal models of periodontitis (Kindlova, 1967, Hock and Kim, 1987) and tissue from gingivitis (Bergstrom, 1992) or treated human periodontitis (Bonakdar et al., 1997) have documented the highly vascular nature of the lesion. Earlier studies have indicated that in the region of the periodontal pocket, there is a relationship between an increase in the numbers of blood vessels and progression of the disease (Zoellner and Hunter, 1991b), with evidence of marked thickening of vascular basement membranes (Pinchback et al., 1996a), especially affecting capillaries and venules (Zoellner and Hunter, 1989).

Subsequent studies demonstrated that untreated advanced periodontitis was associated with vascular expression of matrix metalloproteinase 1 (Pinchback et al., 1996b). These and other studies have also identified the presence of remnants of basement membrane apparently not associated with intact blood vessels (Pinchback et al., 1996a, Haapasalmi et al., 1995).

In the present study vascular remodelling in periodontitis is defined and quantified, using immunohistochemistry and morphometric analysis.

7.2 MATERIALS AND METHODS

7.2.1 Tissue Specimens

Gingival tissue samples were obtained, with approval by the Human Ethics Committee of the Central Sydney Area Health Authority and with informed consent from 22 adult patients who were attending the United Dental Hospital, Sydney, for
dental extractions. These patients had no record of previous periodontal therapy or known significant associated diseases, such as diabetes, or a history of relevant drug usage, including tobacco smoking.

The tissues collected were classified using clinical and histological criteria as either minimally inflamed or advanced periodontitis. Twelve biopsies were collected from teeth with no loss of gingival attachment and with no clinical signs of inflammation, as determined by the Löe and Silness gingival index as well as by a lack of bleeding within 30 seconds of gentle probing. Ten gingival specimens were obtained from patients diagnosed with advanced adult type periodontitis, with suprabony pocket formation, resulting in probing depths ranging from 7 to 10mm. All biopsies were buccal gingivae taken from teeth anterior to the second molars before extraction. The donor age ranges (41-82 years) for advanced periodontitis and minimally inflamed tissues were matched.

Biopsies were snap frozen in isopentane, mounted with a buccolingual orientation, that is, crest of gingiva to base of pocket/sulcus in OCT embedding compound and 6μm sequential sections cut at a cryostat temperature of -12 to -16°C, then stored at -70°C until required.

A tissue reference library was compiled for each biopsy comprising every 10th sequential slide being stained with haematoxylin and eosin.

### 7.2.2 Fluorescence and immunofluorescence probes

Endothelial cells were identified by staining with a lectin from *Ulex europaeus*, UEA-1 (Holthofer et al., 1982) labelled with either fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) (Sigma Chemical Co., St Louis, MO, USA). Extracellular matrix proteins were identified using monoclonal mouse anti-human collagen IV and polyclonal rabbit anti-laminin (Dako Co., Carpintaria, CA, USA).

### 7.2.3 Fluorescence and immunofluorescence procedures

Prior to immunolabelling, sections were fixed in ice-cold methanol for 4 min, washed in phosphate buffered saline (PBS) and then blocked for 1 hour with 20% horse serum in PBS. For single immunofluorescent labelling, tissues were incubated with
UEA-1 for 1 hour in a dark, humid chamber, then washed in PBS and dried. For double immunofluorescence, sections were fixed, washed and blocked as above. Tissues were incubated with the primary antibody for 1 hour, washed and incubated with UEA-1 and a mouse or rabbit anti-mouse secondary antibody. Secondary antibodies were conjugated with the alternate fluorescein or rhodamine fluorochrome. Labelled sections were mounted in Citifluor (Alltech, NSW, Australia) and coverslipped.

An additional dual immunofluorescence staining procedure was undertaken to determine the co-localisation of type IV collagen and laminin using the sulphonated rhodamine fluorochromes, Alexa 488\(^1\) and Alexa 568 purchased from Molecular Probes (Eugene, OR, USA). The method developed by Kumar et al., 1999, for successful visualisation of dual staining by confocal laser scanning microscopy was employed. Briefly, tissues were fixed in acetone at room temperature for 10 min, then nonspecific binding was blocked with 20% goat serum for 20 min. Appropriate concentrations of antibodies or streptavidin were sequentially applied for 30 min each, with 4 ×30 sec washes after each incubation. For rabbit anti-laminin, detection was with goat anti-rabbit immunoglobulins labelled with Alexa 488, while for mouse anti-type IV collagen, detection was with biotinylated goat anti-mouse immunoglobulins (Dako, Carpinteria, CA, USA) followed by streptavidin-Alexa 568.

All concentrations were determined on the basis of preliminary experiments and the diluent used was 10% FCS.

Negative controls were incubated without the primary antibody application or, where the reporter molecule was labelled streptavidin, without either the primary or the secondary antibody. Additional controls consisted of substitution of the specific antibody with an isotope matched irrelevant antibody or with normal rabbit serum for rabbit antibodies. These controls were negative in all cases. Specificity of staining for UEA-1 was confirmed by a pattern of reactivity confined to epithelial cells and vascular profiles. Anti-type IV collagen and anti-laminin antibodies produced the expected recognition of basement membrane components.

\(^1\) It is worth noting that FITC could have been used as an alternative to Alexa 488.
7.2.3 Immunofluorescence microscopy

Fluorescence staining of tissues was visualised using a Leitz Orthoplan microscope equipped with a xenon arc lamp, appropriate dichroic filters and a ×50, NA 1.0 water-immersion epifluorescence objective. Confocal laser scanning was performed with the Optiscan F900e personal confocal system (Optiscan, Melbourne, VIC, Australia). Accumulated digital images were acquired for morphometric analyses and three-dimensional (3-D) reconstruction using Voxblast software, version 1.3.1. (VayTek Inc., Fairfield, Iowa, USA).

7.2.4 Analysis

7.2.4.1 Blood vessel distribution patterns

Cryostat sections of tissue from 10 advanced untreated cases of periodontitis and 10 minimally inflamed gingivae were examined. Blood vessel profiles were identified by labelling endothelial cells with lectin and vascular basement membrane with antibodies to type IV collagen or laminin.

Using confocal microscopy, images were captured from three non-contiguous fields within each region. For analysis, the tissue sections were divided into regions consisting of connective tissue subjacent to oral epithelium, gingival crest, oral sulcular epithelium and pocket epithelium. Profile density counts were undertaken with vessel lumen diameters measured to quantify vessels into two groups, < 25\(\mu\)m and \(\geq 25\mu\)m. Blood vessel lumens were measured at the narrowest point to overcome exaggerated lumen presentation in obliquely cut vessels. Mean profile density counts for each region of each specimen were used for statistical analysis.

7.2.4.2 Non-vascular basement membrane remnants

Cryostat sections of tissue from 10 patients with advanced periodontitis and 12 with minimally inflamed gingivae were immunostained for non-degraded type IV collagen (Pinchback et al., 1996b) and for laminin, labelled with Alexa 488 and Alex 568 respectively. Endothelial cells were identified by staining for UEA-1 labelled with the alternate fluorochrome. Single and double immunofluorescence using a confocal laser-scanning microscope permitted identification of extracellular basement membrane
components not associated with endothelially lined blood vessels. In each stained section, connective tissue subjacent to the pocket epithelium, oral sulcular epithelium, deep connective tissue and the oral epithelial area was examined. Images were captured from 5 non-contiguous fields within each region.

7.2.5 Statistical analysis

Non-parametric analysis of variance using the Kruskal-Wallis test was followed by Dunn's test to compare all regions in both tissue groups with the minimally inflamed oral epithelial region, which served as a control.

7.3 RESULTS

The profile density of blood vessels in the region adjacent to the pocket epithelium was significantly increased in advanced periodontitis (Figure 19A). This was primarily accounted for by vessels with a luminal diameter greater than 25μm (Figure 19B). A modest increase in the number of smaller vessels (less than 25μm) was also observed in periodontitis, from the crest to the pocket wall, but this was not statistically significant (Figure 19C).

In agreement with other reports (Pinchback et al., 1996a, Haapasalmi et al., 1995, Gavin, 1970) the current study found that periodontitis was associated with thickening of vessel basement membranes (Figure 20). Additionally, this study confirmed the presence of linear accumulations of type IV collagen and laminin resembling basement membrane within the inflamed gingival connective tissue (Haapasalmi et al., 1995). Using 3-D reconstruction of z plane images captured at high magnification, these basement membrane-like structures were demonstrably not continuous with blood vessels or with epithelial basement membranes (Figure 21 and 22). However, they were frequently arranged in interrupted outlines resembling blood vessels or vascular networks (Figure 23). Dual immunostaining for endothelial cells and either undegraded type IV collagen or laminin established that the basement membrane-like material was not associated with endothelial cells and that the dominant component was undegraded type IV collagen (Figure 24A) with punctate accumulation of laminin. Furthermore, dual immunostaining for undegraded type IV collagen and laminin established that there was co-localisation
of these basement membrane components (Figure 24B). Therefore, these structures are designated residual vascular basement membrane (RVBM).

The volume density of the RVBM was quantified by point counting. This demonstrated a significant increase in advanced periodontitis, with the highest volume fraction in the connective tissue subjacent to the oral sulcular epithelium and the pocket wall (Figure 25). There was no correlation between volume density of the RVBM and density of macrophages as determined from data in Chapter 5 (Figure 26).
Figure 19  Profile density of blood vessels in 10 minimally inflamed (Min) and 10 untreated advanced periodontitis (Adv) tissues. Tissue sections were divided into regions consisting of connective tissue subjacent to oral epithelium (OE), gingival crest (CR), oral sulcular epithelium (OSE) and pocket epithelium (PE). Vessel lumen diameters were measured at the narrowest point and vessels were classified into two groups, ≥25μm and <25μm.

A: Total profile density of vessels, B: Profile density of vessels ≥25μm in diameter, C: Profile density of vessels <25μm in diameter. The data are mean values ± SEM; *** P<0.001.
Figure 20 Immunolocalisation of undegraded type IV collagen in untreated advanced periodontitis tissue. The negative image shows linear accumulations which were frequently arranged in interrupted outlines (arrows) resembling blood vessels or vascular networks. Thickening of vessel basement membranes was associated with intact vessels (large arrow head) Bar = 10μm.
Figure 21 A 2-D z plane series (1 to 4) collected at 2μm steps showing non-continuous outlines of residual basement membrane fragments (arrows) and intact vascular structures.
Figure 22 3-D reconstruction showing intact blood vessels and remnants of basement membrane not associated with vessels.

Figure 23 Basement membrane-like structures were frequently arranged in interrupted outlines resembling blood vessels and vascular networks. Fluorescence double immunolabelling showing UEA-1 positive endothelial cells (red) and type IV collagen (green) associated with vessel basement membrane, as well as type IV collagen not associated with vessels (arrows). Original magnification × 400
Figure 24A  Immunolocalisation of undegraded type IV collagen in untreated advanced periodontitis tissue. Fluorescence double immunolabelling showing a UEA-1 positive endothelial cell (red) and type IV collagen (green) associated with vessel basement membrane, as well as type IV collagen not associated with vessels (arrows). Bar = 10μm.

Figure 24B  Co-localisation of staining for undegraded Type IV collagen (red) and laminin (green) in intact microvascular complex and remnant of basement membrane not associated with vessels (arrow). Bar = 5μm.
Figure 25  The volume density of the RVBM was quantified by point counting in advanced periodontitis (n=10) and minimally inflamed gingiva (n=12). Tissue sections were divided into regions consisting of connective tissue subjacent to oral epithelium (OE), deep connective tissue (CT), oral sulcular epithelium (OSE) and pocket epithelium (PE). Data are expressed as mean values ± SEM; * P<0.05 and *** P<0.

Figure 26  There was no correlation between increased density of basement membrane fragments probed for undegraded type IV collagen (red) and MAC3 positive macrophages (green). Image shows intact vessels (block arrows) and remnants (fine arrows).
7.4 DISCUSSION

This study of the vascular changes in untreated advanced chronic periodontitis has extended previous work in this area. The results establish that in this disease, there are both quantitative and qualitative changes in the vasculature of the periodontal connective tissues.

There was evidence for extensive remodelling of the vasculature in the connective tissue subjacent to the periodontal pocket epithelium. The findings confirm that there is a marked increase in the numerical density of vessel profiles (Zoellner and Hunter, 1991b) with a selective increase in vessels of larger diameter (Zoellner and Hunter, 1991b). Increase in vessel profiles along the pocket wall could be a result of angiogenesis, that is, new vessels formed from pre-existing vessels (Risau, 1997), or a consequence of increased length and/or tortuosity of existing vessels. Enlargement of the lumen of vessels in the pocket wall might reflect vasodilation but is more likely to be a result of vascular remodelling with an increase in numbers of endothelial cells, particularly with regard to the extent of the enlargement observed. In this context, it is noteworthy that endothelial cells in dilated capillaries can proliferate (Joris et al., 1992, Majno, 1998) and acquire the functional characteristics of venules.

Vascular remodelling is a well-recognised feature of chronic inflammation, but not all tissues display similar vascular responses. For example, proliferation of new blood vessels occurs in rheumatoid arthritis (Jackson et al., 1997), whereas in psoriasis enlargement and tortuosity of vessels occurs without angiogenesis (Creamer and Barker, 1995). In an elegant study of angiogenesis in chronic inflammation, Thurston and co workers (Thurston et al., 1998) found different patterns of vascular remodelling in the chronically stimulated airways of two strains of mice, respectively resistant or susceptible to Mycoplasma pulmonis infection. Vessel proliferation occurred in the resistant mouse strain as a result of the growth of new capillary- and venule-like vessels. The susceptible strain, however, developed vessel enlargement apparently as a consequence of conversion of existing capillaries into vessels resembling venules, without proliferation. It was concluded that differences in remodelling reflected the host response to the chronic stimulus. Whether the vascular features observed in the current
study are primarily determined by the host response or by environmental stimuli is not known.

A striking feature of chronic inflammation in gingival tissue was the presence of linear accumulations of non-degraded type IV collagen with lesser amounts of laminin. These were often observed in a discontinuous pattern suggestive of the outline of a blood vessel, but were not related to endothelial cells as detected by lectin staining, nor were they continuous with existing vascular structures or epithelial basement membrane. The arrangement was strongly indicative of a vascular origin, and it is speculated that these accumulations of basement membrane material are remnants of former vessels, hence the designation RVBM. Although extravascular type IV collagen and laminin basement membrane components in chronic periodontal disease have been documented (Haapasalmi et al., 1995, Pinchback et al., 1996a), the co-localisation of type IV collagen and laminin and the discrete nature of these structures have now been confirmed, using 3-D reconstruction.

The pathogenesis of RVBM is unclear but it may be significant that the density was greatest subjacent to the oral sulcular epithelium and the pocket wall, which are the regions of greatest inflammatory cellular infiltrate. The biological significance of RVBM is also unknown. However, studies in vitro indicate that newly formed vessels deposit the basement membrane components laminin and type IV collagen independently (Yurchenco et al., 1992). Endothelial cells initially secrete laminin, followed by type IV collagen at the same time as lumen formation occurs (Kramer et al., 1984, Nicosia and Madri, 1987). Normally, basement membrane appears to function as a scaffold for orderly positioning of cells. Laminin and type IV collagen proteins isolated from basement membrane facilitate rapid differentiation of endothelial cells into tube-like structures in culture. It may be that RVBM plays some role in vascular regeneration, but if this is a physiological mechanism then it is surprising that the presence of RVBM has not, to this writer’s knowledge, been described in other chronically inflamed tissues.

In conclusion, evidence has been presented in this chapter to confirm that untreated chronic inflammatory periodontal disease is characterised by extensive remodelling of the vasculature along the pocket wall, resulting in a selective increase in larger diameter vessels and an increase in residual vascular basement remnants. In addition, the co-
localisation of type IV collagen and laminin and the discrete nature of residual vascular basement remnants have now been confirmed.

The following chapter describes an investigation of potential sources of angiogenic activity and the contributing role of the macrophage.
CHAPTER 8

ANGIOGENIC GROWTH FACTORS IN PERIODONTITIS

8.1 INTRODUCTION

In the previous chapter vascular remodelling in periodontitis was defined and quantified using immunohistochemistry and morphometric analysis. In this chapter, potential sources of angiogenic activity are investigated.

Angiogenesis, reviewed in Chapter 2, is a prominent feature of chronic inflammation and remodelling processes (Sunderkotter et al., 1994). Macrophages are a rich source of angiogenic factors (Sunderkotter et al., 1991, 1994) in wound repair (Swift et al., 1999), and in chronic inflammatory diseases, such as rheumatoid arthritis (Koch et al., 1994). The role of macrophages in angiogenesis and vascular modelling has been reviewed in Chapter 2.

Recent studies (Nissen et al., 1998) suggest that basic fibroblast growth factor (bFGF, FGF-2) and vascular endothelial growth factor (VEGF) (reviewed in Chapter 2) are likely to be important in vascular turnover and remodelling in chronic inflammation. Therefore, expression and distribution of these factors are examined by immunohistochemistry. In addition this study includes the investigation of the macrophage as a potential source of VEGF in periodontitis and in minimally inflamed gingival tissue, by employing double immunofluorescence and confocal microscopy.

8.2 MATERIALS AND METHODS

8.2.1 Tissue specimens

Gingival tissues used for this study were from the same bank of cut tissue sections used for the previously described study, on vascular remodelling in untreated advanced periodontitis, approved by the Human Ethics Committee of the Central Sydney Area Health Authority.

Briefly, gingival tissues were collected from 22 consenting donors who had no record of previous periodontal therapy, significant associated diseases, including diabetes, or a history of relevant drug usage. The tissues collected were classified using
clinical and histological criteria as either minimally inflamed or advanced periodontitis. Twelve biopsies, collected from teeth with no loss of gingival attachment, probing depth ≤ 3mm and with no clinical signs of inflammation, were classified as minimally inflamed. Ten gingival specimens were obtained from patients diagnosed with advanced adult type periodontitis, with bone loss and pocket formation, resulting in probing depths ranging from 7 to 10mm. All biopsies were buccal gingivae taken from teeth anterior to the second molars before extraction. The donor age ranges (41-82 years) for the advanced periodontitis and minimally inflamed tissues were matched.

Biopsies were frozen and processed as described by Pinchback et al., (1996a).

8.2.2 Immunohistochemistry

Expression of angiogenic growth factors was assessed by labelling with antibodies to vascular endothelial growth factor (VEGF) (Ab2, Calbiochem, La Jolla, CA, USA) and basic fibroblast growth factor (bFGF) (Santa Cruz Biotech Inc., Santa Cruz, CA, USA).

Prior to immunolabelling for bFGF, sections were fixed in ice-cold methanol for 4 min, washed in PBS and then blocked for 1 hour with 20 per cent horse serum in PBS. A two step immunoperoxidase technique previously described by Pinchback et al., (1996a) was used to probe for bFGF.

For immunohistochemical labelling for VEGF, tissue sections were fixed with acetone at room temperature for 10 min, then blocked with 50 per cent goat serum in PBS for 20 min. Incubation with polyclonal anti-VEGF for 1 hour was followed by 30 min incubations with biotinylated goat anti-rabbit bridging antibody (Vector Laboratories, Burlingham, CA, USA.), and then streptavidin-biotinylated horse radish peroxidase complex (Dako). Liquid DAB+ substrate solution (Dako) was prepared and developed for 7 min, followed by a light counterstain with Mayer's haematoxylin.

Negative controls were incubated without the primary antibody application. Additional controls consisted of substitution of the specific antibody with an isotope matched irrelevant antibody or with normal rabbit serum for rabbit antibodies. These controls were negative in all cases. Anti-VEGF and anti-bFGF antibodies stained tissue elements reported to be reactive in other studies.
8.2.3 Immunofluorescence

Cryostat sections of tissue from 5 patients with advanced periodontitis and 5 with minimally inflamed gingiva were dual immunostained to determine coincidence of VEGF protein with a specific macrophage population. The macrophage markers MAC3 (Dako) and RM3/1 (Dianova, Hamburg, Germany) and VEGF, were labelled with streptavidin Alexa 568 (Molecular Probes) and FITC (Sigma), respectively using a technique developed for a single argon ion confocal laser scanning microscope described by Kumar et al., 1999.

Briefly, sections were fixed in acetone at room temperature for 10 min, then blocked with 50% goat serum in PBS for 20 min. Incubation with the primary antibodies against MAC3 was overnight at 4°C. This was followed by 45 min incubations with biotinylated goat anti-mouse bridging antibody (Dako) and polyclonal anti-VEGF antibody (Calbiochem), and then 30 min incubations with streptavidin-Alexa 568 (Molecular Probes, Eugene, OR, USA) and swine anti-rabbit conjugated FITC (Dako), respectively. After each incubation, slides were washed 4 times in PBS for 5 min each.

All antibodies, streptavidin and fluorochromes were diluted in 0.1% BSA in PBS to the appropriate concentrations which were determined on the basis of preliminary experiments.

Positive controls were single stained gingival tissues. Cryostat sections of a pterygium, kindly donated by Associate Professor RK Kumar (Dept of Pathology, University of NSW, Australia), was an additional positive control for VEGF. Single anti-MAC3 and anti-VEGF antibody staining for gingival tissue elements displayed typical patterns as previously reported. Negative controls were incorporated to test each stage of the double staining process. Negative controls were incubated without the primary antibody application. Additional controls consisted of substitution of the specific antibody with an isotope matched irrelevant antibody or where the reporter molecule was labelled streptavidin, without either the primary or the secondary antibody. These controls were negative in all cases.

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8.2.4 Analysis

8.2.4.1 Vascular endothelial growth factor

Cryostat sections of tissue from 10 patients with advanced periodontitis and 9 patients with minimally inflamed gingival tissue were stained for VEGF under the same conditions and at the same time. The slides were semi-quantitatively analysed by grading the staining intensity within the epithelium as follows: 0 = no staining; 1 = occasional positive cell staining; 2 = moderate number of cells staining; 3 = majority of cells displaying strong staining. The slides were examined by 2 independent observers and a consensus reached. Negative controls showed no specific/relevant staining.

8.2.5 Statistical analysis

Non-parametric analysis of variance using the Kruskal-Wallis test was followed by Dunn's test to compare all regions in both tissue groups with the minimally inflamed oral epithelial region, which served as a control.

8.3 RESULTS

Basic FGF was consistently localised to the basement membrane of blood vessels (Figure 27), but showed no regional distribution or change in level of expression between periodontitis and minimally inflamed gingiva.

Immunoreactivity for VEGF was also associated with blood vessels and macrophage-like cells of the inflammatory infiltrate but was most prominent in the epithelium, which exhibited marked regional variation in the intensity of staining (Figure 28A and B). The oral epithelium displayed strong cytoplasmic staining often with intense staining in the basement membrane (Figure 29A). There was, however, significantly reduced expression of VEGF along the epithelium of the pocket wall (Figure 29B) with only occasional rete peg tips staining positive for this protein.

Some CD163 positive cells identified as MAC3 and RM3/1 showed staining for VEGF protein within their cytoplasm (Figure 30A, B). In the advanced tissue sections VEGF positive macrophages were present in low numbers along the pocket wall. In minimally inflamed tissue dual labelled cells were present in low numbers scattered throughout the probed sections.
Figure 27  Tissue from a patient with advanced periodontitis showing bFGF associated with blood vessels.
Figure 28A  Ten advanced and 9 minimally inflamed gingival tissue sections stained for VEGF were semi-quantitatively analysed by grading the staining intensity within the epithelium. Tissue sections were divided into regions consisting of connective tissue subjacent to oral epithelium (OE), gingival crest (CR), oral sulcular epithelium (OSE) and pocket epithelium (PE). Data are expressed as mean values ± SEM; * P<0.05 and *** P<0.001

Figure 28B  Advanced periodontitis tissue section stained for VEGF and counterstained with haematoxylin showing staining intensity of VEGF in oral epithelium (OE), crest (CR), oral sulcular epithelium (OSE) and pocket epithelium (PE).
Figure 29A  Advanced periodontitis tissue stained for VEGF and counterstained with haematoxylin in oral epithelium (OE). In Figure 29B pocket epithelium (PE) showing reduced expression of VEGF except in the tips of some rete pegs (thick arrow head) and blood vessels (arrow), original magnification ×300.
Figure 30A  Fluorescence double immunolabelling in a biopsy section of advanced periodontitis tissue showing MAC3 positive macrophages (red) and VEGF (green) localised within the cytoplasm (arrows) as well as a MAC3 positive macrophage not associated with VEGF (block arrow).

Figure 30B  shows the inverted image of Figure A.
8.4 DISCUSSION

As vascular remodelling was determined to be a significant component of the lesion of advanced periodontitis, possible signalling mechanisms that could account for such changes were investigated. Expression of bFGF was consistently localised to the basement membrane of blood vessels but showed no regional distribution or changes in level of expression between periodontitis and minimally inflamed gingiva.

Basic fibroblast growth factor (bFGF, FGF-2) is important in all aspects of angiogenesis (Sunderkotter et al., 1994) and also in tissue repair. This growth factor was consistently localised to the basement membrane of blood vessels but not to macrophages irrespective of the level of inflammation. Others (Hughes and Hall, 1993, Cordon-Cardo et al., 1990) have also found bFGF associated with the vascular basement membrane. Immunohistochemical evidence indicates that macrophages are a major source of bFGF in a variety of chronic inflammatory states, including rheumatoid arthritis and endometriosis (Sunderkotter et al., 1991).

Gao et al., (1996) studied the presence of bFGF in healthy and inflamed human periodontal ligament tissue pellets, identifying the stained extracellular matrix, fibroblasts and endothelial cells by histological features. A few stained macrophages were observed although location relative to other structures within the tissues could not be determined. In a related study, Murata et al., (1997) using dual immunofluorescence found that the presence of bFGF in reparative granulation tissue of gingiva was associated with macrophages, mast cells and vascular endothelial cells. Arguably, it would be expected that appropriately activated macrophages would display this growth factor in the lesion of periodontitis; however, no supportive evidence was obtained in our study and by this criterion, the macrophages where not activated for angiogenesis.

In contrast to the pattern of expression for bFGF, there was marked regional variation in the intensity of immunostaining for VEGF, with high levels of expression of VEGF in keratinocytes of the oral epithelium and markedly reduced levels in the pocket epithelium. VEGF expression was also localised to the basement membrane along parts of the pocket epithelium. VEGF protein has been localised to foetal airway epithelial cells and in basement membrane subjacent to the airway epithelial cells (Acarregui et
al., 1999). This suggests that translocation of VEGF protein occurs after its synthesis in the epithelium (Acarregui et al., 1999). Localisation of VEGF to the basement membrane may be important for directing capillary development immediately subjacent to the pocket epithelium.

VEGF was also detected in occasional blood vessels and macrophage-like cells. In this context, macrophages are reported to be a major source of VEGF (Nagashima et al., 1995). Investigation of the expression of VEGF protein by macrophages in gingival tissues was undertaken using dual immunofluorescence. VEGF was found to be expressed within the cytoplasm of macrophages identified by MAC3 and RM3/1. The staining pattern for VEGF within the cytoplasm was localised, a finding confirmed by 3-D reconstruction of optical sections. Perusal of the literature has indicated that the intracellular localisation of VEGF in macrophages has not been previously reported, although in a study by Park and coworkers, VEGF165 was observed largely in Golgi apparatus-like structures in human embryonic kidney CEN4 cells (Park et al., 1993). Macrophages are a source of VEGF in gingival health and disease, however, the level of contribution of VEGF in angiogenic responses is not known.

The VEGF family of growth factors is currently the focus of much research (Nicosia, 1998). VEGF has been detected in various periodontal tissues and in saliva and has been postulated to play a role in tissue maintenance/homeostasis and in wound healing in the oral mucosa (Booth et al., 1998, Pammer et al., 1998, Taichman et al., 1998). Booth et al., (1998) documented that the concentration of VEGF in gingival crevicular fluid is higher than that in plasma, consistent with local production of this growth factor. Interestingly, they also reported that the concentration of VEGF in gingival crevicular fluid was higher in relatively healthy sites than in sites with more severe disease, an observation that is consistent with the findings of the present study. It is recognised that the reduced immunostaining in the pocket epithelium could result from an increased rate of transport of VEGF out of the cytoplasm of the epithelial cells. Investigation of the expression of mRNA for VEGF by the epithelial cells will be required to exclude this possibility.

If the reduced immunostaining for VEGF in the pocket epithelium does indeed represent reduced local synthesis, questions arise concerning the mechanisms of such a
reduction. The periodontal pocket is a unique environment where a zone of highly vascular tissue is situated in close proximity to a diverse, complex and organised microbial biofilm (Darveau et al., 1997). Evidence is accumulating to indicate that the epithelium lining the pocket has been altered, including changes in the expression of adhesion molecules and their receptors as well as degradation of the epithelial basement membrane (Haapasalmi et al., 1995). Such changes may reflect a response to the inflammatory reaction but could also in part constitute a response to the microbial flora, and indeed there is evidence for direct entry of bacteria such as P. gingivalis and A. actinomycetemcomitans into gingival epithelial cells (Darveau et al., 1997). The relation between microbial products, regulation of epithelial expression of growth factors and the associated vascular response pattern requires further investigation.

The studies described in this chapter and the previous, of the vascular changes in untreated advanced chronic periodontitis have extended earlier published work on vascular changes in chronic inflammatory periodontal disease (Zoellner and Hunter, 1989, 1991a, b). The results establish that in this disease, there are both quantitative and qualitative changes in the vasculature of the periodontal connective tissues. In addition, evidence is presented for disturbed regulation of angiogenic activity in this environment, manifest as altered epithelial expression of vascular endothelial growth factor. VEGF expression has been localised principally to specific regions of epithelium; however certain macrophage sub-populations also express this growth factor.

The following chapter examines the achievements, limitations and future direction of the investigation of the macrophage in untreated periodontitis.
CHAPTER 9
GENERAL DISCUSSION

ACHIEVEMENTS

The research presented in this thesis demonstrates the distribution and functional status of macrophages in relation to observed pathological features in the untreated lesion of destructive periodontitis while the studies on vascular changes in untreated advanced chronic periodontitis have extended previous observations on the pathology of this disease.

Macrophages within the untreated lesion of periodontitis displayed phenotypic heterogeneity indicating different developmental and functional stages as identified by the antibodies to CD163 (MAC3 and RM3/1) and the macrophage subset antibodies 27E10 and 25F9. Macrophage subsets in advanced periodontitis and minimally inflamed tissues displayed similar distribution patterns and densities. All of the macrophage phenotypes were strongly represented in the tissue adjacent to the oral epithelium whereas there was reduced density of macrophages adjacent to the pocket epithelium. This is notably different from observations in other chronic inflammatory diseases where there is an increase in density of macrophages in association with the intensity of the host response.

In the study of functional activation of macrophages there were few macrophages activated for antigen presentation. There was also little evidence of macrophage activation for tartrate-resistant acid phosphatase. Macrophages expressing acid phosphatase showed a distinct regional distribution; however, this was not associated with some foci of degenerate plasma cells. These findings indicate a lack of appropriate activation in macrophages in untreated periodontitis.

The study on vascular remodelling in destructive periodontitis established that there are both quantitative and qualitative changes in the vasculature of the periodontal connective tissues immediately subjacent to the altered epithelium lining the periodontal pocket. These changes include the selective increase in vessels of larger diameter, and the accumulation of extravascular basement membrane, which has been named residual vascular basement membrane (RVBM). Using a combination of dual
immunofluorescence, laser confocal microscopy and 3-D reconstruction, it was found that RVBM, primarily composed of type IV collagen and laminin, strongly resembled disrupted blood vessel structures. There was no correlation between increased density of RVBM and macrophage numbers.

The distribution of major angiogenic growth factors in periodontitis was investigated by immunohistochemistry. Basic fibroblast growth factor, although consistently associated with blood vessels, showed no regional variation in distribution. There was no evidence for macrophage expression of bFGF. In contrast, there was marked regional variation in the intensity of immunostaining for vascular endothelial growth factor, with significantly reduced staining of the pocket epithelium. Some macrophages within the CD163 population did express this growth factor.

It is speculated that the changes in the vascularity of the periodontal connective tissues in untreated advanced periodontitis are, in part, a consequence of altered expression of angiogenic activity by the pocket epithelium. In turn, this may reflect the epithelial response to microbial flora in the microenvironment of the periodontal pocket.

The apparent failure of recruitment and activation of macrophages may in part be both a cause and a consequence of the pathological features of this disease. Possible mechanisms for lack of recruitment and anergy in macrophages may arise from immunosuppressive effects induced by either an altered host or by bacterial products or by a combination of both. Macrophage recruitment is dependent on signals from the environment inducing upregulation of adhesion molecules on endothelium. The profile of the expression of leukocyte adhesion molecules P-selectin, E-selectin and PECAM-1 (CD31) observed in a collaborative study was not demonstrably different in gingival tissues representing the extremes of the disease spectrum. In addition, there was only trace reactivity for VCAM-1, the adhesion molecule with specificity for mononuclear leukocytes, in both advanced disease and minimally inflamed specimens. This implies a similar recruitment level for macrophages in both tissues, as shown in the thesis study. The adhesion molecules expressed in minimally inflamed tissues could be considered to be appropriate for successful defence of the tissues. In contrast, the destructive lesion of advanced periodontitis is characterised by a manifest failure of protective responses and yet the adhesion molecules expressed are comparable to those in minimal inflammation.
The capacity of the underlying vasculature to support a protective inflammatory response appears to be reduced.

Migration of newly recruited cells may be impeded by pathologically altered blood vessels. Others (Zoellner and Hunter, 1989, Pinchback et al., 1996a) have found marked thickening of the basement membrane as a result of deposition of type IV collagen and laminin, a finding confirmed and extended in the present study of vascular remodelling.

Recently, it has been demonstrated by studies in vitro that cysteine proteinases from *P. gingivalis* can inhibit expression of MHC class II proteins on stimulated endothelial cells by cleavage of IFN-γ, resulting in potential disruption of recruitment and activation of leukocytes in the inflammatory response (Yun et al., 1999). Whether the reduced antigen presenting capacity of macrophages observed in the activation study is associated with immnosuppression by bacterial products remains to be determined. In untreated periodontitis tissue macrophages express TGF-β1 (Steinsvoll et al., 1999, writer’s data), which can exert immunosuppressive effects. TGF-β1 down-regulates MHC class II expression on macrophages (Czarniecki et al., 1988), diminishes phagocytic activity through suppression of FcγRIII (Wahl et al., 1992), and suppresses recruitment of monocytes.

Pocket epithelium has potentially, a strategic role in the chemotactic attraction and activation of macrophages. There is evidence however, indicating the pathological lining epithelium does not function as an effective barrier against the ingress of microbial products into the tissues. Thus, the ability of pocket epithelium to support an effective immune response is severely compromised.

Pocket epithelium in untreated chronic periodontitis expresses an intermediate filament profile that reflects an origin from developmental epithelium of the enamel organ and cell rests of Malassez. In untreated periodontitis this primitive epithelium displays a striking reduction in staining for E-cadherin, involucrin and both connexins and marked alterations of F-actin expression, collectively indicating profound perturbation of epithelial structure.

The loss of E-cadherin, a protein responsible for the structural integrity may contribute to the lack of structural organisation of pocket epithelium. Also, observed
patterns of involucrin expression indicate a failure of the superficial cells of pocket epithelium to undergo terminal differentiation. In particular, there is the implication that the microbial flora of the pocket can interact directly with a responsive epithelial surface. This adds credibility to the studies in vitro that have demonstrated the capacity of the periodontal pathogen *P. gingivalis* to infect gingival epithelial cells. Also, scattered and low levels of connexins 26 and 43 suggest a communication deficiency between epithelial cells in pocket epithelia of advanced periodontitis. This change may provide an altered environment favourable for invasion by bacteria.

The manifest perturbation of structure of the pocket epithelium could be linked to either a disruption of F-actin leading to altered intercellular adhesion and communication, or to a disruption of cell adhesion. In this context, the periodontal pathogens *P. gingivalis* and *A. actinomycetemcomitans* as well as *Treponema denticola* have been demonstrated to alter F-actin expression in gingival epithelial cells in vitro (Lamont and Jenkinson 1998, Fives-Taylor et al., 1999, De Filippo et al., 1995). In addition, the virulence of *P. gingivalis* is mediated by cysteine proteinases with trypsin-like specificity that have the capacity to degrade a wide variety of protein substrates, possibly including cell adhesion molecules. Therefore, a linked perturbation of essential structural units could relate to either the endogenous action of invading bacteria or the exogenous effects of bacterial products.

Studies in vitro have demonstrated that gingival epithelial cells when challenged with invasive as well as non-invasive bacteria secrete pro-inflammatory cytokines (reviewed by Wilson et al., 1996, Lamont and Jenkinson, 1998). Bacterial products can also cause immunosuppression, for example by induction of TGF-β (Wahl et al., 1988, Wahl, 1991) which has strong deactivating effects on differentiated macrophages (Bogdan and Nathan, 1993) and suppresses further monocyte recruitment. The overexpression of TGF-β2 and -β3 observed in pocket epithelium in vivo, strongly suggests an immunosuppressive role which could significantly reduce macrophage recruitment and activation in the region of the pocket wall. TGF-β1 expression in macrophages has been detected in the connective tissue and this may contribute further to immunosuppressive effects on macrophages.
There is evidence that, of the 300 species that can inhabit the pocket, some of these organisms have the capacity to invade (Lamont et al., 1992, Sandros et al., 1994, Papapanou et al., 1994) and replicate within epithelial cells (Lamont et al., 1995, Madianos et al., 1996), and colonise intercellularly in the epithelium (Saglie et al., 1988).

Studies *in vitro* have revealed that *A. actinomycetemcomitans* produces a leukotoxin, a member of the RTX toxin family of pore-forming hemolysins/leukotoxins (Lally et al., 1989), that kills cells of monomyelocytic lineage (Taichman et al., 1980, Rabie et al., 1988). The cell surface receptor for RTX toxins on target cells (Lally et al., 1997) is leukocyte function-associate antigen-1 (LFA-1), a member of the beta 2-integrin family. The ability of *A. actinomycetemcomitans* to utilize this critical cell adhesion molecule to destroy host immune cells at epithelial/connective tissue sites would thus allow microbial colonisation to proceed undeterred. Whether this toxin operates *in vivo* has yet to be determined, although it has been recently demonstrated that *A. actinomycetemcomitans* can adhere to and invade epithelial cells *in vitro* and has the capacity to penetrate the deeper connective tissue (Meyer and Fives-Taylor, 1997).

Recent studies have identified *P. gingivalis* within pocket epithelium and in the deeper layers of the pocket wall *in vivo* (Noiri et al., 1997). Studies *in vitro* have also revealed the ability of *P. gingivalis* to disrupt epithelial cell functions (Fravalo et al., 1996), and to invade and replicate within gingival epithelial cells (Lamont and Jenkinson 1998) as well as in endothelial cells (Deshpande et al., 1998).

In chronic inflammation concurrent tissue destruction and ongoing repair occurs. This repair is manifest as connective tissue matrix turnover and vascular remodelling. The dominant growth factors involved in vascular turnover as part of the repair response are those of the VEGF and FGF families. However, an important observation from the studies described in the thesis is a striking lack of, or reduced macrophage activation for, the growth factors bFGF and VEGF in the untreated lesion. At present little is known about the role of macrophages in regulating the process of tissue repair and remodelling in chronic inflammation or the manner in which bacterial products modulate these processes. Tissue maintenance and repair requires debridement by macrophage and yet the persistence of RVBM and foci of degenerate plasma cells indicates a lack of tissue
debridement. Evidence from studies *in vitro* supports an anti-inflammatory or reparative role for the subpopulation of RM3/1, anti-CD163+ macrophages. This subset was the predominant macrophage type found in both periodontitis and minimally inflamed tissue. This supports the role of the macrophage in tissue maintenance in minimally inflamed gingiva. It also implies that the process of ongoing repair in untreated periodontitis is an important role for macrophages.

Macrophage anergy and vascular disruption observed in the described studies, together with altered vascular adhesion molecule expression and altered epithelium, found in the related collaborative studies, are most likely to be a consequence of interactions with bacterial products. Substantial data has been generated from studies *in vitro* on the gingival tissue response to bacterial products and yet little is known about macrophage response to bacteria and bacterial products in periodontitis.

**LIMITATIONS**

The studies described in this thesis are based on investigations of the pathology of adult periodontitis using untreated human tissues considered to reflect the natural history of disease progression. The approach taken was to compare minimally inflamed tissues with advanced destructive disease, that is the extremes of presentation, and hence there was no attempt to study the pathogenesis of periodontitis.

Observation drives research, however limitations are imposed on observational investigations *in vivo*. The interpretation of observations, although based on prevailing theories, is speculative and hence, mechanisms cannot be determined. In order to investigate mechanisms an appropriate animal model is needed, however there is none that represents the natural progression of human adult periodontitis.

The sophistication of an observation is determined largely by the technological tools used. For example, optical sectioning and reconstruction by laser scanning microscopy can reveal not only 3-D structural aspects of macrophages but also the distribution and spatial relation of these cells to the surrounding environment. The development of high specificity monoclonal antibodies against macrophage subpopulations has made it possible to characterise in some detail these cell populations in normal and diseased
gingival tissues. There are limitations on the interpretation of these data as the functions of these markers have not been fully elucidated.

Increasing awareness of the importance of using untreated tissue has not alleviated the difficulties in procuring tissue representative of native disease. For example, there is always the possibility that a donor may have a subclinical or undiagnosed systemic condition. Also, interpretation of pooled data from different subjects could also be misleading as there may be wide variations in the host response due to differences in microbiological challenge and unidentified disease modifiers, both environmental and genetic or acquired. Studies using tissue samples, representative of different stages of the disease and taken from the same individual may in part address this problem.

**RECOMMENDATIONS FOR FUTURE RESEARCH**

Macrophage anergy and vascular disruption observed in the described studies, together with altered vascular adhesion molecule expression and altered epithelium, found in the related collaborative studies, are most likely to be a consequence of interactions with bacterial products. Substantial data has been generated from studies *in vitro* on the gingival tissue response to bacterial products and yet little is known about macrophage response to bacteria and bacterial products in periodontitis.

Therefore, in future studies the hypothesis to be tested is that macrophage activation and function are critically modulated by the products of pathogens, such as *P. gingivalis* and *A. actinomycetemcomitans*. Also, the function of the pocket lining epithelium, with essential consequences for the barrier function, and the capacity of the underlying vasculature to support a protective inflammatory response including macrophage recruitment, are regulated by bacterial products.

There are few direct data that address the changes in populations of macrophages during challenge and the role that macrophage heterogeneity may play in resolution of disease. Although there is no satisfactory experimental model for human periodontitis, the effects of resolution by manipulation of the tissue through periodontal therapy could be studied, providing data on heterogeneity, distribution and activation of macrophage populations in tissue associated with a reduction in microbial exposure.
As a corollary, in effective therapy there is a release from the direct effects of bacteria and their products through reduction in microbial load, allowing effective host defences to be re-established.
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