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ACTINOBACILLUS ACTINOMYCETEMCOMITANS Induces Expression of Interleukin-8 in Human Gingival Epithelial Cells: Implications for the Pathogenesis of Periodontal

Andreas Sfakianakis

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ACTINOBACILLUS ACTINOMYCETEMCOMITANS INDUCES EXPRESSION OF INTERLEUKIN-8 IN HUMAN GINGIVAL EPITHELIAL CELLS: IMPLICATIONS FOR THE PATHOGENESIS OF PERIODONTAL DISEASE

Andreas Sfakianakis

D.D.S., University of Athens, School of Dentistry, 1995
ACTINOBACILLUS ACTINOMYCETEMCOMITANS INDUCES EXPRESSION OF INTERLEUKIN-8 IN HUMAN GINGIVAL EPITHELIAL CELLS: IMPLICATIONS FOR THE PATHOGENESIS OF PERIODONTAL DISEASE

Presented by

Andreas Sfakianakis, D.D.S.
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## TABLE OF CONTENTS

**OVERVIEW** ........................................................................................................ 1
ABSTRACT ................................................................................................................. 1
INTRODUCTION ......................................................................................................... 3
OVERALL HYPOTHESIS ........................................................................................... 4
THESIS HYPOTHESIS ............................................................................................... 4
SPECIFIC AIMS/OBJECTIVES ................................................................................... 4
BACKGROUND ........................................................................................................... 5
  Bacteria - Host interactions ..................................................................................... 5
  *Actinobacillus actinomycetemcomitans* ................................................................... 6
  CXC Chemokines ..................................................................................................... 6
  Interleukin-8 ............................................................................................................. 7
  CXC-receptors ......................................................................................................... 8
  Epithelial cells and IL-8 ............................................................................................ 8
REFERENCES ............................................................................................................. 10

### CHAPTER 1  Localization of interleukin-8 and interleukin-8 receptors in human gingival tissues and cultured human gingival epithelial cells ........................................ 13
ABSTRACT ................................................................................................................. 13
1. INTRODUCTION .................................................................................................... 15
2. METHODS .............................................................................................................. 19
  2.1. Gingival biopsies ............................................................................................... 19
  2.2. Immunohistochemical technique ....................................................................... 19
  2.3. Culture of gingival keratinocytes and immunocytochemistry ......................... 21
  2.4. Slide analysis .................................................................................................... 22
3. RESULTS ................................................................................................................ 23
  3.1. Expression of IL-8 in gingival tissue and epithelial cells in vitro ...................... 23
  3.2. Expression of CXCR-1 in gingival tissue and epithelial cells in vitro ............... 25
  3.3. Expression of CXCR-2 in gingival tissue and epithelial cells in vitro ............... 27
4. DISCUSSION .......................................................................................................... 29
REFERENCES ............................................................................................................ 36

### CHAPTER 2  Characterization of *A. actinomycetemcomitans*-induced interleukin-8 from gingival epithelial cells ............................................................ 38
ABSTRACT ................................................................................................................. 38
1. INTRODUCTION .................................................................................................... 41
2. METHODS .............................................................................................................. 50
  2.1. Culture of gingival keratinocytes ....................................................................... 50
  2.2. Preparation of PBMC ....................................................................................... 50
  2.3. *A. actinomycetemcomitans* challenge ............................................................. 51
  2.4. Treatment of cells with specific p38 MAP kinase inhibitor ............................... 52
LIST OF TABLES

CHAPTER 2

Table 2.1 Proinflammatory cytokine expression induced by various bacterial components 49
LIST OF FIGURES

OVERVIEW

Figure 1 Three dimensional structure of human Interleukin-8 .................................................. 9

CHAPTER 1

Figure 1.1 Hypothetical model of IL-8 functions in the periodontal environment .................................. 18
Figure 1.2 Immunohistochemical staining of gingival tissue for IL-8 expression ........................................ 24
Figure 1.3 Immunocytochemical staining of gingival epithelial cells (GECs) for IL-8 expression .......... 24
Figure 1.4 Immunohistochemical staining of gingival tissue for CXCR-1 expression ............................... 26
Figure 1.5 Immunocytochemical staining of GECs for CXCR-1 expression ........................................... 26
Figure 1.6 Immunohistochemical staining of gingival tissue for CXCR-2 expression .............................. 28
Figure 1.7 Immunocytochemical staining of GECs for CXCR-2 expression ........................................... 28
Figure 1.8 Summary of immunohistochemistry/immunocytochemistry .............................................. 35

CHAPTER 2

Figure 2.1 Dose-response study for A.a.-induced IL-8 production by GECs ........................................... 57
Figure 2.2 Stimulation Index (S.I.) for A.a.-induced IL-8 production by GECs ........................................... 57
Figure 2.3 Time-response study of A.a.-induced IL-8 expression by GECs ........................................... 59
Figure 2.4 Stimulation index (S.I.) of time-response study ................................................................. 59
Figure 2.5 Effect of p38 MAPK inhibitor on IL-8 expression by GECs ..................................................... 61
Figure 2.6 Percent inhibition of A.a.-induced IL-8 expression due to p38 MAPK inhibitor ..................... 62
Figure 2.7 Dose-response study of IL-8 expression by GECs challenged with E. coli LPS ......................... 64
Figure 2.8 Effect of Polymyxin B on the A.a.-induced IL-8 expression in GECs ..................................... 65
Figure 2.9 Effect of E. coli LPS and A.a. Extracts on IL-8 expression by GECs, with or without Polymyxin B .... 66
Figure 2.10 Effect of E. coli LPS and A.a. Extracts on IL-8 expression by PBMCs, with or without Polymyxin B ... 67
Figure 2.11 P38 MAPK regulates signal transduction pathway for IL-8 expression ................................. 75
Figure 2.12 A.a.-induced IL-8 expression in GECs is LPS-independent ............................................... 79

CHAPTER 3

Figure 3.1 Hypothesized IL-1 autocrine regulation of IL-8 expression in GECs ........................................ 95
Figure 3.2 A.a. induces IL-1alpha expression in GECs ................................................................. 103
Figure 3.3 Stimulation Index (SI) of IL-1alpha expression induced by A.a. ........................................... 104
Figure 3.4 Comparison between SI of IL-1alpha and IL-8 induced by A.a. ........................................... 105
Figure 3.5 A.a. induced IL-1beta expression in GECs ........................................................................... 107
Figure 3.6 Comparison between kinetics of IL-1beta and kinetics of IL-8 .............................................. 108
Figure 3.7 Kinetics of IL-1alpha and IL-1beta induced by A.a. ............................................................ 109
Figure 3.8 Dose-response study of IL-8 expression by GECs following IL-1alpha challenge ....................... 111
Figure 3.9 Time-course of IL-8 expression by GECs following IL-1alpha challenge .................................. 113
Figure 3.10 Comparison of the effect of IL-1alpha and A.a. sonicates in the expression of IL-8 ............... 114
Figure 3.11 Stimulation Index of IL-8 expression induced by IL-1alpha and A.a. sonicates ...................... 115
Figure 3.12 Dose-dependent expression of IL-8 after stimulation with IL-1beta .................................... 117
Figure 3.13 Time-course of IL-8 expression following IL-1beta challenge ........................................... 119
Figure 3.14 Comparison of IL-8 expression induced by IL-1beta with that induced by A.a. ................. 120
Figure 3.15 Stimulation Index of IL-8 expression induced by IL-1beta and A.a. sonicates ....................... 121
Figure 3.16 Effect of anti-IL-1alpha antibodies on IL-1alpha-induced IL-8 expression ......................... 123
Figure 3.17 Effect of anti-IL-1beta antibodies on IL-1beta-induced IL-8 expression

Figure 3.18 Effect of IL-1RA on IL-1alpha-induced expression of IL-8

Figure 3.19 Effect of IL-1RA on IL-1beta-induced expression of IL-8

Figure 3.20 Effect of single anti-IL-1 antibodies and IL-1RA on the [IL-1alpha+IL-1beta]-induced IL-8 expression

Figure 3.21 Effect of combined anti-IL-1 antibodies and IL-1RA on the [IL-1alpha+IL-1beta]-induced IL-8 expression

Figure 3.22 Effect of antibodies and IL-1RA on IL-1-induced IL-8 expression

Figure 3.23 Effect of single IL-1 inhibitors on A.a.-induced IL-8 expression (T=12h)

Figure 3.24 Effect of combined IL-1 inhibitors on A.a.-induced IL-8 expression (T=12h)

Figure 3.25 Effect of single IL-1 inhibitors on A.a.-induced IL-8 expression (T=18h)

Figure 3.26 Effect of combined IL-1 inhibitors on A.a.-induced IL-8 expression (T=18h)

Figure 3.27 Possible mechanisms of IL-8 regulation by bacterial induced-IL-1
OVERVIEW

ACTINOBACILLUS ACTINOMYCETEMCOMITANS
INDUCES EXPRESSION OF INTERLEUKIN-8 IN HUMAN
GINGIVAL EPITHELIAL CELLS: IMPLICATIONS FOR
THE PATHOGENESIS OF PERIODONTAL DISEASE

ABSTRACT

Introduction: The chemokine Interleukin 8 (IL-8) has been implicated in
inflammation and wound healing, but its role(s) and source(s) in periodontal disease are
not clearly understood. We hypothesized that: 1) in vivo, periodontopathic organisms
such as A. actinomycetemcomitans (A.a.) induce IL-8 expression in gingival epithelial
cells (GEC) and 2) this local expression of GEC-derived IL-8 enhances leukocyte,
vascular endothelial cell (VEC) and GEC migration via specific IL-8 receptors (IL-8R).

Objectives: To: 1) characterize the in vivo IL-8 and IL-8R expression in gingiva;
2) demonstrate the ability of A.a.-derived factors (A.a.DF) to induce IL-8 expression in
GEC in vitro; and 3) characterize the mechanisms by which A.a.DF induce IL-8
expression in GEC in vitro.

Methods: For in vivo studies, gingival specimens from patients with periodontitis
were evaluated for IL-8 and IL-8R expression using immunohistochemical (IHC)
techniques. For in vitro studies, we utilized the established GEC line PP. Cells were
cultured under various conditions and the IL-8 expression in the cell lysates and
supernatants was determined by ELISA.
Results: *In vivo*, IL-8 and IL-8R expression was seen, in varying degrees, on GECs, leukocytes and VECs. *In vitro*, *A.a.* extracts induced a time- and dose- dependent expression of IL-8 from PP cells (7-fold, p<0.01). *A.a.* also induced expression of IL-1α and IL-1β, known IL-8 inducers. IL-8 expression was not inhibited by the LPS inhibitor polymyxin B (p>0.10) or anti-IL-1antibodies (p>0.10). Interestingly the MAP kinase inhibitor SB203580 markedly inhibited (>75%, p<0.01) *A.a.*-induced expression of IL-8. In parallel studies, we have also demonstrated that *A.a.* can induce IL-6 expression from PP cells.

Conclusions: GECs are a major source of IL-8 *in vivo* and *in vitro*. *A.a.*-derived factors can induce IL-8 expression from GECs, *in vitro*. Although *A.a.*-induced IL-8 expression involves a MAP kinase signal transducing pathway, it is not the direct result of known IL-8 inducers (i.e. LPS or IL-1). Our observation that *A.a.* can also induce IL-1 and IL-6 expression suggests that GEC may be a source of a variety of proinflammatory cytokines, and therefore a potential target for therapeutic intervention in the future.
INTRODUCTION

The most common cause of periodontal diseases is bacterial infection. *Actinobacillus actinomycetemcomitans* (A.a.) is an important periodontal pathogen that has been implicated in the pathogenesis of periodontitis. Prior studies have demonstrated that this organism is found in periodontal pockets in patients with Localized Juvenile Periodontitis (LJP) and has also been shown to invade human epithelial cells. Gingival epithelium is the first tissue that encounters the bacterial invasion and is considered part of the host’s innate immunity.

Currently little is known about the role of the gingival epithelium in response the specific infection with A.a. In fact, few studies have systematically examined the epithelial chemokine responses, that are essential for the recruitment of leukocytes in the periodontal pocket and the initiation of an inflammatory response.

In gingival biopsies, we were able to demonstrate the existence of Interleukin-8 (IL-8) and IL-8 receptors CXCR-1 and CXCR-2. Based on these observations it is hypothesized that bacterial infection induces significant upregulation of Interleukin-8 expression in the gingival epithelium, that may have chemotactic, angiogenic and proliferative effects. To test this hypothesis, cultures of human gingival epithelial cells will be infected with sonicated extracts of A.a. A systematic investigation of the characteristics of the expression of IL-8 will be undertaken, in both unstimulated (resting) and stimulated (infected) cells. Additionally, the role of Interleukin-1 in the regulation of Interleukin-8 will also be investigated. The results from these studies will not only provide important information about the pathogenesis of microbial periodontal diseases,
but will also provide the foundation for future therapeutic approaches based on modification of cytokine responses of the host.

**OVERALL HYPOTHESIS**

During periodontal disease, periodontal pathogens such as *A.a.* induce expression of proinflammatory cytokines in GECs. This local expression of GEC-derived cytokines, controls inflammation and repair within the gingival tissue.

**THESIS HYPOTHESIS**

IL-8 and IL-8 receptors (IL-8R) are expressed by gingival epithelial cells (GECs) in vivo and in vitro and *A.a.* extracts can induce IL-8 expression in GECs in vitro

**SPECIFIC AIMS/OBJECTIVES**

Our objective was to:

1) characterize the IL-8 and IL-8R expression in gingiva;

2) characterize the IL-8 and IL-8R expression in GEC, in vitro;

3) demonstrate the ability of *A.a.*-derived factors (*A.a.DF*) to induce IL-8 expression in GEC in vitro; and

4) characterize the mechanisms by which *A.a.DF* induce IL-8 expression in GEC in vitro.
BACKGROUND

Bacteria - Host interactions

Gingival tissues and especially the sulcular epithelium, encounters a wide spectrum of oral microorganisms, that form the bacterial plaque, which is the main causative factor of periodontal disease. Depending on the severity, the disease can be classified into gingivitis, characterized by superficial inflammation and periodontitis, characterized by attachment loss, alveolar bone loss and eventually tooth loss.

Recent microbiological studies of periodontal disease in humans have supported the concept of a specific bacterial etiology. Among more than 300 species identified in the oral cavity, a relatively small group of gram-negative organisms, are most frequently isolated from infected periodontal pockets and are thus recognized as potential periodontal pathogens. These include A.a., Bacteroides gingivalis, Eikenella corrodens and Wolinella recta [1]. Etiologic role for Bacteroides forsythus, Treponema denticola and Prevotella intermedia has also been suggested [2].

A.a. is probably one of the few microorganisms, that have been directly related to periodontal lesions. Currently there is very little known about the interaction of this bacterium with the gingival epithelium, except the fact that A.a. is capable of invading oral epithelial cells, in vitro [3]. In addition, the cytokine functions of gingival epithelium in response to bacterial infection have not been explored yet. The focus of our studies is to provide further information about the role of the gingival keratinocyte in the early chemokine signaling that will attract leukocytes in the gingival sulcus, as part of the host defense. In order to describe a model for cellular interactions, we will study the
expression of Interleukin-8 as a representative chemokine of the CXC family, having in mind that similar conditions may apply for the other members of this family, too.

**Actinobacillus actinomycetemcomitans**

*Actinobacillus actinomycetemcomitans* (A.a.) is a small gram-negative coccobacillus that has been implicated in the pathogenesis of adult periodontitis [4-6] and localized juvenile periodontitis (LJP) [7]. A.a. has been shown to adhere to and invade oral epithelial cells and some strains of A.a. express a toxin that enables them to kill leukocytes [3] [8]. Several authors have investigated the inflammatory mediators and cytokines produced by mononuclear cells and fibroblasts in response to A.a. challenge [9-12]. However, little is known regarding the cytokine profile of GECs following their interaction with this pathogen. As stated above, we hypothesized that GECs behave as an integral component of the immune system by increasing their constitutive expression of IL-8 in response to A.a. infection.

**CXC Chemokines**

The a-subfamily of chemokines is characterized by a conserved tri-peptide near the N-terminus, containing cysteines (C-X-C). CXC chemokines function as chemoattractants and activators of leukocytes. They are involved in many physiological processes, such as migration and release of leukocytes, inflammation, angiogenesis, tumor growth and HIV-suppression [13, 14]. Until now, about 15 human CXC chemokines have been discovered, including interleukin-8 (IL-8), GRO proteins (GRO-alpha, GRO-beta and GRO-gamma), neutrophil-activating peptide-2 (NAP-2), platelet factor 4(PF4) and gamma-interferon-inducible protein (IP10) [13].
**Interleukin-8**

**Sources:** IL-8 is probably the best characterized C-X-C chemokine. IL-8 is produced by macrophages, fibroblasts, endothelial cells, keratinocytes, melanocytes, hepatocytes, chondrocytes, and a number of tumor cell lines [15].

**Protein characteristics:** IL-8 is a non-glycosylated protein of 8 kDa (72 amino acids). It is produced by the processing of a precursor protein of 99 amino acids [16]. The three-dimensional structure of IL-8 has been determined by NMR spectroscopy and X-ray crystallography (Figure 1) [31] [32].

**Biological activities:** The biological activities of IL-8 resemble those of a related protein, NAP-2 (neutrophil-activating peptide-2). IL-8 increases Chemotaxis and the enhanced expression of adhesion molecules [14]. IL-8 is chemotactic for all known types of migratory immune cells. IL-8 is a mitogen for epidermal cells. Macrophage-derived IL-8 supports angiogenesis and may play a role in angiogenesis-dependent conditions such as rheumatoid arthritis, tumor growth, and wound healing [17].

**Clinical significance:** IL-8 may be of clinical relevance in psoriasis and rheumatoid arthritis [18, 19]. Elevated concentrations are observed in psoriatic scales and this may explain the high proliferation rate observed in these cells. IL-8 may be also a marker of different inflammatory processes. IL-8 probably plays a role in the pathogenesis of chronic polyarthritis since excessive amounts of this factor are found in synovial fluids. IL-8 has been detected in the gingival crevicular fluid (GCF) and in gingival tissue sections of adult periodontitis patients and IL-8 mRNA has been identified in healthy and diseased periodontal tissues [20-23].
**CXC-receptors**

Chemokines exert their biological functions via cell-surface receptors. Two chemokine receptors, CXCR-1 and CXCR-2, have been identified on human neutrophils [24, 25]. CXC chemokines function as chemoattractants and activators of neutrophils through high-affinity binding to at least one of the two receptors. It has been demonstrated that CXCR-2 has a high affinity for IL-8 and all the other CXC chemokines, while CXCR-1 has a high affinity for IL-8 only [25]. These receptors are present in a variety of cells, including neutrophils, monocytes, keratinocytes, endothelial cells, fibroblasts, smooth muscle cells and T cells.

**Epithelial cells and IL-8**

The gingival mucosa is composed of three types of epithelium: oral gingival epithelium, oral sulcular epithelium and junctional epithelium. The predominant cell type in these epithelia is the gingival keratinocyte (or gingival epithelial cell). This cell shares many properties with keratinocytes located in other types of stratified epithelia like epidermis. These properties include replication and differentiation from a basal cell layer and synthesis of cytokeratin [26]. However, the role of the gingival keratinocyte in periodontal disease has been studied in a limited basis. This is not the case in Dermatology, where the role of epidermal keratinocyte in psoriasis has been established. Psoriasis is a chronic inflammatory disease of the skin, characterized by erythematous scaling plaques, affecting the skin of the knees, elbows or scalp [27]. Psoriasis and periodontitis share common histological characteristics, like the dense accumulation of neutrophils and T-lymphocytes below the epithelium. An important characteristic of psoriasis is the secretion of IL-8 by epidermal keratinocytes, which upregulates leukocyte
chemotaxis [28]. One other well-characterized system, regarding epithelial interactions with pathogens, is the intestinal system. The intestinal epithelial cells express and secrete high levels of the chemoattractant cytokines IL-8, GRO (alpha, beta and gamma) in response to bacterial invasion [29, 30].

Based on the above reports we hypothesize that epithelial cells in the oral mucosa behave similarly regarding their cytokine responses. We test the hypothesis that infection with an oral pathogen (A. a.), can upregulate the gingival epithelial expression of IL-8.

Figure 1 Three dimensional structure of human Interleukin-8
(From Cytokines Web : http://www.psynix.co.uk/cytweb/cyt_strucs/il8.gif)
The three-dimensional structure of IL-8 has been determined by NMR spectroscopy and X-ray crystallography [31] [32].
REFERENCES


CHAPTER 1

Localization of interleukin-8 and interleukin-8 receptors in human gingival tissues and cultured human gingival epithelial cells

ABSTRACT

Interleukin-8 (IL-8) belongs in the CXC chemokine family and is involved in processes, such as leukocyte chemotaxis, inflammation, angiogenesis, wound healing and tumor growth. It has been detected in the gingival crevicular fluid (GCF) and IL-8 mRNA has been identified in periodontal tissues. The existing literature is not conclusive, however, about the exact source and localization of this cytokine in the gingival tissues. Currently, nothing is known about the localization of IL-8 receptors in the gingiva. Therefore, our purpose was to characterize the localization of IL-8 and IL-8 receptors in gingival tissues and cultured human gingival epithelial cells.

We hypothesized that in vivo, periodontal pathogens induce IL-8 expression from gingival epithelial cells (GEC). We further hypothesized that this local expression of GEC derived IL-8 enhances leukocyte, vascular endothelial cell (VEC) and GEC migration via specific IL-8 receptors present on these cells.

Standard immunohistochemical techniques were applied in order to localize IL-8 and its receptors, CXCR-1 and CXCR-2, in archival specimens of gingival tissues from periodontitis patients. Cultured gingival epithelial cells, were also examined, using immunocytochemical techniques, for expression of IL-8 and IL-8 receptors. The results of these studies demonstrated that both IL-8 and the receptors CXCR-1 and CXCR-2 are
present in gingiva, fact that supports our hypothesis. CXCR-1 and CXCR-2 were expressed in the epithelium, microvascular endothelial cells and leukocytes within the inflamed tissues. *In vitro* studies verified the above results, by showing expression of IL-8, CXCR-1 and CXCR-2 in cultured epithelial cells.

It is concluded that IL-8 and the receptors CXCR-1 and CXCR-2 are expressed in the gingival epithelium both *in vivo* and *in vitro*. This suggests that epithelium plays critical role in the host defense against invading pathogens. The existence of IL-8R indicates that epithelial cells can actively respond to IL-8 and other host cytokines, like GRO, and regulate their own cytokine functions. Understanding the mechanisms of gingival chemokine expression against periopathogens will clarify the pathogenesis of specific and non-specific bacterial infections of the periodontium.
1. INTRODUCTION

Periodontitis is a common oral inflammatory condition in humans [1]. Periodontitis includes a group of clinical entities, that are generally characterized by a dynamic influx of neutrophils and T lymphocytes, initially, and plasma cells in later stages [2, 3]. CXC chemokines function as chemoattractants and activators of leukocytes. They are involved in many processes, such as migration and release of leukocytes, inflammation, angiogenesis, and tumor growth. Interleukin-8 (IL-8) belongs in the CXC chemokine subfamily. IL-8 has been detected in the gingival crevicular fluid (GCF) and both IL-8 and IL-8 mRNA have been identified, by our laboratory, in gingival tissue sections of periodontitis patients [4-6].

To date there are two types of IL-8 receptors: CXCR-1 and CXCR-2 [7]. They are present in a variety of cells, including neutrophils, monocytes, keratinocytes, endothelial cells, fibroblasts, smooth muscle cells and T cells. CXCR-1 is highly specific for IL-8. CXCR-2 is not highly specific for IL-8 and can bind other chemokines like MGSA (Melanoma growth stimulatory activity) also known as GRO (Growth related oncogene), MIP-2 (Macrophage inflammatory protein-2), and NAP-2 (Neutrophil-activating protein-2).

It has been shown that IL-8 is mediating inflammatory and immune conditions including psoriasis and rheumatoid arthritis [8-10]. Most of the documentation about keratinocytes derives from epidermal keratinocytes and their role in psoriasis. Although, there are histopathological and etiologic differences between psoriasis and periodontitis, they share some common characteristics like the dense accumulation of T-cells and PMNs beneath the epithelial layer. In both of these conditions there is a neutrophil-
mediated tissue damage. This is also a characteristic of periodontitis. There are contradictory views in the dental literature concerning the relation of IL-8 with the inflammatory status of the gingiva.

IL-8 has been associated with increased gingival inflammation. We have previously reported that interleukin-8 antigen and IL-8mRNA are elevated in inflamed gingiva [6]. Tonetti et al. detected IL-8mRNA in both healthy and diseased samples and reported that the localization of cells expressing IL-8mRNA paralleled the PMN infiltration of inflamed gingiva [5]. Finally, Dongari and Ebersole reported higher levels of IL-8 in connective tissue from periodontitis patients versus healthy patients [11].

Mc Gee et al., stated that IL-8 concentration is relatively higher in healthy gingiva and that it becomes reduced as the periodontal disease progresses [12]. This concept is also supported by Tonetti et al., who reported that IL-8 gene transcription was detectable at healthy sites, but was below detectable levels in 77% of the periodontitis sites [13].

Generally, little is known about the cells producing IL-8 in gingiva, or about their mechanism and sites of production in vivo. IL-8 mRNA-expressing cells in inflamed gingiva, were initially considered to be mainly macrophages [14]. Later, it was shown that IL-8 secreting fibroblast subpopulations may play more important role in the total IL-8 expression in inflamed tissues [11]. Localization of IL-8 within the gingival epithelium has previously been demonstrated, by our laboratory. Tonetti reported that periodontal pocket epithelium and junctional epithelium express IL-8 mRNA [5, 6]. Currently nothing is known about the expression of the IL-8 receptors (CXCR-1 and CXCR-2) in gingiva.
In our study, we hypothesized that periodontal bacteria induce IL-8 expression in gingival epithelial cells. We further hypothesized that this local expression of GEC-derived IL-8 enhances leukocyte, vascular endothelial cell (VEC) and GEC migration via specific IL-8 receptors (CXCR-1 and CXCR-2).

In this chapter (Chapter 1) we determined the distribution of IL-8 and IL-8 receptors CXCR-1 and CXCR-2 in human gingival tissues, using standard immunohistochemical techniques. We also determined the localization of IL-8 and its receptors in cultured GECs using standard immunocytochemical procedures.

Our results clearly demonstrate that IL-8 is predominantly localized in the gingival epithelium, since 12/12 specimens stained positive. IL-8 receptor expression was seen, in varying degrees in 12/12 specimens, on GECs, leukocytes, VECs and smooth muscle cells and was increased in samples with inflammation. These findings support our hypothesis that GECs are a source of IL-8 in vivo and in vitro and that epithelial-expressed IL-8 enhances leukocyte, vascular endothelial cell (VEC) and GEC migration via specific IL-8 receptors (CXCR-1 and CXCR-2). Our study provides new evidence showing that GECs are multifunctional units that interact with a variety of cells, causing chemotactic, angiogenic and mitogenic signals.
Figure 1.1 Hypothetical model of IL-8 functions in the periodontal environment

Periodontal pathogens induce expression of IL-8 in GECs, which can either bind back to the same cell, in an autocrine fashion, or act in a paracrine way. IL-8 can mediate epithelial proliferation and repair, angiogenesis or leukocyte chemotaxis/inflammation. Although inflammation aims to destroy the pathogen, it causes tissue destruction in the same time, resulting in loss of periodontal attachment and alveolar bone. In this chapter we are going to examine the localization of IL-8 and IL-8 receptors.
2. METHODS

2.1. Gingival biopsies

Paraffin-embedded gingival specimens (n=12) were obtained from the archives of Oral Pathology, University of Connecticut Health Center. 8 out of 12 specimens were originally obtained during periodontal surgery, from 8 patients, 3 males and 5 females ranging from 31 to 67 years old. All the patients had undergone routine initial therapy, which consisted of oral hygiene instructions and supra- and sub-gingival debridement. Specimens were selected after histological review of hematoxylin-eosin stained slides to contain both high degree of inflammation. 4 out of 12 were non-inflammed gingival specimens used as controls. The paraffin-embedded tissue was cut into 4-um sections and mounted on slides for further immunohistochemical evaluation.

2.2. Immunohistochemical technique

Immunohistochemical analysis of the tissue slides was performed for IL-8, CXCR-1 and CXCR-2 antigen. The tissue was deparaffinized in xylene and rehydrated sequentially in graded alcohol (100%, 95%, 70% and 50%). To inhibit endogenous peroxidase, the sections were immersed in 100% methanol containing 0.01% hydrogen peroxide at room temperature (RT) for 20 minutes. Antigen unmasking using was then performed. The slides were immersed in 0.01M sodium citrate at 90°C for 5 min and then rinsed in PBS. This procedure unmasked the epitopes from formalin bonds, allowing better antibody recognition. The sections were then blocked with 0.5% normal goat serum (Vector, Burlington, California) at RT for 1 hour. The sections were then washed x3 with phosphate-buffered saline (PBS, pH 7.4) and then rabbit anti-human IL-8, CXCR-1 or CXCR-2 (diluted of PBS with 0.5% goat serum) were added. Those primary
antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used at the following dilutions. For IL-8, 1:100, for CXCR-1, 1:25 and for CXCR-2, 1:800. An equal concentration of goat non-immune IgG was used as control, to verify the specificity of the antibody reaction. After incubation for 2h, biotinylated goat anti-rabbit antibody at a 1:200 dilution in PBS with 0.5% goat serum, was applied to the sections and allowed to incubate for one hour at RT. Sections were washed with PBS between each of the following steps. HRP-streptavidin (Zymed, S.F., CA) at a 1:100 dilution PBS was applied to the sections and incubated at RT for 45 minutes. Especially for IL-8 we used 1:400 poly-HRP-streptavidin. The sections were incubated sequentially with 3-amino-9-ethylbarbazole in 0.1M sodium acetate buffer (pH 5.2) and 0.03% hydrogen peroxide for 20 minutes at RT. Sections were then counter stained with Mayer’s hematoxylin (Sigma, St. Louis, Missouri) for 5 minutes, washed extensively in water, dipped in dilute ammonium hydroxide and mounted in crystal mounting solution (Biomeda, Foster City, CA). The flow diagram summarizes the procedure:

Gingival Tissue (N=12)  
↓  
10% Formalin  
↓  
Paraffin  
↓  
Unstained Slides  
↓  
@IL-8 or @IL-8R Antibodies or N IgG  
↓  
Immunohistochemistry  
↓  
Microscopic evaluation
2.3. Culture of gingival keratinocytes and immunocytochemistry

The human gingival epithelial cells used were immortalized with the Human Papilloma Virus type 16, the E6/E7 gene. The cell line was kindly provided by Dr. Oda, Department of Oral Biology, University of Washington, Seattle. The cells were kept frozen in liquid N2. Before every experiment the cells were thawed, plated into tissue culture flasks (Falcon, Franklin Lakes NJ) and fed every 48 hours. The medium used was Keratinocyte Serum-Free Medium (KSFM, Gibco).

The cells were grown up to the required number (3-4 x 10^6 cells) into 75 cm² tissue culture flasks (Costar). They were trypsinized using 0.025% bovine trypsin in 0.01% EDTA (Clonetics, Walkersville, MD). The cells were then plated into 4-well Lab-Tek chamber slides (Marsh Bio Products, Rochester, NY) at a concentration of 5x10^4 cells/0.5ml medium/well and were incubated for 6 hours in order to adhere and to reach a confluence 60-70%. Then, they were fixed with 10% phosphate buffered formalin. Immunocytochemistry was performed using the indirect immunoperoxidase staining technique as in the immunohistochemistry procedure. However, all the steps of deparaffinization and rehydration were omitted. The cells were immersed in 100% methanol containing 0.01% hydrogen peroxide at room temperature (RT) for 20 minutes, in order to permeabilize the cell membrane and to allow access of the antibodies into the cytoplasm. The sodium citrate unmasking step was also omitted. The flow diagram below describes the procedure:

```
GECs (PP cells)  ↓ 37°C, 5%CO2
Slide Chambers  ↓
10% Formalin  ↓
```
2.4. Slide analysis

Slides were evaluated using light microscopy in magnifications ranging from 40x to 600x using an Olympus Microscope. The degree of staining was graded on a scale of 0 to 3.
3. RESULTS

3.1. Expression of IL-8 in gingival tissue and epithelial cells in vitro

3.1.1. IL-8 and gingival tissue

All the slides stained positive for IL-8. The anti-IL-8 antibody was localized mainly in the epithelial layer of the gingival tissues. The intensity of antibody binding was higher near the keratin layer and it was decreasing in a corono-apical direction (Fig. 1.2,a). On a scale of 0 to 3, the keratin and granular layer stained 3, the spinous layer 1-2 and the basal layer 0. Therefore, the basal epithelial layer presented minimal IL-8 expression. Within the epithelium, there were areas with more intense staining than others. The underlying stroma (connective tissue) and VECs showed little evidence of IL-8 expression ranging from 0 to 1. The infiltrating inflammatory cells stained for IL-8 with a degree 1-2. The same samples were incubated with goat IgG, as specificity control, and no staining was observed (Fig.1.2,b).

3.1.2. IL-8 and gingival epithelial cells

After demonstrating the localization of IL-8 in the gingival biopsies, we hypothesized that IL-8 is produced locally and that GECs are the source of IL-8. In order to test our hypothesis, we stained cultured GEC with anti-IL-8 antibodies. The color difference was compared to the controls, where goat IgG was added instead of anti-IL-8 antibody. 100% of the cultured GECs stained positive for IL-8, with a degree 3 intensity (Fig. 1.3, a). This presented as bright red color that occupied the cytoplasm, where the IL-8 antigen was localized. The pattern of the staining was granular, indicating local condensations of IL-8 antigen, which might correspond to either secretory vesicles or membrane bound IL-8. This indicated that GECs express IL-8 in vitro.
Figure 1.2 Immunohistochemical staining of gingival tissue for IL-8 expression
a: Gingival expression of IL-8 (red color) using anti-IL-8 antibody (200x). IL-8 antigen is localized in the superficial epithelial layers. The epithelial surface is on the upper right corner. b: Negative control staining using IgG in the same concentration as the specific anti-IL-8 antibody (200x).

Figure 1.3 Immunocytochemical staining of gingival epithelial cells (GECs) for IL-8 expression
a: GEC expression of IL-8 (red color) using anti-IL-8 antibody (600x). 100% of the cells stained positive. b: Negative control staining using IgG (600x).
3.2. Expression of CXCR-1 in gingival tissue and epithelial cells in vitro

3.1.1. CXCR-1 and gingival tissue

The localization of CXCR-1 antigen was examined using anti-CXCR-1 antibodies in 12 gingival biopsies. The specificity of the method was tested by incubating the same samples with goat IgG. 100% of the samples (12/12) stained positive, but the intensity of CXCR-1 antigen varied according to the degree of inflammation. Non-inflamed samples presented degree 1 staining, whereas inflamed samples presented degree 2 staining (Fig 1.4, a). CXCR-1 antigen, in all the samples, was detected more in the keratin and granular and less in the spinous layer of the epithelium, indicating CXCR-1 reduction in a corono-apical direction. The basal layer of the epithelium, showed degree 0 staining, indicating absence of CXCR-1 antigen. In inflamed samples, staining (degree 2) was also observed in the underlining stroma, associated with inflammatory cells and extracellular matrix.

3.1.2. CXCR-1 and gingival epithelial cells

Immunocytochemical localization of CXCR-1 antigen was performed in cultured GECs. All the cells stained positive for CXCR-1 (Figure 1.5, a). The intensity of the staining was between 2-3 and was more diffuse compared to IL-8 staining. No staining was seen in the negative control slides (Fig.1.5, b) indicating the specificity of CXCR-1 staining technique.
Figure 1.4 Immunohistochemical staining of gingival tissue for CXCR-1 expression
a: Gingival expression of CXCR-1 (red color) (200x). CXCR-1 antigen is localized in both the epithelium and the inflammatory infiltrate of the underlying stroma. b: Negative control staining using non-immune IgG (200x).

Figure 1.5 Immunocytochemical staining of GECs for CXCR-1 expression
a: GEC expression of CXCR-1 (red color) (600x). 100% of the cells stained positive. The localization of CXCR-1 antigen was diffuse. b: Negative control staining using non-immune IgG (600x).
3.3. Expression of CXCR-2 in gingival tissue and epithelial cells in vitro

3.3.1. CXCR-2 and gingival tissue

The localization of CXCR-2 was examined using anti-CXCR-2 antibodies. The specificity of the procedure was tested by incubating the samples with goat IgG. 100% of the samples stained positive for CXCR-2, with a degree of staining ranging between 2-3. Again, similar to CXCR-1, non-inflamed samples (based on the degree of inflammatory infiltration) stained less intense (degree 2) than inflamed (degree 3). In all the samples, CXCR-2 antigen was detected in the gingival epithelium and the underlying stroma. Within the epithelium, the more intense staining was seen in the apical part of keratin layer and the spinous and granular layers. No staining was seen in the basal layer. Within the stroma, significant staining was associated with leukocytes, smooth muscle cells and microvascular endothelial cells (MVEC), especially in the areas with dense inflammatory infiltrates (Fig.1.6, c). It should be noted that the antibody concentration that we used for CXCR-2 was 32 times lower than the anti-CXCR-1 antibody, in order to obtain the similar degree of staining.

3.3.2. CXCR-2 and gingival epithelial cells

In order to test the hypothesis that the CXCR-2 antigen derives from the epithelial cells, immunocytochemical localization of this antigen was performed in cultured GECs. CXCR-2 were found in 100% of the cells (Fig.1.7, a). The distribution of the receptors presented a unique pattern. Although they were seen throughout the cytoplasm, there was a more intense staining in the perinuclear area, especially in fully developed cells. No staining was seen in the negative control slides (Fig.1.6, b), indicating the specificity of the technique for CXCR-2 binding.
Figure 1.6 Immunohistochemical staining of gingival tissue for CXCR-2 expression
a: Gingival expression of CXCR-2 (200x). CXCR-2 antigen was detected in the epithelium and the inflamed stroma. b: Negative control staining using non-immune IgG (200x). c: MVECs (small black arrow), smooth muscle cells (big black arrow) and leukocytes (red arrow) expressed CXCR-2 (600x). d: Negative control staining using non-immune IgG (400x).

Figure 1.7 Immunocytochemical staining of GECs for CXCR-2 expression
a: GEC expression of CXCR-2 (600x). 100% of the cells stained positive (arrow) b: GEC negative control staining using non-immune IgG (600x)
4. DISCUSSION

Localization of IL-8 in gingival tissues

IL-8 is a chemotactic cytokine for all known types of migratory immune cells. IL-8 is a mitogen for epidermal cells. IL-8 has been implicated in angiogenesis and may play a role in angiogenesis-dependent conditions such as rheumatoid arthritis, tumor growth, and wound healing. IL-8 may be also a marker of different inflammatory diseases. IL-8 may be of clinical relevance in psoriasis. Elevated concentrations are observed in psoriatic scales and this may explain the high proliferation rate observed in these cells. IL-8 mRNA levels were shown to correspond to the severity of periodontitis [5] but its role(s) and source(s) in periodontal disease are not clearly understood.

We hypothesized that in vivo, periodontal pathogens release factors that induce IL-8 expression from gingival epithelial cells (GEC). We further hypothesized that this local expression of GEC-derived IL-8 enhances leukocyte, vascular endothelial cells (VEC) and GEC migration via specific IL-8 receptors present on these cells. Therefore, our aim was to characterize the in vivo IL-8 and IL-8 receptor expression in tissues from patients with periodontal disease; we also studied the localization of IL-8 and IL-8 receptors in gingival epithelial cells, in vitro.

The epithelial localization of IL-8 was demonstrated in our study. In all biopsies the IL-8 antigen was found within the spinous, granular and apical part of keratin layer. Minimal or no staining was seen in the basal layer and in the underlying connective tissue. There was some variation, however, in the intensity of the staining. This possibly reflects inter-patient variation in the degree of inflammation or in the degree of cytokine response to the bacterial challenge [11]. Where the connective tissue was inflamed, large
"rete" processes were observed. No polymorphonuclear leukocytes were seen within the epithelium. However, where the normal epithelial structure was altered, GECs showed strong positive reaction for IL-8, suggesting that IL-8 might have biological activity within the epithelium and it might contribute to the observed epithelial proliferation.

The localization of IL-8 has been studied by Fitzgerald and Kreutzer [6]. They found that both interleukin-8 antigen and mRNA are elevated in chronically inflamed gingiva and that interleukin-8 antigen is detected only in the epithelial cell layer, which is consistent with our findings. Mc Gee et al., on the other hand, have stated that IL-8 concentration is relatively higher in healthy gingiva (compared to IL-6) and that it becomes progressively reduced as the gingival sulcus increases in depth and the periodontal disease progresses [12]. This concept is also supported by Tonetti et al., who reported that IL-8 gene transcription was detectable at healthy sites, but was below detectable levels in 77% of the periodontitis sites [13]. The same author has also examined the localization of IL-8 mRNA+ cells in samples from healthy and periodontal sites, in another publication the following year (1994) [5]. They reported that IL-8 mRNA positive cells were found in the junctional and pocket epithelium, but not in the inflamed connective tissue. In the healthy and gingivitis specimens the number of positive cells increased in an apico-coronal direction and was maximal in the superficial layers. In the periodontitis specimens, the positive cells were prominent in the pocket epithelium, adjacent to the infecting plaque organisms. PMNs were found mainly in the junctional epithelium, pocket epithelium and in the underlying connective tissue. They also observed that IL-8 expression, in general, paralleled PMNs infiltration. Matsuki et al. have identified the IL-8 mRNA expressing cells, in inflamed gingiva, mainly as
macrophages [14]. The authors reported a lower IL-8 mRNA expression in endothelial cells and fibroblasts, but gave no data about epithelial cells.

From the above data, it is suggested that GECs form a barrier between the oral environment and the underlying connective tissues and they modulate the leukocyte infiltration and activation by producing IL-8 after interaction with periodontal pathogens like *A. actinomycetemcomitans*. Since IL-8 has also been detected in healthy gingiva, it may play a role in the constant migration of neutrophils through the gingival tissues and in the establishment of equilibrium between the continuous bacterial challenge and the host defenses.

**Localization of IL-8 in cultured gingival epithelial cells**

Although several investigators have studied the localization of IL-8 in gingival tissues the localization of IL-8 in gingival epithelial cells (GECs) in culture has not been reported to date. After demonstrating the epithelial expression of IL-8 in gingival biopsies, we wanted to further examine the localization of IL-8 on GECs. We hypothesized that GECs are genetically programmed to produce IL-8 as part of innate immunity. Using standard immunocytochemical procedures we showed that IL-8 antigen is, indeed, expressed in a gingival epithelial cell line, whether the cells are exposed to pathogens or not. IL-8 could be in two possible compartments: 1) Intracellular, since the cell membranes were permeabilized with methanol or 2) membrane-bound (connected with IL-8 receptors).

The localization of IL-8 can be seen in the Figure 1.3 and is characterized as diffuse, although in higher magnification some localized condensations were observed. We postulated that part of the excreted IL-8 binds back to its receptors in GECs initiating
an autocrine loop. We have demonstrated that both of the IL-8 receptors (CXCR-1 and CXCR-2) are present in GECs and therefore our hypothesis for autocrine regulation and IL-8-induced epithelial cell proliferation can be valid.

**Localization of IL-8 receptors in gingival tissues**

The receptors that bind C-X-C Chemokines are designated CXCR followed by a number (e.g. CXCR-1, CXCR-2, CXCR-3 etc.). Both IL-8 and GRO belong in that cytokine family. To date there are two types of IL-8 receptors that have been isolated, characterized and cloned: CXCR-1 and CXCR-2 [7]. IL-8 receptors (previously called IL-8RA and IL-8RB) are present in a variety of cells. Neutrophils, monocytes, keratinocytes, endothelial cells, fibroblasts, smooth muscle cells and T cells have been shown to express one or both of these receptors. The receptors show 77% amino acid sequence homology between themselves. CXCR-1 is highly specific for IL-8. CXCR-2 is not specific for IL-8 and also binds other chemokines like MGSA (Melanoma growth stimulatory activity) also known as GRO (Growth related oncogene), MIP-2 (Macrophage inflammatory protein-2), and NAP-2 (Neutrophil-activating protein-2). In neutrophils, the affinity of CXCR-2 for IL-8 is 2-5 times greater compared to that of CXCR-1. It has been proposed that the 2 receptors have different functions. In neutrophils, CXCR-2 may play as active role in the initiation of migration distant from the site of inflammation, where the concentration of IL-8 is at the picomolar level. The low affinity CXCR-1 may play a more active role in mediating IL-8 signal at the site of inflammation, where the concentration of IL-8 is high.

We have shown that IL-8 is present within the periodontal microenvironment and specifically in the oral epithelium. We then hypothesized the existence of IL-8 receptors
in GECs, VECs and leukocytes, which means that those cells are responsive to IL-8 stimulus. We examined the existence and distribution of IL-8 receptors in the same gingival samples that we used for IL-8 localization. It is clear from our results that both IL-8 receptors are expressed in gingival tissues. The existence of those receptors has also been shown in samples from head and neck squamous cell carcinoma by Richards et al [15].

Expression of both CXCR-1 and CXCR-2 was detected within the epithelium, with the exception of the basal layer. The basal epithelial layer lacked staining for CXCR-1 or CXCR-2 in all the gingival samples that we examined. Intense staining for CXCR-2 was seen in microvascular endothelial cells (MVECs), and smooth muscle cells in 100% the gingival biopsies. However, MVEC staining for CXCR-1 was only seen in 50% of the gingival samples, and was less intense. The MVEC staining was the major difference between the patterns seen with the two receptors. This finding was in agreement with Richards et al [15]. They reported that 93% of oral tissues had positive MVEC staining for CXCR-2, but only 42% had positive staining for CXCR-1.

Another difference that we observed was the different amount of antibody that was required in order to stain the 2 receptors in the gingival samples. For the receptor CXCR-1 we used 32 times higher concentration of antibody (8 ug/ml) than for the receptor CXCR-2 (0.25 ug/ml). Although we cannot directly compare the efficacy of 2 different antibodies, there is a suggestion that receptors CXCR-2 are more prominent in gingival tissues.
Localization of IL-8 receptors in cultured gingival epithelial cells

After demonstrating the presence of the receptor antigen in gingival biopsies, we hypothesized that GECs in culture also express both receptors of IL-8. We showed that CXCR-1 and CXCR-2 antigens are, indeed, expressed in the gingival epithelial cell line PP. The localization of CXCR-1 can be seen in the Figure 1.5 and is characterized as diffused and equally distributed. Localization of CXCR-2 was different with apparent perinuclear condensations. However, it is difficult from the 2-dimensionssional images to determine the exact location of the receptors. We were not able to define with certainty whether the receptors were located intracellular, or on the plasma membrane. Future studies should aim in this direction. Clarifying the exact location of IL-8 receptors could help understanding the regulatory events that take place after IL-8 excretion.

It can be concluded from our data that the gingival epithelium participates actively in inflammatory processes. IL-8 is expressed by GECs during inflammation and regulates PMN chemotaxis, angiogenesis and epithelial proliferation and expression of IL-8 in an autocrine fashion. It is the first time that the expression of IL-8 receptors has been characterized in human gingiva. It was shown that CXCR-1 and CXCR-2 are expressed in gingival epithelial cells, MVECs, leukocytes and smooth muscle cells Fig.1.8). The present studies clearly support our hypothesis that GEC are a source of IL-8 in vivo and in vitro and that epithelial produced IL-8 plays a multifunctional role in the periodontal tissues. Future studies are needed to determine the clinical relevance of these observations and the future therapeutic applications that they might have.
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<th>IL-8⁺</th>
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Figure 1.8 Summary of immunohistochemistry and immunocytochemistry
REFERENCES


CHAPTER 2

Characterization of A. actinomycetemcomitans-induced interleukin-8 from gingival epithelial cells

ABSTRACT

Periodontitis is a group of inflammatory conditions that result mainly from the interaction of pathogenic microorganisms with the periodontal tissues. Gingival epithelial cells are the first cells of the host that encounter the bacterial challenge and therefore their role in the initiation of the immune response is critical. Among the periodontal pathogens, Actinobacillus actinomycetemcomitans (A.a.) is a key microorganism that adheres to and invades oral epithelial cells. Currently there is limited knowledge about the role of gingival epithelial cells in the initiation of host defense after encountering A.a..

We hypothesized that sonicated extracts of this bacterium would be able to upregulate IL-8 expression by GECs. We further hypothesized that the IL-8 expression induced by this pathogen is mediated by MAP kinase in the intracellular level. Finally, we examined whether the A.a.-induced upregulation of IL-8 by GECs is related to the A.a.-derived LPS.

An oral epithelial cell line was co-cultured with A.a. sonicated extracts at various concentrations and for various time periods. The supernatants were collected for IL-8 detection with ELISA. For the MAP kinase studies, a potent inhibitor of p38 MAP kinase (SB203580) was added in the cell cultures and 18 hours later the supernatants were
assayed for IL-8. For the LPS studies, Polymyxin B was used to neutralize the LPS content of the bacterial sonicates before stimulating GECs.

Dose-response studies showed that the highest IL-8 secretion was at the dose of 50ug/ml of A.a. protein. Time-course studies revealed that IL-8 secretion reached a maximum level 24 hours after A.a. challenge and remained at those levels until 48 hours. We found that the specific p38 MAP kinase inhibitor inhibited A.a.-induced IL-8 expression, but did not inhibit IL-1beta-induced IL-8 expression. It was also found that the ability of A.a. sonicates to induce IL-8 in epithelial cells was not diminished by Polymyxin B neutralization of LPS, which means that the stimulatory potential of A.a. was not due to the LPS content of the sonicated extracts. In addition, it shown that E. coli lipopolysaccharide (LPS), which was able to stimulate IL-8 release from peripheral blood mononuclear cells (PBMC), did not release IL-8 from gingival epithelial cells.

These data indicate that gingival epithelial cells are capable of upregulating IL-8 expression in response to A.a. and thus may facilitate the recruitment of neutrophils as a host defense mechanism. Our findings provide new evidence on the mechanism responsible for the upregulation of IL-8. It was clearly shown that p38 MAP kinase is involved in the signal transduction pathway that regulates A.a.-induced IL-8 expression.

It is concluded that other factors than A.a. LPS are responsible for the up-regulation of the IL-8 expression in GECs. These might include polysaccharides, surface-associated material (SAM), lipid A-associated proteins (LAP), porins, fimbriae and cell wall polymers (CWP). The ability of A.a. to elicit IL-8 release from gingival epithelial cells may be important in the pathogenesis of periodontal conditions. Knowledge of the host and bacterial factors that induce IL-8 expression in GECs may be used in the future
for modification of cytokine expression, as part of a preventive or therapeutic protocol of periodontal conditions.
1. INTRODUCTION

Bacterial induced IL-8 expression

Periodontitis is a group of inflammatory conditions elicited mainly by bacterial colonization of the gingival sulcus. Normally, gingival epithelial cells form a barrier that prevents microbes from invading internal periodontal tissues like periodontal ligament, connective tissue and alveolar bone. However, *Actinobacillus actinomycetemcomitans* (*A.a.*), an important periodontal pathogen, has been shown to invade oral epithelial cells and has been implicated in forms of periodontal disease like Localized Juvenile Periodontitis (LJP) and adult periodontitis (AP) [1-3]. Oral epithelial cells, as part of the bacterial-host interaction, have been shown to produce several cytokines, including the potent chemoattractor Interleukin-8 [4, 5]. IL-8 belongs in the family of Chemokines. Chemokines are a family of small polypeptides that have chemoattractant properties for inflammatory cells [6] and act as a signal for the emigration of blood cells. Several reports have suggested that cytokines, including IL-8, play important roles in the pathogenesis of periodontitis [7, 8].

Interleukin-8 (IL-8) is one of the C-X-C chemokines, which are considered the most important mediators of the accumulation of granulocytes [9]. IL-8 has been detected in the gingival crevicular fluid (GCF) [10] and in gingival tissue sections of adult periodontitis patients [11] and IL-8 mRNA has been identified in healthy and diseased periodontal tissues [12, 13]. Several studies have shown that unstimulated oral keratinocytes, in culture, have a detectable basal IL-8 expression. Formanek et al. [14] and Li et al showed that resting cultured normal oral keratinocytes produced small amounts of IL-8 [15].
It is currently known that vital bacteria (in-vitro) can upregulate the basal IL-8 production of oral keratinocytes. Vernier et al. have shown that *Streptococci viridans* are potent stimulators of interleukin-8 production by a human oral epithelial cell line (KB line) [16]. It has been shown that KB cells upregulate their basal IL-8 production following infection with the periodontopathic bacterium *Eikenella corrodens*, too [17]. It should be noted that bacterial invasion or even adhesion is not always necessary for cytokine up-regulation. Yumoto et al examined how adhesion of the bacterium affects the cytokine produced by epithelial cells [17]. The results of this study suggest that the direct contact of *E. corrodens* with oral epithelial cells is not necessarily required for the stimulation of IL-8 expression.

Infection with non-vital bacteria can also induce proinflammatory cytokine expression. Huang et al. demonstrated that IL-8 expression of both primary and immortalized oral keratinocytes is upregulated after in-vitro infection with vital and non-vital *A.a.* [5]. The highest IL-8 expression was at the multiplicity of infection (MOI) of 1,000:1 with vital *A.a.* Time-course studies revealed that IL-8 expression rapidly reached a maximum level 6 hours after bacterial infection. 24 hours after vital bacterial challenge a 7-fold increase was demonstrated in the IL-8 expression. In an other publication by the same author it was shown that dead strains of *P. gingivalis* can induce more IL-8 secretion by human gingival epithelial cells compared to live strains [18]. These data indicate that oral epithelial cells are capable of upregulating IL-8 expression in response to bacterial challenge and thus may facilitate the recruitment of neutrophils as a host defense mechanism.
In our study we hypothesized that sonicated extracts of *A. a.* upregulate IL-8 expression in GECs. We exposed GECs, in vitro, to the bacterial stimulus at various concentrations and for various time periods. The medium was then collected and assayed for interleukin-8, using ELISA. The results indicated a dose-dependant relation if IL-8 to the bacterial concentration, up to a maximum of 50ug/ml. Higher concentrations of *A. a.* caused reduction of IL-8. The kinetic study revealed that IL-8 is secreted as early as 2h after the bacterial exposure, peaks at 24h and remains stable until 48h. That corresponded to a 7-fold increase compared to the unstimulated cells.

The above findings support our hypothesis about the role of GECs. They show that vitality of bacterial stains is not required for immune response, since bacterial products can activate cytokine responses. This has significant implications in clinical periodontics because we have demonstrated that killing the periopathogens is not enough for the elimination of periodontitis. Bacterial products that remain in the periodontal pocket can activate immune responses with detrimental consequences for the periodontium.

The role of gingival keratinocyte was also stressed. Their immediate chemokine response seems to be critical for neutrophil influx and activation against the invading pathogens.

**MAP kinase mediation of IL-8 expression**

At present, little is known about the mechanisms by which *A. a.* induces the expression of pro-inflammatory cytokines in the cellular level. A number of recent studies implicate mitogen activated protein (MAP) kinases in the signal transduction pathway of IL-8 expression by several different cell systems like intestinal epithelial cells.
MAP kinases are a family of cell signaling molecules. MAP kinases can be activated by a wide variety of extracellular stimuli and transmit signals from the cell surface to the nucleus to regulate gene expression. Cellular functions that are regulated, at least in part, by MAP kinases include cell proliferation, cell survival, and cytokine production. There are three main groups of MAP kinases:

- the p38 kinases
- the extracellular signal-regulated kinases (ERK),
- the c-Jun N-terminal kinases (JNK).

These MAP kinase subfamilies form three parallel cascades that can be activated simultaneously or independently. P38 and JNK are stimulated by inflammatory cytokines and stress stimuli, but minimally by growth factors. ERK are strongly activated by growth factors, but weakly by inflammatory stimuli.

There is no information to date about MAP kinase mediation of cytokine expression on oral-derived cell systems and specifically gingival epithelial cells. We hypothesized that A.a.-induced expression of IL-8 in GECs is mediated by p38 MAP kinase.

The specific inhibitor of p38 MAP kinase SB 203580 was used to elucidate the biologic function of p38 MAP kinase in A.a.-induced IL-8 expression. The inhibitor was added to the cell cultures prior to the bacterial challenge. The IL-8 expressed by GECs was then determined. SB 203580 significantly reduced IL-8 expression in A.a.-stimulated cells, in a dose-dependent way, whereas it did not affect IL-8 production in unstimulated cells. These results support our hypothesis and showed that A.a. interaction with GECs
induced activation of p38 MAP kinase, which in turn, mediated IL-8 expression and protein production and excretion. Understanding the intracellular mechanisms of IL-8 regulation is of great interest, as it may lead to the development of novel therapeutic approaches for periodontitis, in addition to the traditional scaling and root planing and periodontal surgery.

**Virulence factors of A.a. that induce cytokines**

In this study it was shown that A.a. sonicates are able to induce IL-8 expression and that viability of A.a. was not necessary for the pathogenicity of this bacterium. The question remains however: “What is the virulence factor of A.a. that upregulates GEC IL-8 expression?” In the past many authors considered lipopolysaccharide (LPS) as the major pathogenic substance. LPS is an integral component of the Gram-negative bacterial outer membrane [28]. If released into the systemic circulation, LPS activates specialized inflammatory cells to secrete proinflammatory cytokines including interleukin-1 (IL-1), tumor necrosis factor alpha (TNFα) and IL-6. In the serum, LPS binds LBP (LPS binding protein) and is transferred to the cellular receptor CD14 [29]. Even cells without this receptor can be activated by LPS, because soluble CD14 in serum can bind LPS and LBP [30]. However, the potent effects of LPS on monocytes and macrophages, cannot be generalized to other sites or cells in the infected host [31].

It is only during in the past decade that the concept has arisen that bacterial derived molecules, other than LPS, can induce cytokine expression. A large number of components and products have been investigated for their ability to stimulate cytokine release by mammalian cells and especially peripheral blood mononuclear cells (PBMCs)
and fibroblasts. Some of these components have been proven to be more potent than LPS. It is clear from the current literature that oral bacteria contain lipid A-associated proteins, capsular polysaccharides, fimbriae and cell wall polymers (CWP). We are going to review, briefly the basic pieces of evidence about the virulence factors of periopathogenic bacteria.

Serotype specific A.a. polysaccharides have been shown to stimulate IL-1 release from murine macrophages [32]. Yamaguchi et al. stimulated human monocytes with serotype b-specific polysaccharide antigen (SPA) from A.a. SPA increased the released of IL-1, IL-6, TNF-α and the mRNA expression of IL-1α, IL-1β, IL-6, TNF-α and IL-8 [33].

Saline-extractable surface-associated material (SAM) from A.a. is, according to Reddi et al, a potent cytokine-stimulating agent in stimulating IL-6 release by PBMCs [34]. The same authors showed that lipid A-associated proteins (LAP) from A.a. had cytokine stimulating activity in cultures of human gingival fibroblasts (HGFs) and human PBMCs. A.a. LPS was less potent than the other surface extracts in stimulating release of IL-1 beta, IL-6 and TNF-alpha. LAP and LPS from periopathogens stimulate IL-6 release from human gingival fibroblasts in a dose-dependent manner, although LPS is less potent than LAP [35]. Others have shown that porins of gram negative bacteria are able to induce the release of tumor necrosis factor alpha (TNF-α), interleukin-1 alpha (IL-1α), and IL-6 by human monocytes and of gamma interferon (IFNγ) and IL-4 by human lymphocytes [36].

Tani et al. found that extracellular antigens of A.a. contain a 37-kDa protein. This protein induced IL-1β, IL-6, and TNF-α release from murine macrophages. The IL-6-
inducing activity of the 37-kDa protein was higher than that of LPS. They concluded that the 37-kDa protein plays a significant role in *A.a.*-associated periodontitis [37].

Fimbriae from periodontal pathogens have been shown to induce IL-6 and TNFα from PBMCs [38]. It has also been demonstrated that IL-8 response of human uroepithelial cells to P-fimbriated *E. coli* is CD14 and LPS independent, and that attaching pathogens can overcome the LPS unresponsiveness of epithelial cells by fimbriae-dependent activation mechanisms [31, 39]. Woolverton et al, found that an LPS–free cell wall polymer (CWP) preparation from *A.a.* was able to stimulate IL-1 from mouse splenocytes. That preparation appeared to consist of peptidoglycan, carbohydrates and proteins [40].

An association between attachment and virulence has been suggested for the periothogenic organisms *A.a.* and *Porphyromonas gingivalis* (*P.g.* [41] It has been shown, however, that non-vital bacteria are able to trigger cytokine response as well, which implies that invasive ability is only responsible for part of the pathogenicity and that dead bacteria can initiate a similar responses [5]. Our data also support this concept since we have shown high levels of cytokine expression in response to sonicated extracts from *A.a.*.

It has been postulated that the ability of Gram-negative pathogens to trigger cytokine expression by epithelial cells may relate to other bacterial components than LPS [31]. Most of the data that we have are derived from bronchial epithelial and uroepithelial cell models. Palfreyman et al have demonstrated that *B. cepacia* lipopolysaccharide, which was able to stimulate IL-8 release from monocytes, did not release IL-8 from a bronchial epithelial cell line [39].
Based on the above, we hypothesized that A.a. LPS is not necessary for upregulation of IL-8 expression by GECs. Polymyxin B was used to neutralize the LPS content of the bacterial sonicates before stimulating GECs. We also used PBMCs as controls for LPS stimulation. Our results showed that LPS is a major factor in the PBMC IL-8 expression, but not in the gingival epithelial cells. Other bacterial factors may play more important role in the initiation of proinflammatory cytokine response. However, we believe that more studies are necessary in epithelial cells in order to elucidate the cytokine stimulatory components periodontal pathogens like A.a.
<table>
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<tr>
<th>Cytokine</th>
<th>Bacterial component</th>
<th>Cells</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-1α</strong></td>
<td>LPS(^1), fimbriae, SPA(^2), porins</td>
<td>Human Monocytes</td>
<td>[33, 36]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Murine Splenocytes</td>
<td>[40]</td>
</tr>
<tr>
<td><strong>IL-1β</strong></td>
<td>LPS, fimbriae, polysaccharides, LAP(^3), SAM(^4), SPA, 37kDa protein</td>
<td>Murine Macrophages</td>
<td>[32, 37]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HGFs(^5)</td>
<td>[34]</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>LPS, LAP, SAM, fimbriae, SPA, porins, 37kDa protein</td>
<td>Human Monocytes</td>
<td>[33, 36]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Murine Macrophages</td>
<td>[37]</td>
</tr>
<tr>
<td><strong>IL-8</strong></td>
<td>LPS, fimbriae, SPA</td>
<td>Human Monocytes</td>
<td>[33, 39]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human Uroepithelial</td>
<td>[31]</td>
</tr>
<tr>
<td><strong>TNFα</strong></td>
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<td>Human Monocytes</td>
<td>[33, 36]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Murine Macrophages</td>
<td>[37, 43]</td>
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<tr>
<td></td>
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<td>HPBMCs</td>
<td>[34, 38, 44]</td>
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<td>HGFs</td>
<td>[34]</td>
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</table>

Table 2.1 Proinflammatory cytokine expression induced by various bacterial components

This table summarizes the bacterial virulence factors that can induce cytokine responses.

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\(^{1}\) Lipopolysaccharides  
\(^{2}\) Serotype b specific Polysaccharide Antigen (from *A. actinomycetemcomitans*)  
\(^{3}\) Lipid A-associated Proteins (from *A. actinomycetemcomitans*)  
\(^{4}\) Saline-extractable Surface-associated Material (from *A. actinomycetemcomitans*)  
\(^{5}\) Human Gingival Fibroblasts
2. METHODS

2.1. Culture of gingival keratinocytes

For our experiments we used human gingival epithelial cells immortalized with the Human Papilloma Virus type 16, the E6/E7 gene [45]. The cell line was kindly provided by Dr. Oda, University of Washington, Seattle. The cells were kept frozen in liquid N2. Before every experiment the cells were thawed, plated into tissue culture flask (Falcon, Franklin Lakes NJ) and fed every 48 hours. The gingival epithelial cell line was maintained in Keratinocyte Serum Free Medium (KSFM, Gibco BRL Life Technologies) supplemented with penicillin-streptomycin and amphotericin B. The cells were grown up to the required number (3-4 x 10^6 cells) into 75 cm^2 tissue culture flasks (Costar). They were trypsinized using 0.025% bovine trypsin in 0.01% EDTA (Clonetics, Walkersville, MD). The cells were then plated into 12-well tissue culture plates (Costar, Cambridge, MA), 2 x 10^5 cells, in 1 ml medium per well, and were incubated for 4-6 hours in order to adhere and reach confluence. Then, they were exposed to either A.a., or medium only. There were triplicate wells for each condition. Cell cultures were maintained at 37°C and 5% CO₂. At the end of every experiment, the supernatants were collected, centrifuged and assayed for IL-8. After the supernatants were collected, the cells were solubilized using 0.1% Triton, centrifuged and the cell lysates were assayed for cytokines.

2.2. Preparation of PBMC

Venous blood was collected from a healthy donor (A.S.) in 60 ml syringes containing 5 ml Na-citrate 3.8%. Briefly, 20 ml aliquots of blood were mixed with an equal volume of PBS. 35 ml of the blood-PBS suspension was carefully layered onto 15 ml of Ficoll-Paque (Pharmacia) and centrifuged at 450 g for 30 min at room temperature.
The mononuclear cell layer was collected into Falcon tubes, washed with PBS and centrifuged at 500 g for 15 min. The wash step was repeated and the pellet resuspended in RPMI-1640 medium containing 1% Fetal Calf Serum. Following assessment of cell viability by trypan blue, 1ml PBMC cultures in triplicate were established in 24-well, flat-bottom microtiter plates (Falcon) at approximately 10^6 cells/well. Plates were incubated for 2 h, washed with normal saline to remove non-adherent cells and then 1ml medium containing bacterial constituents with or without polymyxin B, was added to the cells. The PBMCs were stimulated for 24 h at 37°C in an atmosphere of 5%CO_2 and 95% air. Following culture, supernatants were collected, centrifuged and stored at −70°C for use in cytokine quantitation studies. The flow diagram summarizes the procedure:

\[
\begin{align*}
\text{A.a. Extract or E.coli LPS or Media} & \quad \downarrow \\
\pm \text{Polymyxin B} & \quad \downarrow \\
\text{PBMCs} & \quad \downarrow \\
24\text{h, }37^\circ\text{C, }5\%\text{CO}_2 & \\
\text{Supernatants/Lysates} & \quad \downarrow \\
\text{IL-8 (ELISA)} &
\end{align*}
\]

2.3. A.a. challenge

\textit{A.a.} strains (ATCC 33384), were kindly provided by Dr. Zadeh, University of Southern California School of Dentistry, Los Angeles CA, USA. The bacteria were grown for two 3-4 day periods and subcultured after each growth period to ensure strain purity. Cells were harvested by washing and suspension in 3 ml sterile phosphate buffered saline (PBS), followed by sonication to extract soluble bacterial components. Briefly, cells suspended in PBS were subjected to 10 repetitions of sonication at 5-minute intervals at a 10% duty cycle. The cell free supernatant was then collected by
centrifugation at 8000 rpm for 30 minutes at 4° C and dialyzed with sterile PBS at 4° C. Bacterial protein concentration was determined by Bradford protein assay (Bio-Rad, Richmond CA). The extracts were aliquoted and stored at −70° C. Sonicated extracts were used as bacterial challenge in various concentrations in the culture medium. The flow diagram below describes the procedure:

```
A.a. (Dr. Zadeh, USC)  ↓
Grow for 3-4 days  ↓
Sonicate: 5min x 10  ↓
Spin: 8000 RPM, 30min  ↓
Dialysis with PBS  ↓
A.a. Protein Determination
(Bradford Assay)
↓
A.a. Extracts
```

2.4. Treatment of GECs with specific p38 MAP kinase inhibitor

The aim of this study was to determine whether activation of p 38 MAP kinase is required for A.a.-mediated IL-8 production. Cell culture experiments were conducted in 96-well polypropylene tissue culture plates (Corning Costar, Cambridge, MA). The bacterial challenge consisted of A.a. sonicates with protein concentration 20 ug/ml. The sonicates were prepared from A.a., strain ATCC 33384, as described in section 2.3.

For the blockade of p38 MAP kinase we used the specific p38 MAP kinase inhibitor SB 203580 (Calbiochem, La Jolla, CA) It is a pyridinyl-imidazole compound, the properties of which have been described in the literature [27, 47, 48]. The gingival epithelial cells were treated with the specific p38 MAP kinase inhibitor for 60 min before exposure to A.a. and during the incubation period of the experiment. The MAPK inhibitor
was added at progressively increasing concentrations ranging from 0 to 100 uM. Specifically, the following concentrations were used: 0, 0.1, 0.3, 1, 3, 10, 30 and 100 uM. The cells were incubated for 18 hours and the culture supernatants were then harvested, centrifuged and stored at -20°C until assayed. The flow diagram describes the procedure:

\[ \text{GECs} \downarrow \]
\[ \text{12-well Plates} \downarrow \]
\[ \pm \text{p38 MAPK Inhibitor} \downarrow \]
\[ \text{A.a. Extract or rhlL-1beta or Media} \downarrow \]
\[ 18h / 37°C, 5\%CO_2 \]
\[ \text{Supernatants/Lysates} \downarrow \]
\[ \text{IL-8 ELISA} \]

2.5. LPS stimulation and neutralization of LPS

LPS from *Escherichia coli* 0111:B4 was purchased from Sigma Chemical, St.Louis, MO. It was prepared by phenolic extraction and gel filtration chromatography. LPS was diluted in the culture medium at various concentrations. Cells were grown until confluent (approximately 4 x10^4 cells/well) in 96-well plates. Test agents (A.a. sonicates and purified *E. coli* LPS) were added to replicate wells, in the presence or absence of Polymyxin B (purchased from Sigma Chemicals) and the cultures were incubated for 24h before collection of cell supernatants. For neutralization of LPS activity, the stimuli *E. coli* LPS and A.a. sonicates were incubated for 30 minutes with Polymyxin B before every experiment. The flow diagram describes the set-up:

\[ \text{A.a. Extract or E. coli LPS or Media} \downarrow \]
\[ \pm \text{Polymyxin B (100ug/ml)} \downarrow \]
\[ \text{Add to GECs} \downarrow \]
\[ 24h, 37°C, 5\%CO_2 \]
2.6. Measurement of IL-8

Quantification of IL-8 was accomplished using a matched pair enzyme-linked immunoassay (ELISA) (Endogen Inc. Cambridge MA). Measurement of cytokine expression by gingival keratinocytes was conducted on supernatants taken from cultures subjected to various conditions of stimulation and duration of culture. Briefly, 96 well assay plates were treated for 12 hours with 2 ug/ml mouse anti-human IL-8 monoclonal coating antibody (Endogen Inc. Cambridge MA). Then, blocking buffer was added for one hour, followed by 1 hour incubation with 25 ul aliquots of gingival keratinocyte culture supernatant. Assay plates were then incubated with biotinylated mouse anti-human IL-8 monoclonal detecting antibody at 0.1g/ml and linked to horseradish peroxidase-conjugated streptavidin (Zymed Laboratories Inc. San Francisco CA). Assay plates were then incubated with tetramethylbenzidine (TMB) substrate (Dako Inc., Carpenteria CA). The developing reaction was stopped with the addition of 4N sulfuric acid. Absorbance was determined at 450 nm, using a BIORAD 2550 microplate reader and the concentrations of IL-8 were calculated with a software package (Microsoft Excell). Levels were determined from the means of duplicate determinations for each supernatant sample and compared with a standard curve for human recombinant IL-8.

The assay routinely detected IL-8 levels from 24 pg/ml to 6 ng/ml.
2.7. Statistical analysis and display of data

Graphic displays of data were created using Microsoft Excell software. Statistical analyses were performed using JMP software. Cytokine levels were expressed as the mean±standard deviation of results from a representative from 3 identical experiments. The data were compared using paired students t-test with significance considered to be p<0.05.
3. RESULTS

3.1. Dose-response study of IL-8 expression following A. a. challenge

We wanted to determine whether A.a. induces a dose-dependent increase in IL-8 expression from GECs. The following concentrations of bacterial sonicated extracts, diluted in KSFM medium were added: 0, 0.05, 0.5, 5, 50, 500 (ug/ml). The cells were incubated for 24 hours and the supernatants were collected, centrifuged and assayed for IL-8 using matched-pair antibody ELISA. The results of ELISA assay for IL-8 concentration in the cell supernatants suggested a dose-dependent increase in IL-8 expression (Fig. 2.1) in the range of stimulation from 0.05-50 ug/ml A.a. protein. The IL-8 expression induced by 0.05 ug/ml A.a. (1610±296 pg/ml) was not statistically different compared to the basal IL-8 expression (1324±179 pg/ml). However, stimulation with 0.5ug/ml A.a resulted in a 2-fold increase in the IL-8 expression. Similarly, stimulation with 5 and 50 ug/ml A.a. protein resulted in 3- and 7-fold increase (Fig. 2.2). As shown in Fig. 2.1, the maximal IL-8 expression was 9076±2006 pg/ml. Concentrations higher than 50 ug/ml appeared to be cytotoxic, resulting in changes in the morphological characteristics of the PP cell (e.g. rounding). This might explain the observed reduction in the IL-8 expression when the cells were stimulated with concentration 500 ug/ml A.a.. The data points in Fig. 2.1 are mean values of triplicate assay determinations from one experiment representative of 4 replicate experiments with similar results.
Figure 2.1 Dose-response study for A.a.-induced IL-8 production by GECs
The cells were stimulated with increasing concentrations of A.a. sonicated extracts for 24 hours. The IL-8 expression was dose-dependent in the range of 0.05 to 50 ug/ml. Stimulation with 500 ug/ml resulted in decreased IL-8 expression.

Figure 2.2 Stimulation Index (S.I.) for A.a.-induced IL-8 production by GECs
The stimulation index represents the ratio “stimulated/unstimulated” IL-8 expression. Maximum stimulation index was observed when the cells were stimulated with 50 ug/ml A.a. sonicates.
3.2. Time-response study of IL-8 expression following A.a. challenge

For the kinetic study of IL-8 expression, gingival keratinocytes were stimulated with 50μg/ml sonicated extracts of A.a. This concentration was found to induce the maximal IL-8 expression in previous dose-response studies (see section 3.1). Measurements of IL-8 expression in the supernatants of unstimulated or stimulated gingival keratinocytes were conducted at the following time intervals after the beginning of the bacterial challenge: 2, 4, 8, 12, 24 and 48 hours.

As shown in Fig. 2.3, A.a. stimulation induced IL-8 expression early as 2 h after incubation, which increased to a peak concentration of 9927±241 pg/ml, at the end of the 48-hour experimental period, while the unstimulated cells at the same time, exhibited lower IL-8 levels (1839±246 pg/ml). However, it should be noted that during the first 12 hours post-stimulation the levels of IL-8 expression remained under 1200 pg/ml, a value that represents a 2 to 3-fold increase in IL-8 production compared to the basal levels (Fig. 2.4). Between 12 and 24 hours post-stimulation the IL-8 expression rapidly increased, reaching a value of 9127±555 pg/ml, which represents a 7-fold increase compared to the unstimulated levels (see Fig. 2.3). The data points in Fig. 2.3 are mean values of triplicate assay determinations. This experiment is representative of 4 replicate experiments with similar results.
Figure 2.3 Time-response study of A.a.-induced IL-8 expression by GECs
GECs were stimulated with 50 μg/ml A.a. sonicated extracts at time 0 and the IL-8 expression at several time periods was compared to the unstimulated controls. The data are expressed as the mean ± S.D. of a representative experiment out of four identical experiments. (**: p < 0.05 compared with IL-8 expressed by controls).

Figure 2.4 Stimulation index (S.I.) of time-response study
The stimulation index represents the fold increase of stimulated IL-8 expression compared to the unstimulated IL-8 levels for the same time period. The highest S.I. was observed at the 24-hour supernatants.
3.3. Suppression of IL-8 expression by the specific p38 MAP kinase inhibitor SB203580

As others and we have previously demonstrated, A.a. infection is able to upregulate the basal gingival epithelial cell IL-8 production [5]. It has also been reported that MAP kinases regulate upstream signaling events that control IL-8 transcription in other epithelial cell systems [22]. We asked, therefore, whether MAP kinase activation might be involved in A.a.-induced IL-8 production by gingival epithelial cells. In order to evaluate the effect of p38 MAP kinase on the induction of IL-8 expression, the effect of SB203580, a specific p38 MAP kinase inhibitor, was examined. As shown in Fig. 2.5, gingival epithelial cell cultures were stimulated with A.a. sonicates (20 ug/ml) or rhIL-1beta (5 ng/ml), and concentrations of IL-8 in the cell culture supernatant were measured.

After 18 h of stimulation by A.a., the extents of IL-8 augmentation compared to the basal levels were 5.5-fold. Similarly, after 18 h of stimulation by IL-1beta (5 ng/ml), IL-8 production was augmented by 3.5-fold (Figure 2.5). When the cells were treated with various concentrations of SB203580, the extents of induction of IL-8 by A.a. were significantly reduced in a dose-dependent manner. For example, at 10 uM SB203580, the A.a.-stimulated IL-8 production was inhibited by 62% (P<0.01). At 30uM SB203580, the A.a.-induced IL-8 expression was inhibited by 76%. In Figure 2.6 it can be seen that the relation between SB203580 concentration and the percentage of IL-8 inhibition was almost linear in the 0.1 to 30 uM range.
Figure 2.5 Effect of p38 MAPK inhibitor on IL-8 expression by GECs

GECs were stimulated with A.a. (20 μg/ml) or IL-1beta (5 ng/ml), with or without addition of p38 MAP kinase inhibitor. IL-8 levels were measured in medium harvested after 18 h. Data are expressed as the mean ± SD. It can be noted that the p38 MAP kinase inhibitor reduced IL-8 expression by A.a. stimulated cells in a dose-dependent manner. (**: p ≤ 0.05 compared with IL-8 concentrations in cells cultured without inhibitor).

Interestingly, down-regulation of cellular p38 MAP kinase by pretreatment of cells with SB20358 had no inhibitory effect on IL-1beta-induced IL-8 production (Fig. 2.5). The IL-8 level induced by IL-1 beta, without inhibitor, was 3.5-fold the basal (unstimulated) expression. In the presence of 30 μM inhibitor, the level of IL-1beta-induced IL-8 was
3.8-fold the basal level, which was slightly higher, but the difference was not statistically significant.

P38 MAP kinase inhibitor SB 203580 suppressed the levels of IL-8 that were induced by *A. a.*, but not by IL-1beta. These results indicate that multiple signaling pathways are involved in regulating gingival epithelial cell IL-8 production, and that p38 MAP kinase may play different roles in regulating cell function in response to distinct stimulation. Thus, the effect of p38 MAP kinase on IL-8 production appeared to be specific for *A. a.* stimulation only.

![Figure 2.6 Percent inhibition of *A. a.*-induced IL-8 expression due to p38 MAPK inhibitor](image)
3.4. *E. coli* LPS does not induce IL-8 expression in GECs

Purified LPS from *E. coli* is a potent activator of cytokine responses in monocyte models and has been used extensively in the literature as a prototype LPS for cell stimulation [49]. To examine the cellular effects of LPS in our model, gingival epithelial cells were exposed to purified *E. coli* LPS.

A dose-response study was performed with various concentrations of LPS ranging from 0 to 100 µg/ml. The cells were stimulated for 24 hours with the following concentrations of *E. coli* LPS: 0, 0.001, 0.01, 0.1, 1, 10, 100 µg/ml. Then, the supernatants were harvested and assayed for IL-8 using ELISA. In Figure 2.7 we can see that none of the progressively increasing concentrations of LPS resulted in increase of IL-8 expression. The data presented are means from one representative experiment out of three with similar results. There were small, non-significant differences between the different concentrations and overall the samples were in the basal levels (300-500 pg/ml IL-8). Peripheral blood mononuclear cells (PBMCs) were used as controls. *E. coli* LPS with concentration 10 µg/ml were able to stimulate a high IL-8 response as seen in Figure 2.10. There was a 10-fold increase compared to the basal IL-8 expression by PBMCs. That increase was consistent with previous publications [50]. These results show that gingival epithelial cells are unresponsive to purified *E. coli* LPS compared with classical LPS-responsive cells like PBMCs.
Figure 2.7 Dose-response study of IL-8 expression by GECs challenged with *E. coli* LPS

Purified LPS from *E. coli* did not result in significant increase of IL-8 above basal levels. The data are from one experiment out of three experiments with similar results (N.S.: non-significant increase compared with basal IL-8 concentration).

3.5. *A.a.*-induced upregulation of IL-8 expression in GECs is LPS-independent

In order to investigate to what extent the *A.a.* LPS contributed to the *A.a*-induced IL-8 expression, we used Polymyxin B (LPS inhibitor). The concentration of 100 ug/ml was selected after dose-response studies (see Fig. 2.8). It can be seen that higher concentrations of Polymyxin B (500ug/ml and 2000 ug/ml) reduce the IL-8 expression of unstimulated GECs. This implies that they are toxic for the cells. Furthermore, 100 ug/ml of Polymyxin B was considered adequate for LPS blockade, as shown in the PBMC model (Figure 2.10.)
Polymyxin B (100µg/ml) was mixed with *E. coli* LPS or *A.a.* sonicates in order to inactivate LPS. Then the above stimulants were added to GECs. The results of the LPS neutralization are shown in Figure 2.9, where it can be observed that *E. coli* LPS failed to upregulate the basal IL-8 expression of GECs, as we have previously seen in the dose-response study in Fig. 2.7. It can also be seen that *A.a.* extracts induced significant upregulation of IL-8 expression, which is consistent with previous studies. Interestingly, addition of polymyxin B in the *A.a.* extracts caused no reduction in the *A.a.*-induced IL-8 expression. These results indicate that the *A.a.*-induced up-regulation of IL-8 was not due to the LPS part of the extracts.

![Figure 2.8](image_url)

**Figure 2.8 Effect of Polymyxin B on the *A.a.*-induced IL-8 expression in GECs**

IL-8 expression by GECs challenged with *A.a.* sonicates, was not affected with the addition of Polymyxin B 50-100 µg/ml. Concentrations 500 µg/ml and higher were cytotoxic, as can be seen from the decrease in IL-8 expression by the controls. The data are from one experiment out of three experiments with similar results (**: p ≤ 0.05 compared with IL-8 concentrations in cells cultured in medium without Polymyxin B).
Peripheral Blood Mononuclear Cell (PBMC) stimulation was performed in parallel to ensure that the endotoxic activity of *A.a.* LPS had been inactivated. We measured IL-8 production by PBMC after 24 hours of incubation with commercially prepared LPS from *E. coli* and with *A.a.* sonicates (Fig. 2.10). In each case, high levels of IL-8 were measured. IL-8 was abolished by co-incubation with Polymyxin B. The basal IL-8 expression by PBMCs was 100±26 ng/ml. *E. coli* LPS caused a 10-fold increase up to 983±211 ng/ml. Polymyxin B resulted to a 91.6% reduction, down to 174±5 ng/ml. Addition of *A.a.* sonicates resulted to a 12-fold increase up to 1208±98 ng/ml, which was reduced almost 98% by treatment with Polymyxin B (down to 124±11 ng/ml).
Peripheral Blood Mononuclear Cell (PBMC) assay was performed to ensure that the endotoxic activity of LPS had been inactivated by Polymyxin B. Purified LPS from *E. coli* and *A. a. E.* resulted in significant increase of IL-8 above basal levels. Addition of 100 ug/ml Polymyxin B completely inhibited the expression of IL-8, suggesting that the IL-8 expression is due to the bacterial LPS. The data are from one experiment out of three experiments with similar results (** : p ≤ 0.05 compared with IL-8 concentrations in cells cultured in medium without Polymyxin B).
4. DISCUSSION

Characteristics of IL-8 expression induced by *A. a.*

Epithelial cells form a barrier that mechanically prevents bacteria from invading tissues (innate immunity). In addition they can provide signals that are essential for the initiation and amplification of inflammatory responses. Among these signals, production of proinflammatory cytokines is an event that participates to the cascade that leads to the clinical and histological presentation of inflammation. The potent neutrophil chemoattractant Interleukin-8 is one of these cytokines. IL-8 has been detected in the gingival crevicular fluid (GCF) of adult periodontitis patients [10]. IL-8 mRNA has also been identified in healthy and diseased periodontal tissues [12, 13]. Tonetti et al examined gingival biopsies and reported IL-8 expression in the junctional epithelium, adjacent to the infecting microorganisms. They suggested that the observed expression of IL-8 in the sulcular epithelium may be an efficient way for establishment of a cell-type-selective chemotactic gradient within the tissue, that is able to effectively direct polymorphonuclear phagocyte migration toward the infecting bacteria [13].

A large body of information regarding cytokines, including IL-8, in response to periodontal bacteria has been established from experiments with cultures of human gingival fibroblasts (HGF) and human peripheral blood monocytes (PBMCs) [50, 51]. However, little is known regarding the cytokine profiles of gingival epithelial cells and especially the cytokine levels following interaction with periopathogens. Periodontal pathogens are routinely found in pockets of periodontally involved teeth. Among them, *Actinobacillus actinomycetemcomitans* (*A.a.*), is a major contributor in the inflammatory response [1, 2, 52], because of its ability to elicit pro-inflammatory cytokine release by
host cells like keratinocytes, fibroblasts, periodontal ligament (PDL) cells, PMN’s, monocytes and lymphocytes [53, 54]. A.a. has been implicated in the pathogenesis of periodontal conditions like Early Onset Periodontitis (EOP) and Adult Periodontitis (AP) [1, 2, 55]. In this chapter, we analyzed the gingival epithelial cell IL-8 response elicited by sonicated extracts of A. a. For our experiments we used an HPV-16-immortalized cell line. This cell line was preferred over primary gingival epithelial cells for the following reasons:

1. Isolation of primary epithelial cells from biopsies is difficult, since fibroblast contamination can occur. Some authors recommend a reduction in Ca++ concentration in the culture medium, in order to reduce fibroblast growth.

2. There is significant variation in the cytokine profile of primary cells depending of the inflammation status and genetic predisposition of the donor [56].

3. Primary cells cannot be maintained for more than 7 passages, whereas immortalized cells lines can be maintained for long periods [45]. Therefore, primary cells should be derived from the same individual, so that the results could be comparable. This would present technical and ethical problems.

From the above, it is concluded that the use of an epithelial cell line is a convenient and safe way to obtain reproducible results, provided that the experiments are performed with cells from the same passage.

We, then, demonstrated that gingival epithelial cells have a constitutive IL-8 expression that ranged between 1,100 to 1300 pg/ml for 24h of bacterial stimulation. Several studies have shown that unstimulated oral keratinocytes have detectable basal IL-8 expression, which seems to be important for the tissue homeostasis [14]. Huang et al
have also shown that several oral epithelial cell lines have basal IL-8 production that ranges from 645pg/ml to 1,749pg/ml for a 24h time period [5]. The reason of these differences is not currently known. According to McGee presence of IL-8 in non-inflamed sulci may protect the gingiva from the bacterial challenge, by maintaining PMN activity [57].

In our study, we demonstrated that A. a. is a potent stimulator of IL-8 production from gingival epithelial cells and that this ability was dose-related. We used the strain ATCC 33384, which has been shown to induce optimal IL-8 expression in gingival fibroblast cultures [51]. Stimulation with doses from 0.5ug/ml to 50ug/ml resulted in increase of IL-8. As the concentration of A.a. protein increased above 50ug/ml, IL-8 induction was reduced. These results are in agreement with previous observations of a dose-dependant induction of IL-8 with A.a. [5]. Huang et al showed that the highest IL-8 expression occurred at a multiplicity of infection (MOI) of 1000:1. Beyond that bacterial level IL-8 was reduced due to decreased viability of epithelial cells. However, when they used non-viable bacteria they observed considerable IL-8 induction at MOIs up to 5,000:1. Others have reported that high levels of IL-8 secretion are induced by even smaller numbers of periopathogenic bacteria. Yumoto et al have suggested that the optimal level of secretion of IL-8 occurred at a ratio of approximately 100 to 1,000 bacteria (E. corrodens) per oral epithelial cell (KB line) [17].

We reported that the highest IL-8 induction was 7-fold compared to the control levels, at the 24 h timepoint of continuous bacterial stimulation. Huang et al found a 7-fold increase in the IL-8 expressed by their immortalized cell line HOK-16 in response to A.a. challenge at the 24 h timepoint [5]. Other authors, using different bacteria as
stimulants have reported several different increases in IL-8 production. Stimulation of KB cells with oral viridans streptococci has caused a 5-fold increase after 20h [16]. Stimulation of KB cells with *E. corrodens* resulted to a 68-fold increase after 26.5h [17]. It is important to mention that the only bacterium that has been reported to down-regulate IL-8 expression by oral keratinocytes is *P. gingivalis* [18]. Based on the previous findings we suggest that the cytokine response is related to the bacterial stimulant and can vary significantly for the different bacteria.

For our time-course studies we stimulated the cells with 50ug/ml of bacterial sonicates, concentration that elicited the highest IL-8 expression in the dose-response studies. We report significant upregulation of IL-8 expression as early as 2h after continuous exposure to *A.a.* and maximum production of IL-8 between 12 and 24h. After that timepoint the detectable IL-8 reached a plateau. Kinetic studies by other authors have shown a quick IL-8 response of epithelial cells to bacterial stimulus. Huang reported a quick IL-8 increase 6h after *A.a.* challenge that decreased to basal levels 12h after the stimulus [5]. Yumoto reported significant increases of IL-8-specific mRNAs within 60 to 90 min and significant increases of IL-8 secreted protein within 4 h after the exposure of oral epithelial cells to *E. corrodens* [17]. Similar results have been found regarding interaction between bacteria and intestinal epithelial cells. Aihara et al. reported that MKN45 cells started to express IL-8 mRNA rapidly (within 1 h) after the start of coculture with *H. pylori* [58].

Rapid IL-8 expression could benefit the periodontium by recruiting PMNs to the site of the bacterial invasion. It is known that PMNs produce high mounts of IL-8 themselves that can augment the chemotactic response. Thus, the IL-8 upregulation by
the gingival epithelial cells is no longer necessary and the gene expression can be turned off. We have also postulated the existence of an autocrine regulatory mechanism via IL-8 receptors CXCR-1 and CXCR-2.

The bacterial challenge that we used consisted of sonicated extracts from A.a. instead of vital bacteria. Our purpose was to examine the pathogenicity of non-vital bacteria. It is well documented that adhering and invading epithelial cells is a major mechanism of disease induced by A.a.. However, our data show that invasion or vitality of A.a. is not necessary for epithelial upregulation of IL-8. This is in agreement with recent publications by Huang et al who showed IL-8 expression induced by dead A.a., killed by boiling or gentamicin [5]. Sonicates of A.a. have been used in the literature for cytokine induction in gingival fibroblasts [42]. The sonicated extracts have the advantage that the bacterial molecules are maintained relatively unchanged whereas boiling, for example, can denature proteinous components.

In conclusion, we have demonstrated that human epithelial cells may contribute significant levels of IL-8 in the gingival milieu in response to A.a. challenge. IL-8 derived from gingival cells, including gingival epithelial cells can cause neutrophils to accumulate and to be activated in the site of inflammation. Thus, the capacity of the gingival epithelial cells to produce IL-8 in response to A.a. suggests that they play a critical role in the maintenance of the tissue microenvironment. Neutrophil mediated tissue injury is important in the pathogenesis of periodontal diseases.

**MAP Kinase and IL-8 expression**

At present, little is known about the mechanisms by which A.a. induces expression of pro-inflammatory cytokines in the cellular level. Understanding those
mechanisms is of great interest, as it may lead to the development of novel therapeutic approaches for the treatment of periodontitis. These approaches can focus on modification of the cytokine expression of the host in addition to the traditional scaling and root planing and periodontal surgery. The last decade it has been shown that the degree of cytokine response on the bacterial challenge can affect periodontal health. The Periodontitis Susceptibility Test (PST) has been developed by Kornman for clinical use [59]. That test determines, after analyzing the patient’s DNA, if the individual belongs to the 30% of the population with a genetic polymorphism of the IL-1 gene. This polymorphism results in IL-1 overexpression (2- to 4-fold increase) in response to bacterial challenge in the sulcular area. People that test PST (+) are in higher risk to develop periodontitis [59].

In this chapter we hypothesized that IL-8 expression induced by A.a. is mediated by MAP Kinase in the GEC model. Generally, MAP kinases regulate cell proliferation, differentiation, and programmed death, in addition to stress and inflammatory responses. Hence, activation of gingival epithelial cell MAP kinases by A.a. may be instrumental in inducing periodontal inflammation, attachment loss and alveolar bone loss.

MAP kinase signaling also regulates the expression of several proinflammatory cytokines, in other cells [60]. P38 MAPK has been implicated in the pathogenesis of gastrointestinal inflammation due to bacteria like H. pylori and S. typhimurium [19, 46]. It is also known that p38 MAP kinase mediates IL-8 expression by bronchial epithelial cells [22], under certain conditions.

The inhibitor of p38 MAP kinase provides an effective tool for investigating the role of p38 MAP kinase in cellular signaling [27, 48]. SB 203580 inhibits the catalytic
activity of p38 MAP kinase by binding to the ATP site and subsequently phosphorylating its substrate [61]. SB 203580 inhibited p38 MAP kinase activity and significantly reduced IL-8 expression in A.a.-stimulated cells, in a dose-dependant way, whereas it did not affect IL-8 production in unstimulated cells. It is worth noting that, down-regulation of cellular p38 MAP kinase by pretreatment of cells with SB20358 had no inhibitory effect on IL-1beta-induced IL-8 expression (Fig.2.5). That implies that there is an intracellular pathway different of p38 MAP kinase that regulates the expression of IL-8 following exposure to IL-1beta (Fig. 2.11). In agreement with our data, Ridley et al found that IL-8 production by human gingival and dermal fibroblasts in response to IL-1 was independent of p38 MAP kinase activation [62]. These results demonstrate that the activation of cellular p38 MAP kinase is indispensable for the A.a.-mediated cellular functions in gingival epithelial cells, and suggest that p38 MAP kinase may play a different role in response to distinct stimulation.

Several publications have proven the effectiveness of SB 203580 on blocking p38 MAP kinase activity in inflammatory diseases [63]. Although it is currently not known whether SB 203580 is capable of producing beneficial effects on periodontal tissues, inhibiting signal cascade mediated by p38 MAP kinase, may apply in the future for therapy of cytokine related oral conditions. Further laboratory and clinical investigations are needed to clarify this point.

We did not examine whether other MAP kinase pathways are also involved in the IL-8 gene expression. We believe that probably this is the case because blockade of p38 MAP kinase did not result in 100% inhibition of IL-8 expression. That means that there might be parallel pathways for IL-8 regulation, which is consistent with the data that exist
for other epithelial cell systems, like intestinal epithelial cells [19]. More studies are needed in order to elucidate these upstream events and define the specific bacterial and host factors that interact to activate the gingival epithelial cell signaling pathways.

Figure 2.11 P 38 MAPK regulates signal transduction pathway for IL-8 expression
A.a stimulates the expression of IL-8 gene with final result the excretion of IL-8 in the extracellular environment. P38 MAP kinase mediates the events that will lead to DNA transcription.
Role of LPS in A.a.-induced IL-8 expression in GECs

Lipopolysaccharide (LPS) is a basic component of the Gram(-) bacterial outer membrane and is therefore constantly presented to the oral mucosa surfaces. How do tissues react to this constant exposure? We hypothesized that the A.a.-induced upregulation of IL-8 by GECs was due to the LPS content of this bacterium. We demonstrated that gingival epithelial cells released IL-8 after challenge with A.a. sonicates which was both dose- and time-dependent. The effect of A.a. sonicates was not inhibited by Polymyxin B neutralization of LPS. This suggests that LPS was not the causative factor for the epithelial stimulation (see Fig. 2.12). We also demonstrated that gingival epithelial cells were unresponsive to commercially purified E. coli LPS. Similar findings have been reported with other epithelial cell lines, like bronchial and uroepithelial cells [31, 39].

When peripheral blood mononuclear cells (PBMCs) were exposed to A.a. sonicates and E. coli LPS, high levels of IL-8 were detected in the cell supernatant. In contrast with the gingival epithelial cell line, this effect was inhibited by Polymyxin B. It was therefore demonstrated that LPS derived from A.a. and E. coli was the major factor that upregulated IL-8 expression in PBMCs. The PBMC model, except the information about LPS stimulation, validated the effectiveness of LPS blockade by Polymyxin B. Although several investigators have exposed PBMCs to A.a., there are only two publications that have examined the IL-8 response to A.a. LPS:

- Agarwal et al were the first to show IL-8 increase after exposure of monocytes to A.a. LPS [54].
• Yamaguchi et al, one year later, demonstrated increase of IL-8 mRNA in response to A.a. LPS [33].

Finally, in another paper Jiang et al (1996) used whole A.a. bacteria instead of LPS for stimulation of monocytes [50]. They demonstrated a 6-fold increase of IL-8 excretion. However, they did not prove that this increase was due to the LPS component of A.a.. Therefore, only in the first of these publications (Agarwal et al., 1995) has been shown direct cause-effect relation between A.a. LPS and IL-8 protein production. Our results provide additional evidence that A.a. LPS are responsible for the IL-8 upregulation by human PBMCs.

In studies of cytokine inductive properties of stimulatory agents, it is important to control for unstimulated production of cytokines and lack of periodontal inflammation [8]. Therefore, the PBMC donor, in our study, was a systemically and periodontally healthy young adult. Periodontal health was defined as lack of pockets with probing depth more than 3mm and no bleeding on probing (BOP) in any site. There are several lines of evidence showing that PBMCs from periodontally diseased individuals exhibit altered reactivity to plaque bacteria and this is exactly what we wanted to avoid. A study of PBMCs from periodontal patients demonstrated that unstimulated cells and cells stimulated with LPS derived from A.a. produced more IL-1beta than did similarly stimulated cells from healthy controls [64]. Others have shown that upon stimulation with LPS, monocytes from periodontitis patients release more IL-1 beta and PGE2 than monocytes from gingivitis subjects [65].

Our study suggests that epithelial cells differ from cells in the systemic compartment, in that they are extremely poor responders to direct stimulation by LPS,
and that the pathogens overcome this unresponsiveness and trigger similar but LPS-independent cytokine responses. These results also emphasize the difference in LPS response strategy between epithelial cells in the oral lining and the macrophages in the systemic compartment. A number of candidate cellular components and exoproducts from *A.a.* and other periopathogenic bacteria have been examined for their ability to elicit cytokine expression. These include the following: polysaccharides, surface-associated material (SAM), lipid A-associated proteins (LAP), porins, fibriae and cell wall polymers (CWP). A more detailed review has been presented in the introduction section and the findings have been summarized in table 2.1. We do not know the reason of the epithelial unresponsiveness to LPS. Future studies can examine whether normal gingival epithelial cells lack receptors CD14, which could explain this phenomenon. This could be done with immunohistochemical staining of gingival biopsies or with ELISA using homogenates from gingival epithelium.

Although our studies show that gingival epithelial cells, *in vitro*, respond to *A.a.* sonicates in an LPS-independent manner, they do not imply that LPS is inactive in the mucosal compartment or that Gram-negative bacteria elicit totally LPS-independent inflammatory responses at these sites. We do not know if human epithelial cells, *in vivo*, respond similarly in LPS stimulation. If they do, then we can assume that LPS is likely to interact with non-epithelial cells in the connective tissue compartment of the periodontium, since the stimulatory ability of LPS has been already demonstrated. Once LPS reaches CD14-positive or other responsive cells in the subepithelial compartment, a release of pro-inflammatory mediators may be induced with subsequent inflammatory response with clinical and histological signs.
Figure 2.12  A.a.-induced IL-8 expression in GECs is LPS-independent
REFERENCES


CHAPTER 3

Characterization of *A. actinomycetemcomitans*-induced interleukin-1 expression by human gingival epithelial cells. Implications in interleukin-8 expression

ABSTRACT

Gingival epithelial cells are the first cells of the host that encounter the bacterial challenge and therefore their role in the initiation of the immune response is critical. We have already shown that *Actinobacillus actinomycetemcomitans* (*A.a*) can induce IL-8 in human gingival epithelial cells (GEC). In this chapter, we hypothesized that *A.a* is also able to induce IL-1 expression in GECs in vitro. IL-1 is a major cytokine produced in inflamed periodontal tissues. Furthermore, IL-1 has been implicated in the regulation of bacterial-induced IL-8 in endothelial and respiratory epithelial cells. We also hypothesized that exogenous IL-1, when added to a GEC culture can upregulate IL-8 expression. We finally hypothesized that the IL-8 expression that is induced by *A.a* is mediated by endogenous IL-1 in an autocrine fashion.

Sonicated extracts of *A.a* were added to GEC cultures, supernatants were collected at varying time intervals and analyzed for IL-1 expression with ELISA. Similarly rhIL-1alpha and rhIL-1beta were added to the GEC cultures and the supernatants were analyzed for IL-8 expression. Finally, inhibitors of IL-1 were added to the cultures and then the cells were exposed to *A.a* or rhIL-1alpha and/or rhIL-1beta and the IL-8 expression was determined.

Addition of *A.a* extracts caused a significant increase for both IL-1alpha and IL-1beta. Increase of IL-1alpha expression was induced as early as 2h post-stimulus. Within
12h, the IL-1alpha expression corresponded to 6-fold increase compared to the unstimulated levels. IL-1beta, that was initially below detectable levels, increased significantly after 12h of stimulation with A.a. Overall, IL-1alpha levels induced by A.a. were 5 to 20 times higher than IL-1beta, depending the time of sampling. After comparing the time-response curves of the two cytokines, we described an “early stimulatory effect” regarding the induction of IL-1alpha by GEC. When the cells were challenged with either IL-1alpha or IL-1beta, the dose with the highest IL-8 stimulation was 10ng/ml for both of these cytokines. Significant upregulation of IL-8 expression was detected as early as 2h after exposure to IL-1, reached a maximum after 24 hours from the beginning of the experiment and then declined. Incubation of GECs with IL-1 inhibitors blocked the production of IL-8 induced by rhIL-1. This validated the effectiveness of our inhibitors. However, IL-1 inhibitors failed to reduce the A.a.-induced IL-8 expression by GECs after 12 and 18 hours of stimulation.

In conclusion, A.a. is capable of amplifying the local immune response and promoting periodontal tissue inflammation by stimulating GEC to secrete IL-1alpha and IL-1beta. We showed that gingival epithelial cells are capable of upregulating IL-8 expression in response to exogenous IL-1 challenge and thus may facilitate the recruitment of leukocytes. However, there was no evidence that the A.a.-induced expression of IL-8, is mediated by endogenous IL-1 in an autocrine fashion. Rather, it appears that A.a. stimulates epithelium either by a direct signaling mechanism or by induction of a different host-derived proinflammatory cytokine. A third possibility is also discussed, this one of an intracrine IL-1-dependent pathway. Further experiments are needed in order to elucidate these intracellular functions.
The involvement of IL-1 in the intercellular signaling can have clinical implications. Recent data have associated periodontitis-prone individuals with IL-1 hyper-secretory genotypes. Therefore, inhibitors of IL-1 may be useful in suppressing inflammation due to IL-1alpha and IL-1beta and reducing the detrimental effects that their hyper-secretion exerts to the periodontium.
1. INTRODUCTION

IL-1 expression in GECs

Interleukin-1 (IL-1) is a hormone-like cytokine that plays a central role in the regulation of immunologic and inflammatory reactions. There are two functionally almost equivalent forms of IL-1, IL-1alpha (17 kDa, 159 amino acids) and IL-1beta (17 kDa, 153 amino acids) that are encoded by two different genes. At the protein level IL-1alpha and IL-1beta display approximately 27 percent homology. On the other hand the three-dimensional structure of the two IL-1 forms is almost identical and both forms bind to the same receptor [1]. Primary producers in the periodontal lesion are monocytes/macrophages, dendritic cells, Langerhans cells, B lymphocytes, endothelial cells, neutrophils, fibroblasts and epithelial cells. Epidermal keratinocytes have been shown to have receptors for IL-1 and therefore are responsive to IL-1 treatment [2]. The main biological activity of IL-1 is the stimulation of T helper cells, which are induced to secrete IL-2, IL-4, IL-6, IFN-gamma and CSF [1]. IL-1 acts directly on B-cells, promoting their proliferation and the synthesis of immunoglobulins. IL-1 stimulates the proliferation and activation of NK-cells and fibroblasts. It also induces ICAM-1, E-selectin and VCAM-1 on endothelial cells, enabling PMNs, monocytes and lymphocytes to migrate into the tissues [3]. Biological effects of IL-1 that are directly related to periodontal attachment are activation of collagenase, bone resorption and induction of other mediators including PGE₂, PF₄ (platelet factor-4), CSF (colony stimulating factors), IL-6 and IL-8.

Interleukin-1 was the first cytokine shown to be produced by keratinocytes [4]. It is IL-1alpha that is responsible for the biological activity of IL-1 in skin keratinocytes as
assessed by a thymocyte proliferation assay [5]. Formanek et al examined the cytokine expression and corresponding receptor pattern of unstimulated human oral mucosa-derived keratinocytes [6]. IL-1alpha and IL-1alpha receptor antagonist, were detectable at the protein and mRNA level. Johnson et al reported that oral keratinocytes, in culture, express both IL-1alpha and IL-1beta [7]. The levels of IL-1 alpha from the lysed cells, however, were 20 times higher than the levels of IL-1beta, after 24 in culture (120 pg/10^4 cells versus 6pg/10^4 cells respectively). Only IL-1alpha could be detected in the culture supernatant after 24 hours (2-3pg /10^4 cells)[7]. Li et al, (1996) compared the cytokine profile of oral keratinocytes (OK) with that of skin keratinocytes (SK)[8]. Unstimulated OK and SK produced IL-1 alpha constitutively. The IL-1alpha produced by OK (43 pg/ml/10^6 cells/24h) was doubled after stimulation with TNF alpha. Overall there was no significant difference in the IL-1 alpha production between OK and SK.

Yamamoto reported that gingival keratinocytes, in vitro, excrete 30-40 pg/10^5 cells/ml IL-1beta over a 3-day period [9]. Keratinocytes obtained from inflamed gingiva excreted more IL-1beta compared to keratinocytes from non-inflamed sites and exposure to LPS (10ug/ml) increased further the IL-1 beta production.

Several studies have shown the presence of IL-1 in inflamed gingival tissues. Honig et al found IL-1beta present in all tissue extracts from adult periodontitis patients, whereas no IL-1 was found in healthy tissues [10]. Charon et al. demonstrated that IL-1 is present in a greater extent in gingival fluid (GCF) from inflamed sites [11]. Masada et al detected IL-1 alpha and IL-1 beta in the GCF of all 15 patients with untreated periodontitis that they examined [12]. Both forms of IL-1mRNA were detected in gingival tissue samples. Jandinski et al observed that IL-1 beta positive cells were
increased by almost 3-fold in periodontally diseased tissue when compared to normal tissue [13]. Stashenko reported that IL-1beta is the predominant form present in periodontal tissues [14]. Disease-active sites had higher IL-1 beta than inactive and healthy sites and IL-1beta levels were related to attachment loss. Matsuki et al reported that IL-1 activity in gingival crevicular fluid (GCF) from inflamed gingiva was higher than that from healthy gingiva and decreased after periodontal therapy [15]. They also found that IL-1 alpha was the predominant form of IL-1 in GCF. Similarly, Reinhardt et al showed that IL-1alpha was the predominant form of IL-1 in GCF, which was 5-fold higher than IL-1beta [16]. They also showed that presence of A.a. in a site caused a 3-fold increase in both IL-1alpha and IL-1beta in GCF.

The results of the above studies suggest that IL-1 is locally produced in the gingival tissue and that raises the question of the cellular origin of IL-1 in the periodontal microenvironment. Expression of IL-1 has been demonstrated with IHC both in gingival epithelium [17] and gingival connective tissue [15, 18]. When IL-1beta is expressed in inflamed gingiva it usually reflects the density of infiltrating macrophages [19].

We hypothesized that A.a. can induce both IL-1alpha and IL-1beta in human gingival epithelial cells in culture. We exposed GECs to sonicated extracts of A.a. for several time periods and then assayed the cell medium for IL-1alpha and IL-1beta using ELISA. Our results demonstrated that A.a. induced both IL-1alpha and IL-1beta in human GECs in culture. The production of those cytokines was time-dependent. IL-1alpha was expressed as early as 2h post-stimulus. This was described as an “early stimulatory effect” and may have implications in the secondary expression of IL-8. Our next objective in this chapter was to show that IL-1 can induce IL-8 in GECs, in vitro.
IL-1-induced IL-8 in GECs

It has been shown that stimulation with IL-1alpha and TNF-alpha can increase IL-8 production by primary human oral keratinocytes [20]. Stimulation for 24 h with 100-1,000 U/ml of IL-alpha also induced IL-8 production by oral keratinocytes [20]. Bickel et al. studied the influence of IL-1 alpha on chemokine expression of 2 human oral keratinocyte cell lines: SCC-25 (squamous cell Ca) and HEPM (human embryonatal palatal mucosa cells) [21]. IL-1 stimulation resulted in an increase of mRNAs encoding interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and GRO gamma. IL-8 mRNA was expressed as early as 1 hour after stimulation and reached a first peak after 4 hours.

We hypothesized that rh IL-1alpha and rh IL-1beta can induce IL-8 expression in GECs. We performed dose-response and time response studies by adding different concentrations of recombinant human IL-1alpha and IL-1beta in cultures of GECs and then assaying the supernatants for IL-8 using ELISA.

Our results indicate that oral epithelial cells are capable of upregulating IL-8 expression in response to exogenous IL-1 challenge. Since IL-1 is abundant in the periodontal environment during inflammation, this provides an indirect mechanism for IL-8 upregulation and thus may facilitate the recruitment of leukocytes as a host defense mechanism.

Effect of IL-1 inhibitors on bacterial-induced IL-8

We have shown that GECs secrete IL-1 in response to A.a. and that rh IL-1 can induce IL-8 in the same cell model. We then studied whether A.a.-induced IL-1 is involved in the regulation of IL-8 expression in an autocrine manner. IL-1-dependent
autocrine pathways for IL-8 regulation have been described in endothelial and respiratory epithelial cells. Kaplanski et al, found that both IL-8 and IL-6 production by R. conorii-infected human umbilical vein endothelial cells (HUVEC) was mediated, in a large part, by a cell-associated form of IL-1alpha [22]. Production of IL-8 was suppressed by 80% by the addition of IL-1RA and by 60% by anti-IL-1alpha antibodies. IL-1alpha has also been reported by Patel et al. to be an autocrine regulator of IL-8 production by a pulmonary epithelial cell line infected with respiratory syncytial virus (RSV) [23].

Incubation with neutralizing antibodies against IL-1alpha and IL-1beta showed that IL-1alpha was the predominant soluble mediator that enhanced the mRNA expression and synthesis of IL-8. Porat et al examined whether LPS-induced IL-8 production by human peripheral blood mononuclear cells (PBMCs) was due to endogenous IL-1 [24]. The addition of saturating concentrations of IL-1RA reduced the IL-1beta-, LPS-, and B. burgdorferi-induced IL-8 synthesis by 85, 50, and 40%, respectively, indicating that IL-1 is involved in the regulation of IL-8 in PBMCs in an autocrine manner.

Other authors have shown that IL-8 upregulation is independent of endogenous IL-1. Burns et al. found that activation of endothelium by B. burgdorferi, is not mediated through an autocrine action of secreted IL-1, since IL-1 RA had with no effect on the bacterial induced IL-8 [25]. They suggested that B. burgdorferi must stimulate endothelium either directly, or by induction of a novel host-derived proinflammatory cytokine.

Chen et al examined the effects of IL-1alpha, IL-1RA or anti-IL-1 neutralizing antibody upon expression of proinflammatory cytokines in oral squamous Ca (SCC) cell lines [26]. They found that IL-RA or anti-IL-1 neutralizing antibody could completely
inhibit the IL-1alpha-inducible increase in IL-8 expression. Unfortunately, nothing has been published regarding periodontal pathogens and their interactions with gingival epithelial cells. Since our previous studies have shown that A.a.-infected epithelial cells express both IL-1alpha and IL-1beta, we hypothesized that endogenous IL-1 induced by A.a. mediates the expression of IL-8 in GECs.

We used anti-IL-1 neutralizing antibodies and rhIL-1RA to neutralize the endogenous IL-1 expressed by GECs after A.a. infection. The effectiveness of those inhibitors was tested by examining whether they could reduce the IL-1-induced IL-8. First the IL-1 inhibitors were added to the cultures and 30 min later the A.a. stimulus was introduced. The culture medium was collected at various timepoints and assayed for IL-8.

Our results showed that blockade of secreted IL-1 did not result in significant change in the expression of IL-8 in A.a.-stimulated GECs. Although our findings do not support our original hypothesis for autocrine regulation, we cannot rule out the role of IL-1 in the regulation of IL-8. The possibility of an intracrine mechanism, that involves IL-1 expression is still to be tested for possible involvement in the expression of IL-8. The involvement of IL-1 in the intercellular signaling can have clinical implications. Recent data have associated periodontitis-prone individuals with IL-1 hyper-secretory genotypes [27]. Therefore, inhibitors of IL-1 may be useful in suppressing inflammation due to IL-1alpha and IL-1 beta and reducing the detrimental effects that their hyper-secretion exerts to the periodontium.
Figure 3.1 Hypothesized IL-1 autocrine regulation of IL-8 expression in GECs
We hypothesized that *A. a.* induces IL-8 expression in GECs via IL-1 mediation. In this chapter we test our hypothesis by utilizing IL-1 inhibitors for neutralization of endogenous IL-1 that is induced by *A.a.*
2. METHODS

2.1. Culture of GECs

For our experiments we used human gingival epithelial cells immortalized with the Human Papilloma Virus type 16, the E6/E7 gene [28]. This cell line-named PP- was kindly provided by Dr. Oda, Department of Oral Biology, University of Washington, Seattle. The cells were kept frozen in liquid N2. Before every experiment the cells were thawed, plated into tissue culture flask (Falcon, Franklin Lakes NJ) and fed every 48 hours. The medium used was Keratinocyte Serum-Free Medium (KSFM, Gibco). Cell cultures were maintained at 37°C and 5% CO₂. The cells were grown up to the required number (3-4 x 10⁶ cells) into 75 cm² tissue culture flasks (Costar). They were trypsinized using 0.025% bovine trypsin in 0.01% EDTA (Clonetics, Walkersville, MD). The cells were then plated into 12-well tissue culture plates (Costar, Cambridge, MA), 2 x 10⁵ cells, in 1 ml medium per well, and were incubated for 4-6 hours in order to adhere and reach confluence. There were triplicate wells for each condition. For antibody studies triplicate cultures of GECs were plated at 96-well plates at a density of 4x10⁴ cells per well. The cells were left to adhere for 4-6 hours. Then, 100ul of KSF medium that contained the antibodies was added to the wells and 30 minutes later the stimulus was introduced (bacterial extract or recombinant cytokine or plain medium) to a final volume of 200ul. After incubation for various time periods, the supernatants were aspirated, centrifuged, and stored at -70°C until they were assayed for cytokine.

2.2. Bacterial challenge

Actinobacillus actinomycetemcomitans strains (ATCC 33384), were kindly provided by Dr. J. Slots, University of Southern California School of Dentistry, Los
Angeles CA, USA. The bacteria were grown for two 3-4 day periods and subcultured after each growth period to ensure strain purity. Cells were harvested by washing and suspension in 3 ml sterile phosphate buffered saline (PBS), followed by sonication to extract soluble bacterial components. Briefly, cells suspended in PBS were subjected to 10 repetitions of sonication at 5-minute intervals at a 10% duty cycle. The cell free supernatant was then collected by centrifugation at 8000 rpm for 30 minutes at 4°C and dialyzed with sterile PBS at 4°C. Bacterial protein concentration was determined by Bradford protein assay (Bio-Rad, Richmond CA). The extracts were aliquoted and stored at −70°C. Sonicated extracts were used as bacterial challenge in various concentrations in the culture medium.

2.2.1. A.a.-induced IL-1 expression in GECs

In order to demonstrate that A.a.E. induces IL-1 in GECs the cells were stimulated with 50 μg/ml A.a.E. and the supernatants and lysates were assayed at various time-points for IL-1alpha and IL-1beta (ELISA). The flow diagram below describes the procedure:

```
GECs (PP cells)  ↓
96-well Culture Plates  ↓
± A.a. Extracts  ↓ 0-48h / 37°C, 5%CO₂
Supernatant/Lysates  ↓
IL-1alpha / IL-1beta ELISA
```

2.3. Antibodies and proteins

Recombinant human Interleukin-1alpha (rhIL-1alpha) and Interleukin-1beta (rhIL-1beta) was supplied by Endogen (ENDOGEN Inc. Cambridge MA). Goat polyclonal neutralizing antibodies against rhIL-1alpha and rhIL-1beta were purchased
from R&D Systems (Minneapolis, Minn.). Recombinant human IL-1 receptor antagonist (rhIL-1RA) was obtained from R&D Systems, as well. Purified goat non-immune IgG (N IgG), obtained from Sigma (Sigma Chemical Co., St. Louis, Mo.), was used as control antibody.

2.4. Neutralization of exogenous IL-1

In order to confirm the neutralizing activities of the antibodies and IL-1RA, they were mixed with the respective recombinant cytokines and IL-8 expression from GECs stimulated with the above mixture was determined. The cytokine concentration was 5 ng/ml for both rhIL-1alpha and rhIL-1beta. The ND50 for anti-IL-1alpha was 2 ug/ml and for IL-1beta 10 ug/ml, as determined by the manufacturer. The final concentration of antibodies that were used against rhIL-1alpha (2-10 ug/ml) and against rhIL-1beta (10-50 ug/ml) was determined based on the ND50. As control antibodies, similar concentration of goat IgG was used. The final concentration of rhIL-1RA was 800 ng/ml. The flow diagram below describes the procedure:

```
GECs
↓
96-Well Culture Plates
↓
Pre-incubate GECs with @IL-1alpha or @IL-1beta or N IgG or IL-1RA
↓
Add rhIL-1alpha(5ng/ml) or rhIL-1beta(5ng/ml) or Media
↓ 0-18h, 37°C, 5%CO2
Supernatants/Lysates
↓
IL-8 ELISA
```
2.5. Neutralization of \textit{A.a.-induced endogenous IL-1} \\

Based on the quantities of endogenous IL-1alpha and IL-1beta detected in supernatants of PP cells (see section 3.1), neutralizing antibodies against the respective cytokines or rhIL-1RA were added to the medium in more than 10-fold excess of the recommended concentrations. Those concentrations were based on the ND\textsubscript{50} of the antibodies and IL-1RA \textsuperscript{1}. The inhibitors were first added to the cultures and 30 minutes later the bacterial extracts were introduced. The final concentration of antibodies against endogenous IL-1alpha and IL-1beta was 10 \textmu g/ml. As control antibodies, similar concentration of goat IgG was used. The final concentration of rhIL-1RA was 200 ng/ml. The flow diagram below describes the procedure:

\begin{center}
\begin{tikzpicture}
  \node {GECs} [text width=2cm] ;
  \node [below] {96-Well Culture Plates} ;
  \node [below] {Pre-incubate with \textit{IL-1alpha} or \textit{IL-1beta} or \textit{NIGG} or \textit{IL-1RA} } ;
  \node [below] {\textit{A.a.E} / Media} ;
  \node [below] {\textit{0-18h, 37°C, 5\%CO\textsubscript{2}}} ;
  \node [below] {Supernatants /Lysates} ;
  \node [below] {IL-8 ELISA} ;
\end{tikzpicture}
\end{center}

\textsuperscript{1} \textbf{Neutralization Dose}_{50} (ND\textsubscript{50}) for an antibody/inhibitor is defined as the required concentration to yield one-half maximal inhibition of the cytokine activity on a responsive cell line, when that cytokine is present at a concentration high enough to elicit a maximum response. The ND\textsubscript{50} for anti-IL-1alpha was 0.008-0.02 \textmu g/ml in the presence of 50 pg/ml of rh IL-1alpha. The ND\textsubscript{50} for anti-IL-1beta was 0.05-0.1 \textmu g/ml in the presence of 50 pg/ml of rhIL-1beta. The ND\textsubscript{50} for rh IL-1RA was 7-10 ng/ml in the presence of 50 pg/ml of IL-1alpha (according to R&G systems, product specifications).
2.6. Measurement of IL-1alpha and IL-1beta

Quantification of IL-1 was accomplished using a matched pair enzyme-linked immunoassay (ELISA) (Endogen Inc. Cambridge MA). Briefly, 96 well assay plates were treated for 12 hours with 8.0 ug/ml mouse anti-human IL-1alpha or 2.0 ug/ml mouse anti-human IL-1beta monoclonal coating antibody (Endogen Inc. Cambridge MA). Then, blocking buffer was added for one hour, followed by 1-hour incubation with 25 ul aliquots of gingival keratinocyte culture supernatant. Assay plates were then incubated with biotynlated mouse anti-human IL-1 monoclonal detecting antibody at 0.8 ug/ml (anti-IL-1alpha) and 0.5 ug/ml (antiIL-1beta) and linked to horseradish peroxidase-conjugated streptavidin at a dilution 1/8,000. (Zymed Laboratories Inc., San Francisco, CA). Assay plates were then incubated with tetramethylbenzidine (TMB) substrate (Dako Inc., Carpenteria CA). The developing reaction was stopped with the addition of 2N sulfuric acid. Absorbance was determined at 450 nm, using a BIORAD 2550 microplate reader and the concentrations of IL-1alpha and IL-1beta were calculated with a software package (Microsoft Excell). Levels were determined from the means of duplicate determinations for each supernatant sample and compared with a standard curve for human recombinant IL-1alpha and IL-1beta. The assay routinely detected IL-1alpha and IL-1beta levels from 7.8 pg/ml to 500 pg/ml.

2.7. Measurement of IL-8

Quantification of IL-8 was accomplished using a matched pair enzyme-linked immunoassay (ELISA) (Endogen Inc. Cambridge MA) as described above for IL-1alpha and IL-1beta. The concentration of mouse anti-human IL-8 monoclonal coating antibody (Endogen Inc. Cambridge MA) was 2 ug/ml and that of biotinylated mouse anti-human
IL-8 monoclonal was 0.1 g/ml. The assay routinely detected IL-8 levels from 24 pg/ml to 6 ng/ml.

2.8. Statistical analysis

Cytokine levels were expressed as the mean±standard deviation of results from a representative from 3 identical experiments. Statistical analysis of the data was performed using paired students t-test with significance considered to be p<0.05.
3. RESULTS

3.1. *A. a.* induces IL-1 expression in GECs *in vitro*

In this section we are going to demonstrate that infection with *A. a.* induced significant expression of both IL-1alpha and IL-1beta by GECs. The kinetics of those cytokines are going to be presented in detail and they are going to be compared with the kinetics of IL-8 expression induced by *A. a.* in an attempt to detect associations.

3.1.1. *A. a.* induces IL-1alpha expression in gingival epithelial cells (GECs)

In order to determine the kinetics of the *A. a.*-induced-IL-1alpha expression by GECs, cell monolayers were exposed to *A. a.* sonicated extracts and the cytokine expression was compared to the unexposed controls. Expression of IL-1alpha was determined for each time point by removing the medium from the specific wells and assaying using ELISA. The results shown in Figure 3.2 are from a representative experiment out of four with similar results. The data-points represent mean values from triplicate wells. It is clearly shown that a significant increase in the IL-1alpha expression was observed as early as 2 hours after the bacterial challenge. At that time point, the basal IL-1alpha expression was 30±13pg/ml, whereas the *A. a.*-stimulated IL-1alpha expression was 137±12pg/ml, which was over 4.5 times higher. Between the 4- and 12-hour time-point, there was a linear increase in IL-1alpha concentration in the supernatant, reaching a value of 199±1pg/ml, which corresponds to a 5.7-fold stimulation compared to unstimulated controls. As it can be seen in Figure 3.3, the highest stimulation index (5.7) was observed at the 12-hour time point. Between 12 and 48 hours, IL-1alpha increased gradually and reached the maximum value of 291±11pg/ml. The stimulation index,
however, decreased at 24 hours to a value of 2.2 and remained in that level for the rest of the experimental period.

Figure 3.2 A.a. induces IL-1alpha expression in GECs
Gingival epithelial cells were stimulated with 50 ug/ml A.a. sonicated extracts. IL-1alpha was compared to unstimulated controls (dotted line). Rapid increase of IL-1alpha by the stimulated cells was observed during the first 12 hours. The basal IL-1alpha expression showed a steady increase over time. The data are expressed as the mean ± S.D. of a representative experiment out of four identical experiments. (**: p < 0.05 compared with IL-1alpha expressed by controls).
Figure 3.3 Stimulation Index (SI) of IL-1alpha expression induced by *A.a.*
The stimulation index represents the fold increase in IL-1alpha by gingival epithelial cells stimulated with *A.a.* sonicates compared to the unstimulated controls. The data are expressed as the mean ± S.D. of a representative experiment out of four identical experiments.

3.1.2. Comparison of the kinetics of IL-1alpha with the kinetics of IL-8 induced by *A.a.*

In chapter 2 we showed that the level of IL-8 expression induced by *A.a.* sonicates was quite high, reaching 10,000 pg/ml, after 24 hours. The IL-1alpha levels, however, that were detected in the same supernatants and for the same time period were 40 times lower. The time-course study of both cytokines is presented in Figure 3.4. It is important to note that the increase in IL-1alpha production occurred as early as 2 hours post-stimulation and peaked at 12 hours. The peak IL-8 expression occurred at 24 hours and the levels of this cytokine remained high until the end of the experiment. In Fig.3.4, it can be seen that the peak of the IL-8 response to the bacterial challenge lagged significantly compared to the IL-1alpha response, which can be considered “immediate”. Even though,
in absolute concentrations, IL-8 was overwhelmingly the major cytokine detected, during the first 12 hours the relative increase in IL-1alpha expression by the cells is higher than that of IL-8. At the 24 and 48-hour time-points, things were reversed, with a delayed increase in IL-8 expression and decrease in IL-1alpha.

Figure 3.4 Comparison between SI of IL-1alpha and IL-8 induced by A.a. The Stimulation Index is presented here. Notice the delay in peak IL-8 response (interrupted line) compared to the IL-1alpha response (continuous line).
3.1.3. *A.a.* induces Interleukin-1beta expression in gingival epithelial cells.

The kinetic study of IL-1 beta was performed by removing and assaying the supernatants for IL-1beta at the following timepoints: 2h, 4h, 8h, 12h, 24h and 48h. The results presented in Figure 3.5 are from one experiment representative of four experiments with identical results. There are two curves representing a) the IL-1beta expression by the gingival keratinocytes stimulated with sonicates from *A.a.* with protein concentration 50 ug/ml and b) the IL-1 beta expression by unstimulated cells. It is shown that there were no detectable IL-1beta levels in the supernatants from the unstimulated cells. The sensitivity of our assay was 7 pg/ml IL-1beta. Concerning the stimulated cells, it can be noted that until the 8-hour time point there were no detectable levels of IL-1beta. IL-1beta expression was detected at 12 and 24 hours as seen in Figure 3.5. The cytokine expression increased over time and reached the maximum level (29 pg/ml) at 48 hours after stimulation.

3.1.4. Comparison of the kinetics of IL-1beta with the kinetics of IL-8 induced by *A.a.*

The relative cytokine expression was compared for IL-1beta and IL-8 (Figure 3.6). Although IL-8 production was 250 times higher than IL-1beta there are some interesting observations regarding the relative production of these two cytokines over time.

- They both had a limited expression during the first 12 hours. This comes in contrast with the IL-1alpha expression, which reached high levels as early as 2h after the beginning of *A.a.* stimulation.
• Both cytokines increased gradually to their maximum expression at the 48-hour time point. However, the main increase in IL-8 expression (from 7% to 97%) occurred between 12h and 24h, whereas the main increase in IL-1beta (25% to 100%) took place between 12 and 48 hours and was more gradual.

• It is concluded that the kinetics of these two cytokines present several similarities as well as differences. Compared to the kinetics of IL-1alpha they can be characterized as "delayed response cytokines" despite the fact that significant stimulation of IL-8 occurred as soon as 2h the main difference between IL-8 and IL-1beta was that IL-8 reached the maximum expression at 24h, whereas IL-1beta reached the maximum one day later.

![Figure 3.5](image)

**Figure 3.5 A.a. induced IL-1beta expression in GECs**
Gingival keratinocytes were stimulated with 50 ug/ml A.a. sonicates. The basal IL-1beta expression was below detectable levels (dotted line). The data are expressed as the mean ± S.D. of a representative experiment out of four identical experiments. (**: p < 0.01 compared with IL-8 expressed by controls).
Figure 3.6 Comparison between kinetics of IL-1beta and kinetics of IL-8
Both cytokines were produced by A.a.-stimulated gingival epithelial cells. Here, they are presented as cytokine levels above the basal expression. The data are expressed as the mean ± S.D. of a representative experiment out of four identical experiments.

3.1.5. Comparison of the kinetics of IL-1alpha with the kinetics of IL-1beta induced by A.a.

In Figure 3.7 the right axis corresponds to IL-1beta expression whereas the left corresponds to IL-1alpha. IL-1alpha production was approximately 5 times higher that IL-1beta. The following observations were made regarding the relative production of these two cytokines over time. During the first 12 hours IL-1alpha reached a maximum but this did not happen with IL-1beta until 36h later. IL-1alpha was detected as soon as 2h, whereas IL-1beta was first detected 10h later, at 12h-timepoint. After that moment
IL-1beta continued to increase, but IL-1alpha actually reached a plateau. It is concluded that the kinetics of these two cytokines are significantly different.

![Graph showing kinetics of IL-1alpha and IL-1beta](image)

**Figure 3.7 Kinetics of IL-1alpha and IL-1beta induced by A.a.**
Levels above the basal expression are presented here. The data are expressed as the mean ± S.D. of a representative experiment out of four identical experiments.
3.2. IL-1alpha induces IL-8 expression

In this section of the results we demonstrate that IL-1alpha induced significant expression of IL-8 in GECs. The dose-response and kinetic studies are presented and they are compared with the kinetics of IL-8 expression induced by *A. a.* in an attempt to detect associations.

3.2.1. IL-1alpha induces IL-8 in a dose-dependent way

Our goal was to determine how increasing concentrations of rhIL-1alpha would affect the IL-8 expression. In addition, we wanted to determine what concentration of IL-1alpha would elicit the highest IL-8 response by GECs. Therefore, monolayers of cells were incubated for 24 hours in the presence of 0, 0.3, 1, 3, and 10 ng/ml rhIL-1alpha. Then, the supernatants were collected and assayed for IL-8 using ELISA.

Our results demonstrated a dose-dependent relation between IL-1alpha and IL-8. The findings can be seen in figure 3.8. The data presented in that figure are from one representative experiment out of three consecutive experiments with similar results. It can be seen that with increasing concentrations of IL-1alpha, GECs expressed increasing levels of IL-8 in the culture supernatant. Their basal IL-8 expression was 1,372±171 pg/ml. Addition of 0.3ng/ml IL-1alpha resulted in significant increase in the IL-8 production (2,274 ±134pg/ml). When we increased the concentration 10-fold to 3ng/ml the IL-8 expression increased to 4,358±138pg/ml, which was 3.5 times higher than the unstimulated (basal) value of IL-8. The highest IL-8 expression (10,588±420pg/ml) occurred when IL-1alpha was added in the cell culture with concentration 10ng/ml. Therefore, the level of IL-8 expression was dependent on the concentration of IL-1alpha in the range of 0.3 to 10 ng/ml.
Concentrations higher than 10 ng/ml resulted in decreased viability of GECs. That was observed microscopically as rounding of the cells and detachment from the bottom of the tissue culture well. Therefore, the concentration of IL-1 alpha that produced the maximal IL-8 response by GECs was 10 ng/ml for the experimental period of 24 hours.

Figure 3.8 Dose-response study of IL-8 expression by GECs following IL-1 alpha challenge
GECs were challenged for 24 hours with increasing concentrations of IL-1 alpha and the supernatants were assayed for IL-8. For IL-1 alpha values between 1 and 10 ng/ml there was a linear increase in the IL-8 expression. All the IL-8 values were statistically higher than the unstimulated controls. (**: p≤0.05).
3.2.2. IL-1alpha induces IL-8 expression in a time-dependent way.

The purpose of this section was to determine the kinetics of IL-8 expression by GECs after exposure to recombinant human IL-1alpha. Cell monolayers were stimulated with 10 ng/ml IL-1alpha for the following periods: 2h, 4h, 8h, 12h, 24h and 48h. Expression of IL-8 was determined for each time point by removing the medium from the specific wells and assaying using ELISA. The results presented in Figure 3.9, are from one representative experiment out of three.

It is shown that a statistically significant increase in the level of IL-8 protein in the supernatant was observed as early as 2 hours after stimulation. The IL-8 concentration at 4 hours was 546±77 pg/ml and was significantly higher compared to the unstimulated controls. Between the 2 and 12-hour time-point, there was a constant increase in IL-8 concentration, reaching 2,031±261 pg/ml, which was 7-fold higher that the unstimulated controls. The IL-8 expression increased steadily over time and reached the maximum level (10,736±611 pg/ml) at the 24 hour time-point, which was 10-fold greater that the non-exposed control. The detectable IL-8 levels decreased to 9,831±1,221 pg/ml, 48 hours after the beginning of the challenge. This probably reflects a decrease in IL-8 expression. However, part of this decrease might be due to degradation of this cytokine, as well, under the specific experimental conditions (37°C and 100% humidity). A slight increase in IL-8 expressed in the control (unstimulated) cells was evident during the 48-hour observation period. This increase may reflect the natural characteristics of IL-8 expression of the PP cell line.
3.2.3. Comparison of the effect of Interleukin-1alpha and A.a. on the kinetics of Interleukin-8.

Once we determined the kinetics of the IL-1alpha-induced IL-8 expression we compared it with the previously reported kinetic study of the A.a.-induced IL-8 in order to see how GECs respond in different stimuli over time. As shown in Figure 3.10, stimulation of GECs with 10 ng/ml rhIL-1alpha induced higher IL-8 expression than stimulation with 50 ug/ml of A.a. sonicates for the same timepoint. The increase for both...
conditions was time dependent until the 24-hour time point. Between 24 and 48 hours, there were no statistically significant changes in IL-8 expression for both conditions.

![Figure 3.10](image)

**Figure 3.10** Comparison of the effect of IL-1alpha and *A. a.* sonicates in the expression of IL-8

Stimulation of GECs with 10 ng/ml rhIL-1alpha induced higher IL-8 expression than stimulation with 50 µg/ml of *A. a.* sonicates for the same timepoint. The increase for both conditions was time dependent until the 24-hour time point. The data points represent mean values of triplicate assay determinations from one representative experiment. All values were statistically higher than controls. (** : p< 0.05)

In order to evaluate the relative increase in IL-8 expression, we calculated the fold increase compared to the unstimulated controls for the corresponding time-points. This is presented in Figure 3.11, where it can be observed that for the 2 hour time-point both stimuli cause similar stimulation. However, at the 4-hour time-point, IL-1alpha induced a 4.7-fold increase, whereas *A. a.* sonicates induced a 2.5-fold increase. At the 8 and 12-hour time-points, IL-1alpha induced levels of IL-8 three times higher than those induced
by *A. a.* sonicates (Figure 3.11). This “early stimulatory effect” of 10ng/ml IL-1alpha compared to 50ug/ml *A. a.* sonicates was more intense for the first 12 hours. At the 24 and 48-hour points the fold IL-8 increase induced by *A. a.* (7-fold and 6.7-fold respectively) increased and was closer to the IL-8 expression induced by IL-1alpha (10-fold and 8-fold respectively).

![Figure 3.11 Stimulation Index of IL-8 expression induced by IL-1alpha and *A. a.* sonicates](image)

**Figure 3.11 Stimulation Index of IL-8 expression induced by IL-1alpha and *A. a.* sonicates**

Stimulation of GECs with 10 ng/ml rhIL-1alpha induced higher IL-8 stimulation Index than stimulation with 50 ug/ml of *A. a.* sonicates for the same timepoint. The data points represent the fold increase in IL-8 expression compared to the unstimulated cells for the corresponding time-points. Notice the “early stimulatory effect” of IL-1alpha compared to *A. a.* sonicates. At the 8-hour time-point, IL-1alpha had already induced three times higher levels of IL-8 than *A. a.* sonicates had.
3.3. IL-1beta induces IL-8 expression

In this section it is demonstrated that IL-1beta induced significant expression of IL-8 in GECs. The dose-response and kinetic studies are presented and they are compared with IL-8 expression induced by A.a.

3.3.1 IL-1beta induces IL-8 in a dose-dependent way

We have already demonstrated a dose-response relation between the IL-1alpha challenge and the expression of IL-8 by GECs. Based on the existing literature we know that IL-1beta is a potent inducer of IL-8 in several cell types. Our purpose was, as with IL-1alpha, to determine how the concentration of IL-1beta would affect the expression of IL-8 by the PP cell line. Therefore, monolayers of gingival epithelial cells were incubated for 24 hours in the presence of 0, 0.3, 1, 3, and 10 ng/ml rhIL-1beta. Then, the supernatants were collected and assayed for IL-8 using ELISA.

The IL-8 excretion of GECs, stimulated by IL-1beta can be seen in figure 3.12. The data presented are from one representative experiment out of three consecutive experiments with similar results. It can be seen that with increasing concentrations of IL-1beta, cells express more IL-8. The unstimulated IL-8 expression by gingival epithelial cells was 1,372±171 pg/ml. Addition of 0.3 ng/ml IL-1beta resulted in significant increase in the IL-8 expression to 2,095±242 pg/ml. When we increased the concentration 10-fold to 3 ng/ml the IL-8 expression increased to 8,324±1,251 pg/ml, which was 6.6 times higher than the unstimulated (basal) value of IL-8. The maximum IL-8 expression was 11,011±878 pg/ml when the cells were stimulated with 10 ng/ml IL-1beta. This was an 8.7-fold increase compared to the basal IL-8 expression. Therefore, the IL-8 expression can be considered dependent on the concentration of IL-1beta, on the range
0.3 to 10 ng/ml. IL-1beta concentrations higher that 10 ng/ml resulted in reduced viability of the GECs (rounding and detachment from the bottom of the tissue culture well).

Figure 3.12 Dose-dependent expression of IL-8 after stimulation with IL-1beta GECs were challenged for 24 hours with increasing concentrations of rhIL-1beta and the supernatants were assayed for IL-8. All the IL-8 values were statistically higher than the unstimulated controls. (**: p<0.05).
3.3.2. IL-1beta induces IL-8 expression in a time-dependent way

In order to determine the kinetics of IL-8 expression in the culture supernatant after exposure to 10 ng/ml IL-1beta, time-course studies were performed using gingival epithelial cell monolayers. Expression of IL-8 was determined for each time point by removing the medium from the specific wells and assaying using ELISA. The results presented in Figure 3.13, are from one representative experiment out of four. It is shown that an increase in the level of IL-8 protein was observed as early as 2 hours after stimulation. The IL-8 concentration at 2 hours was 427±97 pg/ml and was significantly higher compared to the unstimulated controls (83±22 pg/ml). Between the 2 and 8-hour time-point, there was a linear increase in IL-8 concentration, reaching 3566±268 pg/ml, which was 12 old higher that the unstimulated controls. The IL-8 expression increased steadily over time and reached the maximum level (11943±979 pg/ml) at 24 hours after IL-1beta challenge, which was 9-fold greater that the non-exposed control. The detectable IL-8 decreased 48 hours after the challenge. This probably reflects a decrease in IL-8 expression. However, part of this decrease might be due to degradation of this cytokine, as well, under the specific experimental conditions (37°C and humidity).

A slight increase in IL-8 expressed in the control (unstimulated) cells was evident during the 48-hour observation period. This increase may reflect the natural characteristics of IL-8 expression of the PP cell line.
Figure 3.13 Time-course of IL-8 expression following IL-1beta challenge
Stimulation of GECs with 10 ng/ml rhIL-1β induced an increase in the basal IL-8 expression. The increase was time dependent until the 24-hour time point. The data points represent mean values of triplicate assay determinations from one representative experiment. (**: p< 0.05)

3.3.3. Comparison of the effect of Interleukin-1beta and sonicates of A. a. on the kinetics of Interleukin-8 expression

Once we determined the kinetics of the IL-1beta-induced IL-8 expression we compared it with the previously reported kinetic study of the A.a.-induced IL-8 expression in order to see how the gingival epithelial cells respond in different stimuli over time. In Figure 3.14 it can be seen that stimulation with 10 ng/ml rhIL-1beta induced
higher IL-8 expression than stimulation with 50 μg/ml of A.a. sonicates, until the 24-hour time point. The increase for both conditions was time dependent. However, at 48 hours, IL-1beta-induced IL-8 expression (7620±1472 pg/ml) was significantly lower than the IL-8 expression induced by A.a. (9927±241 pg/ml).

Figure 3.14 Comparison of IL-8 expression induced by IL-1beta with that induced by A.a.
Stimulation of GECs with 10 ng/ml rhIL-1beta induced higher IL-8 expression than stimulation with 50 μg/ml of A.a. sonicates for the first 24 hours. The data points represent mean values of triplicate assay determinations from one representative experiment. All values were statistically higher than controls (** : p< 0.05).

In order to evaluate the relative increase in the basal IL-8 expression, we calculated the fold increase compared to the unstimulated (basal) controls for the corresponding time-points. This is defined as Stimulation Index and is presented in Figure 3.15, where it can be observed that at the 4-hour time-point, IL-1beta induced a 7-
fold increase, whereas *A.a.* sonicates induced a 2.6-fold increase. At the 8 and 12-hour time-points, IL-1beta induced levels of IL-8 much higher than those induced by *A.a.* sonicates. This “early stimulatory effect” of 10 ng/ml IL-1beta compared to 50 ug/ml *A.a.* sonicates was more intense 8 hours after the beginning of the stimulation with a 15-fold increase of IL-8 Vs only a 2.6-fold increase for *A.a.* At the 24 and 48-hour points the fold IL-8 increase induced by IL-1beta (9-fold and 4-fold respectively) was closer to the IL-8 expression induced by *A.a.* (7-fold and 6-fold respectively).

![Figure 3.15 Stimulation Index of IL-8 expression induced by IL-1beta and *A.a.* sonicates](image)

The “early stimulatory effect” that was observed after IL-1alpha challenge, was more intense after IL-1beta challenge. At the 8-hour time-point, IL-1beta had already induced six times higher IL-8 levels compared to *A.a.* sonicates. However, after the 24-hour timepoint, the difference in IL-8 expression was nullified between the two conditions.
3.4. Effect of IL-1 inhibitors on IL-8 expression induced by IL-1 or by *A. a.*

The results of this study will be presented in 4 sections depending on the agent that we used for the cell stimulation and the inhibitors used. We stimulated the GECs with either combinations of IL-1alpha and IL-1beta or *A. a.* sonicates and examined the effect of IL-1 inhibitors on the IL-8 expression.

3.4.1. Effect of anti-IL-1alpha neutralizing antibodies on the IL-1 alpha-induced IL-8 expression by GECs.

We tested the ability of anti-IL-1alpha neutralizing antibody to inhibit the inducible and constitutive expression of IL-8 in the PP cell line. Rh IL-1alpha was added at a concentration of 5 ng/ml, with or without anti-IL-1alpha or N IgG (Ctrl Ab). The addition of rh IL-1alpha induced an increase in expression of IL-8 from 281±41 pg/ml to 1972±61 pg/ml. The inducible response was inhibited in a great degree by anti-IL-1alpha, but not from the same concentration of control antibody. Increasing concentration of anti-IL-1alpha resulted in increased inhibition (Figure 3.16) and specifically, IL-8 expression from 1972±61 pg/ml was decreased to 1149±96 pg/ml (49% inhibition) and 793±70 pg/ml (70% inhibition) with the addition of 2 ug/ml and 10 ug/ml anti-IL-1alpha respectively. This means that anti-IL-1alpha antibody is effective in neutralizing rh IL-1alpha.

Addition of anti-IL-1alpha had no significant effect on the basal level of IL-8 expression by GECs (Figure 3.16). The level of 5ng/ml rh IL-1alpha that was used as
stimulant, was 25 times higher compared to the endogenous IL-1alpha levels (200 pg/ml) that were detected in GEC supernatants, after bacterial stimulation for 24 hours.

Figure 3.16  Effect of anti-IL-1alpha antibodies on IL-1alpha-induced IL-8 expression
Rh IL-1alpha (5 ng/ml) was added in cultures of GECs, with or without anti-IL-1alpha antibody or control antibody (Ctrl Ab). Anti-IL-1alpha antibodies significantly reduced IL-8 induced by rh IL-1alpha. Experimental period was 18 hours. ( ** : p < 0.05).
3.4.2. Effect of anti-IL-1beta neutralizing antibodies on the IL-1beta-induced IL-8 expression by GECs.

After demonstrating the effectiveness of anti-IL-1 neutralizing antibody on rh IL-1alpha induced expression of IL-8 we wanted to determine if similar results could be obtained with anti-IL-1beta neutralizing antibody, as well. Rh IL-1beta was added at a concentration of 5 ng/ml, with or without anti-IL-1beta or N IgG (Ctrl Ab). The addition of IL-1beta induced an increase in IL-8 expression (Fig.3.17) from 281±41 pg/ml to 2879±183 pg/ml (10-fold).

![Figure 3.17](image)

**Figure 3.17 Effect of anti-IL-1beta antibodies on IL-1beta-induced IL-8 expression**

Rh IL-1beta (5ng/ml) was added in cultures of GECs with or without anti-IL-1beta antibody or control antibody (Ctrl Ab). Anti-IL-1beta antibodies significantly reduced IL-8 induced by IL-1beta. Experimental period was 18 hours ( ** : p < 0.05 ).
The inducible response was significantly inhibited by anti-IL-1beta, but not from the same concentration of control antibody. Increasing concentration of anti-IL-1beta resulted in increased inhibition (Figure 3.17) and specifically, IL-8 expression from 2879±183 pg/ml was decreased to 1591±74 pg/ml (49% inhibition) and 1027±178 pg/ml (71% inhibition) with the addition of 10 ug/ml and 50 ug/ml anti-IL-1beta respectively. Addition of anti-IL-1beta had no significant effect upon the basal level of IL-8 expression by GECs (Figure 3.17). It should be noted that the level of 5 ng/ml IL-1beta that was used as stimulant was 250 times higher compared to the endogenous IL-1beta levels (15-20 pg/ml) that were detected in GEC supernatants, after bacterial stimulation.

3.4.3. Effect of IL-1RA on the IL-1-induced IL-8 expression by GECs.

After demonstrating the effectiveness of anti-IL-1 neutralizing antibodies on IL-1alpha/IL-1beta-induced expression of IL-8 we wanted to determine if similar results could be obtained with IL-1RA, as well.

Figure 3.18 Effect of IL-1RA on IL-1alpha-induced expression of IL-8
Figure 3.19 Effect of IL-1RA on IL-1beta-induced expression of IL-8.
Gingival epithelial cells were challenged with 2 ng/ml rhIL-1alpha or rhIL-1beta and 800 ng/ml IL-1RA was added. Experimental period was 18 hours ( ** : p < 0.05 ).

IL-1RA was added at a concentration of 800 ng/ml, with or without IL-1alpha or IL-1beta. The results showed significant inhibition of IL-8 expression, up to 60%, for both IL-1alpha and IL-1beta. This validates the effectiveness of IL-1RA in blocking the receptor for both IL-1alpha and IL-1beta.
3.4.4. Stimulation with IL-1alpha and IL-1beta and addition of IL-1 inhibitors

After demonstrating the effectiveness of anti-IL-1alpha, anti-IL-1beta and IL-1RA on IL-1 induced expression of IL-8 we wanted to see how their combination would affect the IL-8 expression of GECs. Rh IL-1alpha 5 ng/ml and rh IL-1beta 5 ng/ml were added in the cell culture medium, with or without anti-IL-1 antibodies, IL-1 RA, or control antibody (Ctrl Ab). We used concentrations five times higher than ND$_{50}$ for both anti-IL-1alpha and anti-IL-1beta, which was 10 ug/ml and 50 ug/ml, respectively. [The ND$_{50}$ for anti-IL-1alpha and anti-IL-1beta, was 2 ug/ml and 10 ug/ml respectively, for neutralization of 50% of the bioactivity of 5 ng/ml IL-1alpha and IL-1beta].

The addition of IL-1alpha + IL-1beta induced an increase in IL-8 expression (Fig.3.20) from 809±81 pg/ml to 2784±192 pg/ml. The inducible response was not significantly inhibited by 10 ug/ml anti-IL-1alpha (2672±226 pg/ml) or by 50 ug/ml control antibody (2745±276 pg/ml) and was slightly inhibited by 50 ug/ml anti-IL-1beta (2353±166 pg/ml), as seen in Figure 3.20. This suggests that when either IL-1alpha or IL-1beta was neutralized, the other cytokine was adequate for stimulating a high IL-8 response. The inducible IL-8 response was also significantly inhibited by 800 ng/ml IL-1RA (1167±98 pg/ml).

In Figure 3.21 it can be seen that combinations of antibodies with or without IL1RA can effectively inhibit the [IL-1alpha+IL-1beta]-induced IL-8 expression. Specifically the following combinations were tested: a) 10 ug/ml anti-IL-1alpha + 50 ug/ml anti-IL-1beta (1203±192 pg/ml) and b) 10 ug/ml anti-IL-1alpha + 50 ug/ml anti-IL-1beta + 800 ng/ml IL-1RA (1031±187 pg/ml).
The basal level of IL-8 expression by GECs was not significantly changed by the addition of anti-IL-1alpha, anti-IL-1beta, or IL-1RA. (Figures 3.20 and 3.21). However, addition of IL-1RA or control antibody (N IgG) or combination of those, resulted in a small, but statistically significant increase in the basal IL-8 expression.

When we calculated the % inhibition of IL-8 expression induced we reached the following results. The highest inhibition was observed after addition of IL-1RA (82%), combination of the 2 antibodies anti-IL-1alpha and anti-IL-1beta (80%) and combination of the antibodies above with IL-1RA (89%). Also combination of the control antibody with IL-1RA elicited significant inhibition in IL-8 expression (Fig. 3.21).

![Figure 3.20](image.png)  
**Figure 3.20** Effect of single anti-IL-1 antibodies and IL-1RA on the [IL-1alpha + IL-1beta] induced IL-8 expression
Figure 3.21 Effect of combined anti-IL-1 antibodies and IL-1RA on the [IL-1alpha +IL-1beta]-induced IL-8 expression
GECs were stimulated with IL-1alpha (5ng/ml) + IL-1beta (5ng/ml) with or without IL-1 inhibitors. Anti-IL-1 antibodies and IL-1RA significantly reduced IL-8. Experimental period was 18hours ( **:p< 0.05 compared to initial IL-8 expression without antibodies).

Figure 3.22 Effect of antibodies and IL-1RA on IL-1-induced IL-8 expression.
Addition of anti-IL-1 antibodies inhibits the IL-1-induced IL-8 expression in GECs. This validates the experimental system
3.4.5. Inhibitors of IL-1 do not affect IL-8 expression induced by A.a.

We have already shown in section 3.1, that A.a. challenge induced endogenous IL-1alpha expression by gingival epithelial cells (GECs), as early as 2 hours after the exposure. We have also shown in sections 3.2 and 3.3 that rh IL-1alpha and rh IL-1beta, induce high levels of IL-8 expression, as early as 2 hours post-exposure, in a pattern similar to the IL-1alpha expression. Our next objective was to study whether IL-8 expression by GECs, after bacterial challenge, is regulated by an IL-1-dependent autocrine mechanism.

Different combinations of inhibitors were used. The cell supernatants were tested at 12 and 18 hours for IL-8 expression. Addition of A.a. induced an increase in expression of IL-8 (first column in every figure) 562±34 pg/ml and 2043±139 pg/ml for the first 12 and 18 hours respectively. The inhibitors used alone (Fig. 3.23 and 3.25) or in combination (Fig. 3.24 and 3.26), had no effect on the production of IL-8 in response to A.a. after 12 and 18 hours of incubation. Therefore, the IL-8 response does not appear to be regulated by the endogenous IL-1 excretion in an autocrine way, for the two time periods that were tested. We did not test this hypothesis in earlier time points, because the difference between the unstimulated and stimulated IL-8 levels was small and statistically significant results were difficult to be obtained.
Figure 3.23 Effect of single IL-1 inhibitors on A.a.-induced IL-8 expression (T=12h)
Gingival epithelial cells were stimulated with A.a. sonicates 20 ug/ml for 12 hours. No significant inhibition of IL-8 expression was found for any of the IL-1 inhibitors compared to the initial IL-8 expression induced by A.a.

Figure 3.24 Effect of combined IL-1 inhibitors on A.a.-induced IL-8 expression (T=12h)
Gingival epithelial cells were stimulated with A.a. sonicates 20 ug/ml for 12 hours. No significant inhibition of IL-8 expression was detected for any of the combinations of IL-1 inhibitors.
Figure 3.25 Effect of single IL-1 inhibitors on A.a.-induced IL-8 expression (T=18h)
Gingival epithelial cells were stimulated with A.a. sonicates 20 ug/ml for 18 hours. No significant inhibition of IL-8 expression was detected for any of the IL-1 inhibitors.

Figure 3.26 Effect of combined IL-1 inhibitors on A.a.-induced IL-8 expression (T=18h)
GECs were stimulated with A.a. sonicates 20 ug/ml for 18 hours. No significant inhibition of IL-8 expression was detected for any of the combinations of IL-1 inhibitors.
4. DISCUSSION

**GECs express IL-1 constitutively.**

IL-1 is synthesized by a variety of cells and especially by macrophages activated by microbial antigens or other cytokines. Therefore most of the studies in periodontal literature have focused on the monocyte/macrophage reaction to the periodontal pathogens. There are very limited data on GEC expression of IL-1 in response to *A.a.* We showed that under resting conditions IL-1 alpha is constantly expressed at a rate of 200pg/ml/0.3x10⁶cells/24h, whereas IL-1beta is below detectable levels. This finding is in agreement with Woods et al who reported that the ratio of excreted IL-1alpha to IL-1beta is more than 10 to 1 in Squamous Cell Carcinoma and HVP-16 cell lines [29]. Johnson et al were able to detect IL-1alpha in culture supernatant of oral keratinocytes after 24 hours, at a level of 2-3 pg/10⁴ cells. This amount of IL-1alpha was similar to the one we reported [7]. The levels of intracellular IL-1 alpha that they found were 20 times higher than the levels of IL-1beta.

Others have reported lower levels of IL-1alpha compared to our results (43pg/ml/10⁶cells/24h) [8]. However, they used primary keratinocytes, that usually express reduced cytokine responses compared to the immortalized cell lines [30]. Primary keratinocytes seem to be able to excrete IL-1beta. Over a 3-day period, Yamamoto et al were able to detect significant amounts of IL-1beta (30 pg/10⁵cells/ml) produced by primary cultures, derived from healthy gingiva [9]. Future experiments can examine the IL-1 profile of those cells for longer than 48h, which was our experimental period. Overall, our findings, prove that gingival epithelial cells are similar to skin epithelial cells in regard to the predominance of IL-1alpha expression Vs IL-1beta, as
described by Kupper [5]. This is important given the fact that the role of epidermal keratinocytes in inflammatory diseases has been extensively studied in the medical literature [31].

**A.a. induces upregulation of IL-1 expression in GECs**

In this study, we demonstrated that the exposure of gingival epithelial cells (GEC) to *A.a.* (*A.a.*) induces expression of IL-1alpha and IL-1beta (Fig. 3.2 and 3.5). Significant increases of IL-1alpha and IL-1beta secreted proteins were detected within 2h and 12h, respectively, compared to the levels in uninfected controls. Bacterial challenge resulted in rapid increase of IL-1alpha, reaching a 6-fold stimulation compared to the basal levels at the 12h-timepoint. The IL-1alpha expression later declined to a 2-fold stimulation and remained there until the end of the experimental period. This “early stimulatory effect” of IL-1alpha has never been described before and might be important for the induction of other cytokine responses, since IL-1alpha is a potent cytokine inducer.

IL-1beta was also upregulated after exposure to *A.a.* and reached a level of 30pg/ml after 48h of continuous stimulation. The upregulation of IL-1beta excretion that we reported in GECs is not observed in human gingival fibroblasts (HGF) stimulated with *A.a.* Reddi et al reported that *A.a.* stimulation failed to stimulate any release of IL-1beta [32]. Similar results were presented by Dongari [33]. After exposing HGF to *A.a.* they observed no difference in IL-1beta expression.

In our study, the ratio “IL-1alpha:IL-1beta” varied over time. After 24h, the IL-1alpha levels were approximately 20 times higher than the IL-1beta levels (ratio 20:1). After 48h, this ratio became 5:1 and there was a tendency for further decrease. Therefore, we can see that IL-1alpha appears to act as an early mediator after bacterial challenge
with activity peaking at 12h, whereas IL-1beta may act as a late mediator with activity peaking at 48h or even later.

The kinetics of IL-1alpha and IL-8 were then compared. IL-8 peak expression lagged compared to IL-1alpha peak expression. The IL-8 stimulation index was between 2 and 3 until 12h and then it peaked reaching the value of 7, at 24 h. The delayed production of IL-8 can be due to the existence of an additional mediator that is induced by A.a. and stimulates IL-8 expression. In a respiratory epithelial cell line (A549), IL-1alpha has been identified as an autocrine mediator that is produced by the cells and induces the expression of IL-8 [23]. A possible autocrine mediator has not been identified in gingival epithelial cells to date. In clinical conditions, expression of IL-1alpha and IL-beta may take part in the amplification of the immune response, by activating fibroblasts, macrophages and other keratinocytes to produce IL-1beta, IL-6, IL-8 and TNF-alpha. The presence of IL-1alpha and IL-1beta in GCF can also be contributed to GECs in addition to macrophages and fibroblasts. Our results indicate that GECs are actively involved in the regulation of the complex cytokine network, in addition to macrophages and other immune cells.

**IL-1 induced upregulation of IL-8 expression in GECs**

In periodontal diseases IL-1beta has been detected in inflamed gingiva and is shown to be important mediator of the inflammatory process [10, 12, 14]. The local generation of IL-1beta by macrophages in the gingiva after bacterial infection can act in a classical paracrine function to induce the expression of IL-8 by non-immune cells. In our study we examined how rhIL-1 affects the basal IL-8 expression by gingival epithelial cells. A dose-response relation was shown with maximum stimulation of IL-8 by 10
ng/ml IL-1alpha and IL-1beta. The IL-8 levels were comparable with the A.a.-induced levels and ranged between 10,000 and 12,000 pg/ml for a 24h stimulation period. Concentrations as low as 0.3 ng of IL-1alpha or IL-1beta were capable of inducing significant IL-8 response above the basal levels. These results are in agreement with other authors that have demonstrated that IL-1 upregulates the IL-8 produced by oral epithelial cells in a dose-dependent manner [20].

The kinetic study on IL-8 production revealed that significant IL-1 expression occurred as early as 2 hours after the beginning of stimulus. At the 8 hour timepoint the IL-8 expression due to IL-1alpha was 7-fold compared to the basal levels and the IL-8 due to IL-1beta was 15-fold the basal levels. Therefore, we have shown that IL-1beta is more potent stimulator, especially for the early timepoints, inducing twice as much IL-8 than IL-1alpha. Another possible explanation for this phenomenon is that the epithelial cells are genetically predisposed to be more reactive to IL-1beta challenge, reflecting the critical role that this cytokine plays in the inflammatory microenvironment. Bickel et al studied the influence of IL-1 alpha on chemokine expression of 2 human oral keratinocyte cell lines. IL-1 stimulation resulted in an increase of mRNAs encoding IL-8 IL-8 mRNA was expressed as early as 1 hour after stimulation [21]. We also demonstrated that the stimulatory potential of IL-1beta was reduced, after the 24h timepoint, to levels lower than those induced by A.a. This "early stimulatory effect" of IL-1 compared to the bacterial extracts suggests a direct effect of IL-1 on epithelial cells versus a possible indirect effect of A.a. IL-8 derived from gingival cells, including gingival epithelial cells can cause neutrophils to accumulate and to be activated in the site of inflammation. Thus, the capacity of the gingival epithelial cells to produce IL-8 in
response to IL-1alpha, IL-1beta or A.a. suggests that they play a critical role in the maintenance of the tissue microenvironment.

**Effect of IL-1 inhibitors on IL-1-induced expression of IL-8**

In this chapter (Chapter 3) we hypothesize that IL-1 is involved in the A.a.-induced expression of IL-8 in an autocrine manner and we use IL-1 inhibitors (rhIL-1RA and anti-IL-1 neutralizing antibodies) to block the extracellular IL-1 and its receptors. However, it is important to validate our antibodies and antagonist first. Therefore, we test whether they can inhibit IL-1-induced IL-8 expression in GECs.

The level of 5 ng/ml IL-1 that was used as stimulant, was 25 times higher compared to the endogenous (GEC excreted) IL-1alpha levels (200 pg/ml) and approximately 160 times higher compared to the endogenous IL-1beta levels (30 pg/ml) after bacterial stimulation for 24 hours. Therefore, neutralization of those high concentrations of exogenous IL-1 would definitely indicate that the inhibitors are adequate for neutralizing the smaller amounts of endogenous IL-1.

We found that addition of IL-1 inhibitors reduced by 70% the IL-1-induced IL-8 expression. This confirmed that the anti-IL-1 neutralizing antibodies and IL-1RA were active and that they were added in excess. In our experiments we used 2 concentrations of antibodies. The first was equal to the neutralizing dose 50 (ND$_{50}$) determined from the manufacturer (R&D Systems) and the second was 5 times higher. ND$_{50}$ is defined as the concentration of antibody that inhibits 50% of the bioactivity of rhIL-1 that is added to a mouse T-cell system and is calculated based on the amount of rhIL-1 that is being used (R&D Systems). Thus we were able to validate the data provided by R&D Systems, since
addition of concentration equal to ND₅₀ from each antibody, inhibited practically 50% of the IL-1-induced expression of IL-8 in the GEC system.

When we increased the antibody concentration x5, the resulted inhibition of IL-8 increased from 49% to 70% for anti-IL-1alpha and from 49% to 71% for anti-IL-1beta proving the effectiveness of those 2 antibodies. This revealed a dose-dependent effect of anti-IL-1 antibodies.

We also asked whether the combined addition of rhIL-1alpha and rhIL-1beta would have a cumulative effect of IL-8 expression. This simulates closer in vivo conditions since GECs secrete both IL-1alpha and IL-1beta when stimulated with A.a. Therefore, we added a mixture of 5ng/ml IL-1alpha and 5ng/mlIL-1beta to the GEC culture and we examined the effect of anti-IL-1alpha, anti-IL-1beta and IL-1RA when added separate, or in combinations.

IL-1RA was as effective as the combination of anti-IL-1alpha and anti-IL-1beta together in the neutralization of the 2 forms of IL-1. This is direct evidence that IL-1 receptor in gingival epithelial cells is non-specific and binds both IL-1alpha and IL-1beta. It is currently known that there are two kinds of IL-1 receptors [34]. Type-1 receptor is expressed predominantly on T-cells and cells of mesenchymal origin. Type-2 receptor is expressed predominantly on B-cells and cells of the myelomonocytic lineage. Type-1 and Type-2 receptors bind both types of IL-1. We conclude that the use of IL-1RA in experiments provides an effective way to neutralize both forms of IL-1. However, if specific neutralization of IL-1alpha or IL-1beta is required, then antibodies should be used instead.
**Effect of IL-1 inhibitors on *A.a.*-induced IL-8 expression**

Once we demonstrated the effectiveness of our inhibitors, we used them to neutralize the GEC-excreted IL-1alpha and IL-1beta after bacterial challenge. Inhibitors of IL-1 did not prevent activation of GECs by *A.a.*, as measured by production of IL-8 excreted in the culture medium. This was the case for stimulation periods of 12 and 18 hours.

Although we cannot rule out possible involvement of intracellular IL-1alpha [35] our results show that stimulation of epithelium by *A.a.* occurs independently of secreted IL-1. This finding was unexpected, given that GECs are capable of producing IL-1alpha and IL-1beta and respond to these cytokines in the same way as they do to *A.a.* regarding the IL-8 expression. Given the fact that IL-1alpha is the first mediator that reaches peak activation soon after bacterial challenge, it seemed likely that excretion of IL-1alpha would mediate stimulation of GECs by *A.a.*

To our knowledge, the participation of IL-1 in the IL-8 expression has never been tested in GECs. In other cells types such autocrine regulation has been demonstrated. Specifically, in cultured endothelial and pulmonary epithelial cells IL-1alpha has been shown to be an autocrine regulator of IL-8 production [22, 23]. Porat et al showed that in PBMCs endogenous IL-1 induced IL-8 after bacterial stimulation [24].

How *A.a.* effects IL-8 stimulation of GECs is an open question. Here are some possible answers:

- Perhaps *A.a.* elicits production of an unidentified host cytokine with functions that overlap those of IL-1. TNF-alpha seems to be a candidate for this function, but no data have ever been published about that.
• Alternatively, direct binding of A.a antigens to endothelial cells may initiate signaling mechanisms that are used both by IL-8 and by IL-1. That means that IL-8 expression is completely independent to endogenous IL-1. A proposed way to test this hypothesis is to knock-out the gene for IL-1 in GECs and to evaluate the IL-8 production after A.a. stimulation. If IL-8 remains unchanged after that, it means that the above hypothesis is correct and IL-8 and IL-1 are not functionally related. If IL-8 expression is affected (reduced), that leads to the third possibility.

• The third possibility is that IL-8 expression is regulated by an IL-1-dependent intracrine mechanism. Previous studies by others have shown that IL-1 translation product undergoes a unique pattern of processing, giving rise to two proteins. One 17-kDa C-terminal segment, referred to as “mature” or processed IL-1alpha and one 16-kDa N-terminal propiece. The C-terminal segment is secreted and binds with the IL-1 receptors. The N-terminal piece is actively transported to the nucleus [36]. Here, we hypothesize that the N-terminal part of IL-1alpha, that is produced in response to the bacterial challenge, returns to the nucleus and induces the expression of IL-8. As we mentioned above one way to validate this option is to knock-out the IL-1alpha gene and to see if IL-8 is affected after bacterial challenge.

These above hypotheses are presented diagrammatically in Figure 3.26. It can be seen that bacterial stimulation induces IL-1 and IL-8 excretion. The Autocrine hypothesis is indicated with the bigger question mark ( ? ) and interrupted arrow. The Intractine hypothesis is indicated with the smaller question mark ( ? ) and continuous arrow. Based on our results the autocrine hypothesis should be ruled out since IL-1 inhibitors did not reduce IL-8 expression after A.a. challenge. Further experiments should be performed in
order to show which one of the remaining 2 hypotheses is true. This will contribute to a better understanding of the transcriptional activation and autocrine loops involved in expression of proinflammatory cytokines and their role in periodontal disease.

Figure 3.27 Possible mechanisms of IL-8 regulation by bacterial induced-IL-1

- **Intracrine pathway** of IL-8 control involves the direct action of IL-1 within a cell. The IL-1alpha N-terminal segment is transported to the nucleus soon after IL-1alpha is synthesized and induces IL-8 expression (small question mark, small solid arrows).

- **Autocrine pathway** of IL-8 control involves secretion of IL-1 that binds to its receptor, which is expressed also by the cell. This creates an autocrine loop in which IL-1 acts back on the cell and induces IL-8. (large question mark, interrupted arrow).

- **Parallel pathways** indicate that secretion of IL-8 and IL-1 is independent from each other (large solid arrows).
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- **Parallel pathways** indicate that secretion of IL-8 and IL-1 is independent from each other (large solid arrows).
REFERENCES


CHAPTER 4

CONCLUSIONS

Based on our findings we reached the following conclusions:

1. Both IL-8 and IL-8 receptors are present in gingival epithelial cells (GECs) in vivo and in vitro.

2. A. a extract (A.a.E.) can induce dose- and time-dependent IL-8 expression in GECs in vitro.

3. In GECs, A.a.E.-induced expression of IL-8 is not the direct result of major IL-8 induction pathways (i.e. LPS or IL-1).

4. GECs are the only cells studied so far that do not respond to LPS by upregulating IL-8 expression.

5. In GECs, A.a.E.-induced expression of IL-8 involves a p38 MAPK signal transducing pathway unlike the IL-1beta-induced IL-8 expression.

6. GEC are likely a major source of IL-8 in vivo and in vitro.

7. A.a.E.-induced expression of IL-8 in GECs likely contributes to the pathogenesis of periodontal disease.

SIGNIFICANCE

A.a.E.-induced expression of cytokines from GECs likely controls inflammation and repair in periodontal disease. Thus, GECs can be a potential target for therapeutic intervention in the future for the treatment of periodontal disease.
FUTURE DIRECTIONS

Directions for future research relevant to this project include:

- **Identify the mechanisms of A.a.-induced IL-8 expression.** This is an unexplored field since we don’t really know how A.a. induces IL-8 in GECs. We have taken the first step by reporting the role of p38 MAP kinase in the signal transduction pathway.

- **Identify A.a. factors that induce IL-8 expression in GECs.** We have shown that A.a.-induced IL-8 expression is not LPS or IL-1 dependent in GECs. Thus, the mediating factors are yet to be discovered.

- **Suppress in vitro and in vivo IL-8 expression by using antibodies against A.a. virulence factors.** Once the virulence factor(s) of A.a. are discovered, they can be targeted with specific antibodies.

- **Explore the use of p38 MAPK inhibitors to suppress IL-8 expression and inflammation in vivo.** Future clinical trials can utilize cytokine inhibitors to selectively down-regulate the host response to the bacterial antigens without altering basal cytokine expression.

- **Characterize other cytokines induced in GECs by A.a.** We are currently investigating the expression of IL-6 in GECs. However, there is a variety of cytokines to be studied, especially proinflammatory ones, such as TNF-alpha.

- **Determine the role of IL-1 family members in the expression of other cytokines in GECs.** Even though IL-1 does not mediate IL-8 expression in GECs, it may mediate expression of other proinflammatory cytokines. This is yet to be investigated.