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DANIEL NEBEL

FUNCTIONAL IMPORTANCE OF ESTROGEN RECEPTORS IN THE PERIODONTIUM

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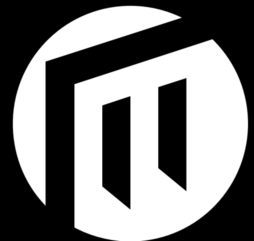

MALMÖ UNIVERSITY

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Cover image:
Gene expression in estrogen treated PDL cells. The microarray chip is displaying the complete human genome on 28,869 genes. Light intensity in each pixel corresponds to level of gene expression in one gene.

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DANIEL NEBEL

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Department of Periodontology
Faculty of Odontology
Malmö University, Sweden 2012

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To Frida, Amandus & Elsie

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LIST OF PAPERS

This thesis is based on the following five papers, which will be referred to in the text by their Roman numerals. The papers are appended at the end of the thesis together with a review paper, not included in the thesis.

I. Daniel Jönsson, **Daniel Nebel,** Gunilla Bratthall, Bengt-Olof Nilsson. LPS-induced MCP-1 and IL-6 production is not reversed by oestrogen in human periodontal ligament cells.
Archives of Oral Biology 2008;**53:**896-902.

II. **Daniel Nebel,** Gunilla Bratthall, Gunnar Warfvinge, Bengt-Olof Nilsson. Effects of ovariectomy and aging on tooth attachment in female mice assessed by morphometric analysis.
Acta Odontologica Scandinavica 2009;**67**:8-12.

III. **Daniel Nebel,** Daniel Jönsson, Ola Norderyd, Gunilla Bratthall and Bengt-Olof Nilsson. Differential regulation of chemokine expression by estrogen in human periodontal ligament cells.
Journal of Periodontal Research 2010;**45:**796-802.

IV. **Daniel Nebel,** Gunilla Bratthall, Eva Ekblad, Ola Norderyd and Bengt-Olof Nilsson. Estrogen regulates DNA synthesis in human gingival epithelial cells displaying strong estrogen receptor β immunoreactivity.
Journal of Periodontal Research 2011;**46:**622-628.

V. **Daniel Nebel,** Joel Arvidsson, Johan Lillqvist and Bengt-Olof Nilsson. Differential effects of LPS from *Escherichia coli* and *Porphyromonas gingivalis* on IL-6 production in human periodontal ligament cells. *Submitted.*

Published paper appended, but not included in the thesis:

Daniel Jönsson, **Daniel Nebel,** Gunilla Bratthall and Bengt-Olof Nilsson. The human periodontal ligament cell: a fibroblast-like cell acting as an immune cell.
Journal of Periodontal Research 2011;**46**:153-7.

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ABSTRACT

The main functions of estrogen are associated with reproduction. However, estrogen has been shown to be of functional importance also in non-classic target organs. Previous studies, especially epidemiologic and clinical ones, have addressed estrogen's influence on periodontitis, suggesting that estrogen has a beneficial effect, but the biological mechanisms have not been identified. Estrogen exerts genomic effects in the target cells by binding to the nuclear receptors, estrogen receptor (ERs), ERα and ERβ. The expression of the two subtypes of ERs varies depending on the tissue. The overall objectives of this thesis were to study the functional importance of estrogen receptors in the periodontium with special focus on inflammation, and stimulators of inflammation and their signaling pathways. The thesis is based on the following five papers.

In **Paper I**, effects of estrogen on *E. coli* LPS-induced PDL cell production of interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and C-reactive protein (CRP) are assessed, by using ELISA. Furthermore, effects of LPS and estrogen on the normal characteristics of the PDL cell such as collagen synthesis and cell proliferation is determined by using L-[3H]proline incorporation and measurement of DNA synthesis, respectively. **Key findings:** *E. coli* LPS stimulates PDL cell IL-6 and MCP-1 production but has no effect on the normal physiological properties of PDL cells. LPS-induced IL-6 and MCP-1 is not reversed by estrogen suggesting that estrogen has no anti-inflammatory effect in these experiments.

In **Paper II,** we investigate the effects of ovariectomy and aging on tooth attachment in female mice by using morphometric analysis. **Key findings:** Withdrawal of female sex hormone production by ovariectomy has no effect on alveolar bone height and apical termination of the junctional epithelium. In a second series of experiments these parameters are similar in mice sacrificed at 8-26 weeks of age, suggesting that tooth attachment is preserved with age in mice within a period of six months.

In **Paper III**, the objective is to investigate the regulation of CCL2/MCP-1, CCL3/MIP-1α, and CCL5/RANTES chemokines by estrogen in human PDL cells by determining mRNA transcript levels (using quantitative real-time PCR) and protein levels (using ELISA). **Key findings:** A physiological concentration of estrogen reduces the expression of CCL3 mRNA by about 40% compared to PDL cells treated with LPS alone. In contrast, inter-individual differences in the effects of estrogen on CCL5 mRNA expression are observed. These findings indicate that estrogen affects chemokine expression in PDL cells showing a complex pattern involving down-regulation as well as up-regulation of chemokines. Estrogen exerts both anti-inflammatory and pro-inflammatory effects through these mechanisms.

In **Paper IV,** ER expression in human gingival biopsies, and effects of estrogen on cultured gingival epithelial cell (HGEP) proliferation, are investigated. Expression of ERα and ERβ is determined by immunohistochemistry and effects of estrogen on HGEP proliferation monitored by measuring DNA synthesis. **Key findings:** HGEP cells show strong ERβ immunoreactivity but low ERα immunoreactivity both *in vivo* and in culture, suggesting that ERβ is the predominant ER subtype in HGEP. High, but not low, concentrations of estrogen attenuates proliferation of gingival epithelial cells, indicating a concentration-dependent mechanism.

In **Paper V**, the objective is to investigate the effects of LPS from *Escherichia coli* and *Porphyromonas gingivalis* on IL-6 production in human PDL cells and endothelial cells, and the signaling mechanisms involved. Quantitative real-time PCR is used to determine IL-6 mRNA levels and ELISA to determine IL-6 protein. **Key findings:** *E. coli* LPS (but not *P. gingivalis* LPS) stimulates IL-6 production in PDL cells. Treatment with the non-selective nitric oxide synthase inhibitor L-NAME reduces IL-6 by 30%, while aminoguanidine, an inhibitor of inducible nitric oxide synthase, does not affect IL-6 levels, showing a mechanism probably involving nitric oxide formation via endothelial nitric oxide synthase. Treatment with the glucocorticoid steroid dexamethasone totally prevents-*E. coli* LPS-induced IL-6 in PDL cells.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Ordet östrogen brukar associeras till det faktum att det är ett kvinnligt könshormon. Östrogen utövar förvisso viktiga effekter i samband med utveckling och utmognad av kvinnans könsorgan samt vid reproduktionen, men har också visat sig ha många andra funktioner i kroppen, hos både kvinnor och män. Östrogen verkar i cellkärnan genom att binda till en östrogenreceptor (ER), som finns i två olika typer, ERα och ERβ.

Parodontit (tandlossning) är en inflammationssjukdom som drabbar tandens fäste som svar på bakterier som normalt finns i munhålan. I cellmembranet hos vissa bakterier finns en molekyl, LPS, som fungerar retande och startar inflammationen genom att binda till TLRs (en mottagarmolekyl) som sitter på vita blodkroppar, men också till vävnadsceller som till exempel PDL celler. Effekten blir att cellerna börjar producera ämnen, bland annat cytokiner och kemokiner, som får fler vita blodkroppar att komma till platsen och inflammationen är då ett faktum. Även om grundorsaken till parodontit är känd vet vi inte fullt ut varför vissa personer drabbas och andra inte. Det är därför viktigt att undersöka om kroppsegna faktorer såsom hormonet östrogen kan påverka mottagligheten för parodontit. Sedan tidigare finns ett flertal studier som visar ett samband mellan förändringar i östrogennivåer och tandlossning. Det övergripande syftet med studierna i denna avhandling var att undersöka ERs betydelse i parodontiet och hur

östrogen via ER påverkar cytokin- och kemokinfrisättning i PDL-celler som aktiveras med bakterietoxinet lipopolysackarid (LPS).

Studie I: LPS får PDL-celler att drastiskt öka produktionen av inflammationsproteinerna, IL-6 och MCP-1. Effekten dämpas inte av östrogen. LPS påverkar inte PDL-cellers normala funktioner, vilket betyder att LPS specifikt stimulerar produktionen av inflammationsproteiner i dessa celler.

Studie II: För att studera effekten av östrogen i parodontiet opererades de östrogenproducerande äggstockarna bort på en grupp av honmöss. Tändernas fästenivå hos denna grupp jämfördes med en kontrollgrupp med normal östrogenproduktion. Efter sex veckor visade det sig inte vara någon skillnad på tandfästet mellan de två grupperna. Då ingen av grupperna utvecklade tandlossning kan ingen säker slutsats dras om östrogens eventuella effekter på tandfästet.

Studie III: Östrogen påverkar PDL-cellers produktion av flera olika inflammationsproteiner, men mönstret är komplext. Ett protein som stimulerar rekrytering av vita blodkroppar (kemokin) hämmas av östrogen medan en annan kemokin förblir oförändrad . För ett tredje inflammationsprotein skilde produktionen sig åt beroende på vilket genetiskt ursprung cellerna hade. Detta tyder på att östrogen verkar både pro- och anti-inflammatoriskt och att det genetiska ursprunget kan påverka östrogenets funktion.

Studie IV: I tandköttet (gingivan) är ERβ den dominerande östrogenreceptorn. Mönstret går igen både på odlade celler och på vävnadsprover från patienter. Tätheten av östrogenreceptorerna skiljer sig inte åt när man jämför vävnadsprov från inflammerade ställen med vävnadsprov från friska ställen. Vid höga doser av östrogen minskar förmågan att dela sig hos gingivala epitelceller. Studien visar att östrogen verkar genom ERβ i tandköttet och att höga östrogenhalter minskar gingivala epitelcellers förmåga att dela sig.

Studie V: PDL-celler producerar olika mängder av inflammationsproteinet IL-6, beroende på vilket LPS de behandlas med. LPS från den parodontitassocierade bakterien, *P. gingivalis* orsakar ingen IL-6 produktion i PDL-celler medan LPS från tarmbakterien *E. coli* ökar IL-6 produktionen med cirka 30 gånger. Effekten verkar involvera kväveoxid (NO). När enzymen, som behövs vid bildandet av NO, blockerades minskade IL-6 produktionen som svar på *E. coli* LPS med 30% vilket indikerar att NO är inblandat i IL-6 produktionen.

Sammanfattningsvis visar studierna att östrogen, sannolikt via ERβ, påverkar parodontiets celler på flera olika sätt. Östrogen utövar både effekter som kan tolkas som skyddande (minskning av produktionen av inflammationsproteiner) men också effekter som kan innebära reducerat skydd (t ex hämning av gingivala epitelcellers celldelning). Studierna bidrar med ny kunskap om den biologiska betydelsen av östrogen i parodontiet. Denna kunskap utgör en viktig grundbult för att förstå hur förändringar i produktion av östrogen kan påverka utveckling av tandlossningssjukdomen.

ABBREVIATIONS USED

CCL	Chemokine ligand
CRP	C-reactive protein
E_2	17β-estradiol
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
GCF	Gingival crevicular fluid
HGEP	Gingival epithelial cells
GPR30	G-protein coupled receptor 30
GRO-α	Growth-regulated oncogene-α
HUVEC	Human umbilical vein endothelial cell
IL	Interleukin
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1 (also CCL2)
MIP-1α	Macrophage inflammatory protein-1α (also CCL3)
MyD88	Myeloid differentiation primary-response gene
NO	Nitric oxide
NOS	Nitric oxide synthase
OvX	Ovariectomy
PCR	Polymerase chain reaction
PDL	Periodontal ligament
P. gingivalis	*Porphyromonas gingivalis*
PMNs	Polymorphonuclear leukocytes
RANKL	Receptor activator of nuclear factor kappa-B ligand

RANTES	Regulated upon activation, normal T-cell expressed and secreted (also CCL5)
SAA	Serum amyloid A
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α

INTRODUCTION

Anatomy of the periodontium

The periodontium comprises different parts: the gingiva, the periodontal ligament (PDL), the root cementum, and the alveolar bone. These are all different types of connective tissues, two of which are mineralized (cementum and bone) and two fibrous (gingiva and PDL). This complex forms an effective support for the teeth. The junction where the gingiva attaches to the tooth is unique. It is the only place where hard tissue penetrates the epithelium, representing a gateway between the inside of the body and the oral cavity that is full of microorganisms. This barrier is of course a vulnerable spot, but works surprisingly well most of the time. Under certain conditions, the barrier weakens and there is a risk of disease in the periodontium.

The gingiva is the soft tissue covering the alveolar bone. It also attaches to the surface of the tooth, with hemidesmosomes forming a relatively tight barrier towards the oral cavity. The gingival tissue consists of an epithelial layer with high turnover of cells and an underlying connective tissue called the lamina propria.

The root surface is covered with hard tissue called cementum, which is formed by the cementoblasts. The cementoblasts first lay down the organic collagen matrix, which later becomes mineralized. The cementum enables the collagen fibers of the PDL to at-

tach to the tooth and is an avascular tissue, which makes it less important in immunological terms.

The periodontal ligament is the connective tissue that attaches to the root cementum on one side and the socket wall of the alveolar bone on the other. The ligament plays an important role in tooth maintenance, tooth mobility, and cementum formation. It also has sensory, nutritive, and homeostatic functions.

The alveolar bone is a specialized mineralized tissue built from an organic matrix of type-I collagen permeated by the mineral hydroxyapatite. In addition, bone consists of noncollagenous proteins e.g. osteocalcin, alkaline phosphatase, and osteonectin.

Cell types

The periodontium harbors a great variety of different cell types that interact with bacteria of the biofilm and the immune system. There are cells derived from all three germ layers: endoderm, mesoderm, and ectoderm. These cells are in direct contact with the bacteria and their products, and form the first line of defense against this threat. Traditionally, the host tissues have only been seen as a target for inflammation, but in recent years more attention has been given to the tissues' role in being a part of the immune response. This thesis focuses on two different types of primary cells found in the periodontium: the periodontal ligament cell (PDL cell) and the gingival epithelial cell (HGEP). Furthermore, primary endothelial cells (HUVEC) and monocytes (THP-1) were used to study endothelial function and immune responses.

Fibroblasts in the periodontium

The fibroblast is a key cell in the periodontal tissues, with its great capacity for synthesis and secretion of fibrous proteins forming the extracellular matrix. It shows a remarkable ability to differentiate and mature into more specialized cell types. Gingival fibroblasts form collagen types I and III, which synthesize the connective tissues of the gingiva. In bone, the matrix is secreted by osteoblasts, a differentiated fibroblast. Embryologically, the fibroblast originates

from the mesodermal germ layer, in contrast to the gingival epithelial cell which is derived from the epidermal germ layer. In addition to collagen formation, fibroblasts may serve other important functions in the periodontium.

Periodontal ligament cells (PDL cells)

PDL cells are fibroblasts, and the most common type of cell in the periodontal ligament. Furthermore, the PDL contains endogenous stem and progenitor cells. These can differentiate into mesenchymal lineages such as osteoblasts, adipocytes, and chondrocytes *in vitro* (Shi *et al.* 2005; Fujii *et al.* 2008). These features make the PDL especially interesting in terms of regeneration after loss of attachment resulting from periodontitis. The collagen in periodontal ligament is formed by the periodontal ligament cells. PDL cells account for about 5–6% of the total volume of the periodontal ligament. PDL cells have a high production of collagen (Overall *et al.* 1987; Somerman *et al.* 1988). Periodontal ligament cells have also been suggested to be osteogenic. In contrast to gingival fibroblasts, PDL cells produce high levels of alkaline phosphatase. Furthermore, PDL cells are capable of producing osteocalcin and form mineral-like nodules (Arceo *et al.* 1991; Nohutcu *et al.* 1997).

PDL cells express both receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG), both of which are important signaling proteins that function in bone and immune cell communication (Kanzaki *et al.* 2001; Hasegawa *et al.* 2002). RANKL is normally expressed in osteoblasts but it serves to differentiate and activate osteoclasts.

Apart from the normal functions of PDL cells, e.g. synthesizing collagen, they have the capacity to initiate inflammation by expressing different cytokines. PDL cells have functional characteristics similar to those of leukocytes and leukocyte-derived cells involved in innate immunity. Interleukins IL-1β, IL-6, and IL-8, and also MCP-1 and TNF-α are examples of cytokines that are expressed by PDL cells after stimulation with bacteria or bacterial LPS (Yamaji *et al.*

1995; Ozaki *et al.* 1996; Okada *et al.* 1997; Agarwal *et al.* 1998; Engels-Deutsch *et al.* 2003; Shu *et al.* 2008; Sun *et al.* 2010;) In addition, PDL cells have also been suggested to participate in immune reactions by expressing surface proteins characteristic of antigen-presenting cells (APCs) (Konermann *et al.* 2011).

Gingival epithelial cells (HGEP)

The gingiva is composed of an outer epithelium and an underlying connective tissue called the lamina propria. These two layers are separated by the basal membrane, which anchors the epithelium to the loose lamina propria. A thin layer of gingival epithelial cells (HGEP or GEC), the basal layer, is situated on the basal membrane. The cells show stem cell properties and are in a constant state of renewal. The proliferation rate is high and the HGEP show high mitotic activity. As new cells proliferate in the basal layer, older cells migrate towards the surface. Shedding of the outermost layers of cells occurs when they reach the surface facing the oral cavity. The desquamation acts as a protective mechanism since bacteria have difficulty in adhering and colonization. The main function of HGEP is to synthesize keratin, and they are therefore often defined as keratinocytes. Depending on the location, the keratin production can be low (e.g. in the cheeks and soft palate) or high (e.g. in the alveolar gingiva and hard palate). When maturing, the HGEP loses many of its functions but the keratin production continues until the cell is shed. Cultured HGEPs are obtained by isolating cells from the basal cell layer. The cells are kept in a highly proliferative and low keratin-producing state.

Human umbilical vein endothelial cells (HUVEC)

Endothelial cells form a thin layer at the interior surface of blood vessels. These cells are responsible for a number of biological functions, e.g. a barrier function by regulating the passage of leucocytes into and out of the bloodstream. Furthermore, endothelial cells have a role in inflammation, angiogenesis, thrombosis, recruitment of white blood cell and fibrinolysis; all important factors in modulating the inflammation response. The function of endothelial cells is therefore of importance regarding periodontitis. Cardiovascular

disease has been shown to be associated with endothelial dysfunction. High levels of dimethylarginine (ADMA) lead to reduction of nitric oxide (NO) concentrations, which in turn leads to hypertension (Deanfield *et al.* 2005). Endothelium of different origin can differ in phenotype. The cells used in paper V are venous endothelial cells derived from the umbilical cord HUVEC.

Periodontitis

There has been little consensus regarding diagnosis and classification of diseases in the periodontium. Historically, different systems have been used, some of them focusing more on the extent of breakdown of the periodontal attachment and some focusing on susceptibility to development of periodontal disease. The fact that periodontitis often has very slow progression, makes it difficult to determine whether there is an ongoing loss of attachment.

In short, a healthy periodontal condition is characterized by a firm non-bleeding gingiva with a shallow (1–3-mm) periodontal pocket. Gingivitis is defined as inflammation of the gingiva without any loss of attachment. Typical signs of inflammation are redness, bleeding on probing, and an increased pocket depth due to swelling. Periodontitis is usually defined as inflammation of the gingiva that extends into the adjacent attachment apparatus. The disease is characterized by loss of clinical attachment due to destruction of periodontal ligament and loss of the adjacent supporting bone. The loss of attachment is usually seen as marginal bone loss in X-rays. In many classification systems, a number of subgroups of gingivitis and periodontitis are defined.

Periodontitis is an inflammatory disease caused by mainly Gram-negative, anaerobic bacteria in a subgingival biofilm, the plaque. Already in the 1960s, Loe and coworkers showed that bacterial plaque causes gingivitis (Loe *et al.* 1965). The mechanism behind gingivitis developing into periodontitis is still not fully understood. Not all patients with gingivitis will go on to develop periodontitis. Periodontitis patients have a wide range of progression rates. While patients with faster-progressing periodontitis will suffer tooth loss,

most patients can, despite the loss of attachment, keep their teeth for the rest of their life. Still, gingival inflammation is seen as a risk factor for the development of periodontitis (Hamp *et al.* 1972). Teeth with consistently inflamed gingiva had a 46-times higher risk of being lost than teeth without gingivitis, within a 26-year period. Gingival inflammation is thus a risk factor for tooth loss (Schatzle *et al.* 2004).

It is now generally accepted that periodontitis is caused by bacteria in the dental plaque (Jenkinson *et al.* 1999). In early periodontal research, much effort was made to identify specific bacterial species that were believed to cause the inflammation. In a periodontal pocket, there are about 500 different bacterial species. Even though several species of bacteria have been isolated and identified as being more likely to be found in a site of periodontitis, the pathogenesis appears to be even more complex. The ecology of the bacterial culture appears to be a more important factor than the specific bacterial species. A study carried out by Loe and coworkers on Sri Lankan laborers who did not get any dental treatment showed that different individuals can respond completely differently to the same bacterial load. It is clear that the patient's susceptibility to periodontitis is crucial to whether attachment loss will occur or not. 8% of the population got periodontitis with rapid progression and subsequent tooth loss, 81% suffered moderate progressive periodontitis, and a group of 11% showed no progression of periodontal disease beyond gingivitis (Loe *et al.* 1986). This study clearly shows that there is a correlation between severity of periodontitis and the susceptibility of the patient. Several other epidemiological studies have shown the same ratio between individuals who develop severe periodontitis, those who develop moderate periodontitis, and those who do not experience any loss of periodontal attachment (Hugoson *et al.* 1998; Albandar *et al.* 1999).

Tobacco smoking and diabetes mellitus with fluctuating blood glucose levels are risk factors for periodontal disease (Jansson *et al.* 2002; Emrich *et al.* 1991) (Figure I). These factors modulate the host response to the bacterial biofilm. This thesis focuses on how estrogen can modulate the host response.

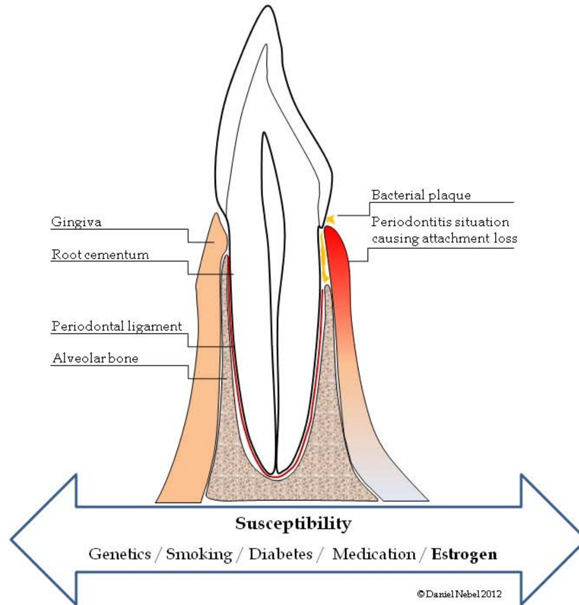

Figure I: Periodontitis is an inflammatory disease initiated by bacterial plaque, but patients do not respond equally to the same bacterial load. It is clear that the patient's susceptibility is a crucial factor for whether or not he/she will develop periodontitis. The factors that affect the patient's susceptibility are not fully understood. This thesis concentrates on the influence of estrogen and on the role of host tissue in initiating inflammation.

The studies conducted and presented in this thesis are experimental basic research and are therefore not directly applicable to any particular periodontal diagnosis.

LPS and TLR signaling

Lipopolysaccharides (LPS) are large molecules found in the outer cell membrane of Gram-negative bacteria. LPS consist of a lipid

and a polysaccharide bound together with a strong, covalent bond. The main function of LPS is to protect the bacteria from chemical substances and to stabilize the structure of the bacteria. LPS also work as an endotoxin by promoting the secretion of pro-inflammatory cytokines e.g. lymphokines, interleukins, and chemokines. Accordingly, LPS trigger a strong immune response. This takes place especially in immune cells, e.g. macrophages and B cells, but also in many other cell types as presented in this thesis.

In short, LPS bind to a complex with LPS-binding protein (LBP), which then binds to the cell-surface receptor CD14. The complex subsequently activates Toll-like receptor 4 (TLR4) (Poltorak *et al.* 1998), which activates multiple intracellular signaling pathways. The intracellular TIR domain (Toll/interleukin-1 receptor-like domain) of TLR4 interacts with other TIR domain-containing intracellular adaptor molecules such as TIRAP (TIR domain-containing adaptor molecule) and MyD88 (myeloid differentiation primary-response protein 88). A signaling cascade continues downstream with activation of several factors, including TRAF6 (TNF receptor-associated factor 6) and IRAK4 (interleukin-1 receptor-associated kinase 4).

Two major TLR4 pathways have been identified: the MyD88-dependent pathway and the MyD88-independent pathway. The early-phase MyD88-dependent pathway involves nuclear factor-κB (NF-κB). Activation of NF-κB leads to production of pro-inflammatory cytokines. The MyD88-independent pathway involves late-phase NF-κB activation and leads to production of interferon beta (IFN-β) (not shown in figure) (Akira *et al.* 2004; Krishnan *et al.* 2007). The signaling via MyD88 leads to stimulation of genes encoding inflammatory cytokines, thus triggering inflammation (Figure II).

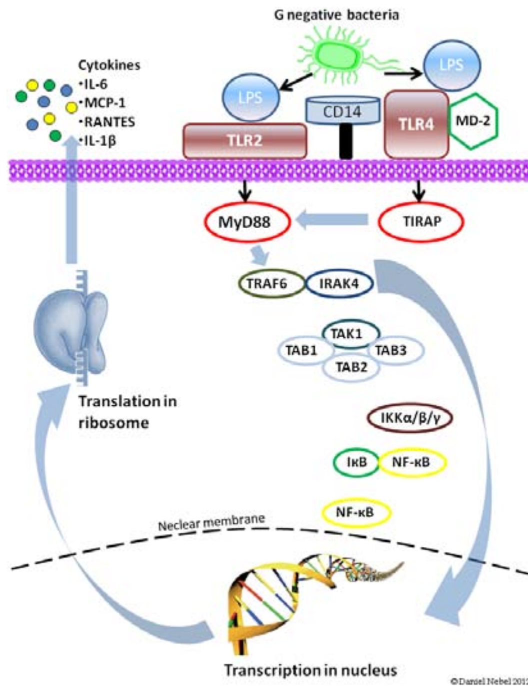

Figure II: LPS from Gram-negative bacteria exert effects on either TLR2 or TLR4. The figure shows the early-phase MyD88-dependent pathway involving a signaling cascade including a number of factors, which ends up in the activation of nuclear factor-κB (NF-κB). The activation leads to upregulation of genes encoding pro-inflammatory cytokines. The MyD88-independent pathway of TLR4 is not shown. (Figure adapted from Akira 2004; Krishnan 2007)

The major transducer of the cellular effects of bacterial LPS is TLR4, but LPS from some bacterial species act through TLR2. The effects of *E. coli* LPS are mediated by TLR4. In contrast, LPS from some periodontal-associated bacteria are recognized by TLR2. *P. gingivalis* LPS-induced IL-6 production is observed in U87 cells

transfected with human TLR2 but not with TLR4, indicating that LPS from *P. gingivalis* signals via TLR2 but not via TLR4 (Hirschfeld *et al.* 2001). LPS from *P. gingivalis* and *C. ochracea* bind to TLR2 but can also work as an antagonist on human TLR4 (Yoshimura *et al.* 2002).

In HUVEC, *E. coli* LPS was found to be a potent inducer of E-selectin, yielding significant expression (Darveau *et al.* 1995). In contrast, LPS obtained from *P. gingivalis* did not induce E-selectin expression, indicating that *E. coli*-derived LPS but not *P. gingivalis-derived* LPS regulates E-selectin expression in this cell type. *P. gingivalis* has also been shown to promote endothelial cell invasion, suggesting that *P. gingivalis* may regulate proliferation of endothelial cells (Deshpande *et al.* 1998). Furthermore, animal experiments have shown that oral injection of *P. gingivalis* accelerates atherosclerotic lesion formation in hyperlipidemic mice (Koizumi *et al.* 2008).

Estrogen

Estrogens are a group of steroid hormones responsible for numerous functions throughout the body. 17β-estradiol (E_2) is the most potent endogenous estrogen, and was used in our studies. In this thesis the term estrogen will be used synonymous with 17β-estradiol.

In 1929, Adolf Butenandt and Edward Adelbert Doisy independently discovered estrogen. They isolated it and determined the structure, and also characterized other steroid hormones including testosterone and progesterone. Butenandt and Doisy were later awarded the Nobel Prize, which illustrates the growing importance of the field of hormone research (Tata 2005).

In 1957, Jensen and Jacobsen came to the conclusion, based on the specific binding of E_2 in the uterus, that the biological effects of estrogen must be mediated by a receptor protein. About thirty years later, two groups reported the cloning of the estrogen receptor (ER) (Green *et al.* 1986; Greene *et al.* 1986). However,

in 1995 a second estrogen receptor, ERβ, was cloned from rat prostate and ovary by a Swedish research group (Kuiper *et al.* 1996).

The expression of ER varies depending on the tissue and organ. Some tissues express one estrogen receptor type exclusively while others express both ERα and ERβ. ERα is widely expressed throughout the reproductive organs such as breast, uterus, and vagina. ERβ has higher expression than ERα in many other organs such as the bladder, intestines, lungs, prostate, salivary glands, and blood vessels (Andersson *et al.* 2001; Matthews *et al.* 2003; Valimaa *et al.* 2004). There are also examples where there can be different expression of ERα and ERβ in the same tissue. Bone has such a pattern. There is higher expression of ERα throughout bone but bone marrow has much higher expression of ERβ (Gustafsson 2003). Importantly, estrogen shows similar affinity for both ER subtypes.

Both men and women produce estrogen. However, estrogen is present at significantly higher levels in women of reproductive age. In men, estrogen is synthesized in the testes while it is the ovaries that are responsible for estrogen production in women. The maturing follicle in the ovary is responsible for the monthly variation in estrogen production during the menstrual cycle. No more follicles mature after menopause, thus causing a dramatic fall in estrogen levels.

Estrogen signaling and functions

Estrogen is synthesized from cholesterol in the gonads. Like other steroid hormones, estrogen works by entering the cell across the cell membrane. In the nucleus, it binds to one or both of the ER's specific domains in the DNA where it can regulate target genes. In this way, estrogen can both stimulate and reduce gene activity, which leads to an increase or decrease in protein synthesis.

Estrogen receptors normally reside in the nucleus but they may also occur in mitochondria. These receptors are inactive in the absence of ligand, but when estrogen enters the nucleus a complex between ligand and receptor is formed, which then regulates transcription. This estrogen-ER complex then connects to specific DNA sites, called estrogen response elements. In turn, this complex binds to coactivator proteins and genes become active or inactive. mRNA is produced by the genes, leading to the synthesis of specific proteins. These proteins influence cell behavior differently, depending on the cell type involved (Dahlman-Wright *et al.* 2006; Nilsson *et al.* 2001).

The main functions of estrogen are associated with reproduction, with effects during the different phases of the menstrual cycle and during pregnancy. Promotion of proliferation of breast cells and cells that form the endometrium of the uterus are just two examples of these functions. However, estrogen has also been shown to have a number of other functions in non-classic target organs. Estrogen receptors are distributed throughout the body, and it has been shown that estrogen affects many processes beside those associated with reproduction.

In the work included in this thesis, we looked more closely at the effects of estrogen on modulation of inflammation in periodontitis.

Estrogen and periodontitis

There have been several epidemiological, clinical, and experimental studies suggesting that estrogen has an impact on gingivitis and periodontitis. Estrogen has both direct and indirect effects on different parameters in periodontitis. Some examples are given below:

- Inhibits release of IL-6 by human marrow cells (Gordon *et al.* 2001)
- Reduces T-cell-mediated inflammation (Josefsson *et al.* 1992)
- Suppresses leukocyte production by the bone marrow (Josefsson *et al.* 1992; Cheleuitte *et al.* 1998)
- Inhibits PMN chemotaxis (Ito *et al.* 1995)

- Reduces gingival inflammation and frequency of clinical attachment loss in osteopenic/osteoporotic women in early menopause (Reinhardt *et al.* 1999)
- Low levels of estrogen allow increased local production of the bone-active cytokine IL-1β in GCF (Reinhardt *et al.* 1994)

Estrogen levels fluctuate throughout the different phases of a woman's life. In childhood estrogen levels are low; during puberty, they rise and then become cyclic with the highest peak when ovulation occurs. During pregnancy, the plasma levels of E_2 rise dramatically during the second and third trimesters, to as much as 10 ng/ml. The serum levels of E_2 during the third trimester of pregnancy are 30–40 times higher than their peak levels during the menstrual cycle (Tulchinsky *et al.* 1972). Figure III shows the estrogen levels during the different stages of a woman's life. Puberty, pregnancy, and menopause—phases associated with dramatic alterations in estrogen levels—may affect periodontal tissues by altering the host response.

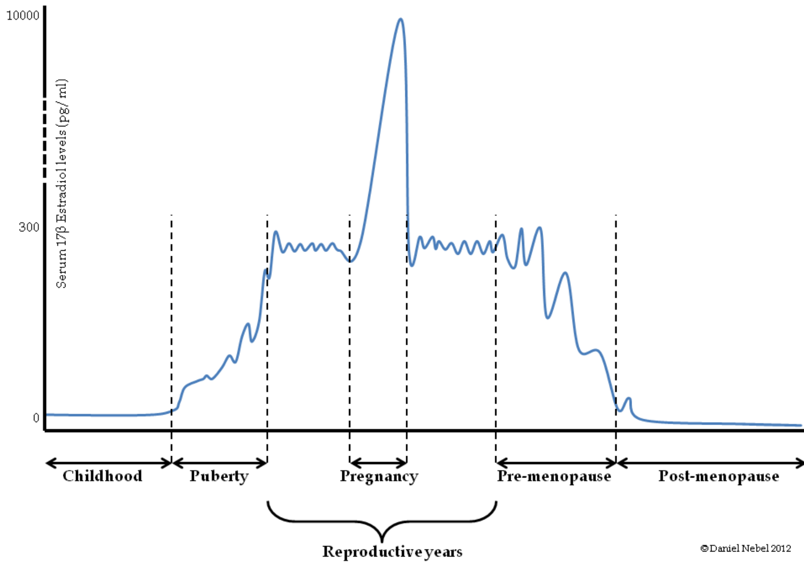

Figure III: Schematic overview of fluctuating estrogen levels in women throughout life. In childhood, estrogen levels are low and stable. During puberty, estrogen levels rise, leading to female characteristics such as breast development. When the menstrual cycle starts, estrogen levels cycle and reach their peak just before ovulation. In pregnancy, the level of estrogen rises dramatically during all trimesters and then reverts post partum. In the pre-menopausal stage, estrogen levels can be both raised and lowered, and then decrease and remain at a very low level during menopause. These levels are significantly lower than in men.

After menopause, E_2 levels decrease to a minimum. Menopause causes permanent cessation of the functioning of the ovaries, and occurs on average at 51 years of age in the western world (Kato et al. 1998). It has been reported that post-menopausal women (without hormone replacement therapy) have a greater risk of developing periodontitis than pre-menopausal women (Haas et al. 2009).

17β-estradiol was found to promote human cementoblast cell proliferation *in vitro* and periodontal regeneration in an experimental periodontitis model in beagle dogs (Nunez *et al.* 2010).

Even though estrogen has been considered a female hormone, levels of serum estradiol in elderly men are higher than those in postmenopausal women. There have only been a few studies addressing the effect of estrogens on periodontitis in men. Estrogen concentrations are not related to periodontal status or number of teeth in elderly men (Orwoll *et al.* 2009).

Importantly, the role of estrogen in bone homeostatis should also be taken into account. It is well known that estrogen stimulates osteoblast function but inhibits osteoclast function, thereby promoting bone formation. At menopause, the low levels of estrogen result in osteoporosis, which has severe effects on the health of women (Lerner 2006).

The general hypothesis is that estrogen has a protective effect via anti-inflammatory mechanisms. However, the picture is complex: some studies have supported the hypothesis while others have not. It can be stated that estrogen exerts effects on the periodontium under both physiological and pathological conditions, but the mechanisms are not fully known. In addition, it has not yet been proven that estrogen has a protective effect against periodontitis as claimed.

Nitric oxide and periodontitis

Nitric oxide (NO) is synthesized endogenously by a group of enzymes called nitric oxide synthase (NOS). The amino acid arginine (L-arginine) functions as a substrate for the reaction (Moncada *et al.* 1991). There are three isoforms of NOS: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). iNOS is present in several cell types such as macrophages and polymorphonuclear cells, and is expressed in response to inflammatory stimuli e.g. IL-1 and LPS (Kendall *et al.* 2001). Furthermore, alveolar bone loss has been shown to be prevented by inhibi-

tors of nitric oxide synthase (NOS) in an experimental periodontitis model, suggesting that nitric NO modulates inflammation and bone resorption in periodontitis (Leitao *et al.* 2005; Herrera *et al.* 2011). Inflammatory cells have the capacity to stimulate NO production in the periodontal lesion. There are higher levels of L-arginine in inflamed gingival tissue than in healthy tissue (Matejka *et al.* 1998), and increased iNOS expression is seen in macrophages and endothelial cells in periodontitis (Lappin *et al.* 2000). Thus, NO appears to have an important function in periodontal disease.

Animal models of periodontitis

For investigation of the etiology and pathogenesis of periodontitis and for the pursuit of new types of treatment, having a good model is crucial. It is not always possible to do research on humans. Periodontal disease often progresses slowly, which complicates the evaluation of treatment results.

Various animal species have been used for the study of periodontal disease, e.g. mice, rats, dogs, ferrets, and pigs. Anatomically, all these animals have a periodontium similar to that of humans with alveolar bone, PDL, root cementum, and gingiva. The number of teeth varies, and of course the size and shape is a not unimportant factor. In rodents, the teeth migrate throughout life. Although dogs have more similarities regarding the human periodontium than mice, murine models are used most, for several reasons. The possibility of using transgenic or gene knockout mice in experimental models creates new opportunities. The number of transgenic and gene knockout mouse models is increasing successively, and will undoubtedly be of great importance for future periodontal research. In knockout mice lacking the lysosomal-associated membrane protein-2 (LAMP-2) gene, periodontitis develops spontaneously (Beertsen *et al.* 2008). This is an example of how the functions of a single gene can be studied in detail.

In order to induce periodontitis in experimental animals, a ligature can be placed in the periodontal pocket. The ligature accumulates bacteria and the loss of attachment is rapid. This method is not

suitable for mice because of the small size of their teeth. It has therefore been important to find other models to study periodontitis in mice. A different method to induce periodontitis in mice is to administer components that initiate inflammation. Injection of LPS adjacent to the teeth has been used for this purpose (Ukai *et al.* 1996).

OBJECTIVES

The specific objectives were:

- To investigate whether LPS from *Escherichia coli* affects production of IL-6, MCP-1, and CRP by PDL cells and/or the normal functional characteristics of PDL cells, and whether estrogen modulates the effects of LPS (paper I).

- To investigate the effects of ovariectomy, i.e. loss of estrogen production, and aging on tooth attachment in female mice (paper II).

- To investigate the regulation of CCL2/MCP-1, CCL3/MIP-1α, and CCL5/RANTES chemokines by estrogen in human PDL cells (paper III).

- To investigate gingival ERα and ERβ expression in health and disease, and the effects of estrogen on gingival epithelial cell proliferation (paper IV).

- To investigate the effects of LPS from *Escherichia coli* and *Porphyromonas gingivalis* on IL-6 production in human PDL cells and endothelial cells, and the signaling mechanisms involved (paper V).

METHODOLOGY

Cells

In four out of five papers included in this thesis, human primary cells were used—i.e. PDL cells, endothelial cells, gingival epithelial cells, and monocytes. There are many positive aspects of using primary cells. The fact that the cells are directly derived from the location you want to study is of course crucial. Culture itself may change the phenotype of the cells, but the cells are probably very similar to the cells in tissue, as opposed to animal cell lines or immortalized cell lines which may differ a great deal from native human cells.

The disadvantage of using human primary cells is mainly that they are more challenging to culture than immortalized cell lines. After a number of population doublings, cells undergo senescence and stop dividing. Consequently, donors are required who consent to donate tissue for this purpose. The process by which cells are obtained is time-consuming and complicated. In recent years, primary cell lines have become commercially available which facilitates laboratory work.

In our laboratory, PDL cells were obtained using an explant culture technique. Both female and male patients aged between 12 and 16 years were PDL cell donors. The patients and their parents were informed and the parents gave written consent. The periodontal ligament is scraped from teeth extracted for orthodontic indications. Only the middle third is used, to avoid contamination from gingival or apical tissues and to avoid possible pathology. The small pieces of connective periodontal tissue are placed under a co-

verslip and then cultured under growth-stimulating conditions, i.e. in the presence of 10% fetal calf serum. The cells divide and migrate from the explant, and large numbers of cells are obtained after just a few passages (Figure IV). The cells show typical features of PDL cells and fibroblast morphology: spindle-shaped cells with a long, branched cytoplasm and one or two nucleoli. When crowded together, PDL cells align themselves locally in parallel clusters. PDL cells were used at passages 3–5 in all experiments. This procedure is widely used, and was first described by Somerman (Somerman *et al.* 1988).

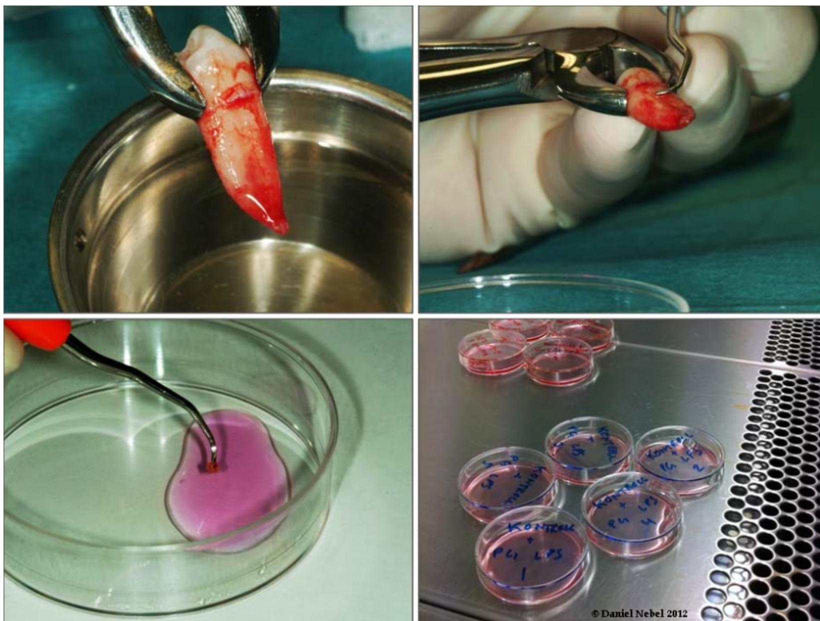

Figure IV: Teeth extracted for orthodontic indications are used to obtain PDL cells. Directly after extraction, the tooth is washed in phosphate-buffered saline and the middle third of the PDL is scraped off with a sterile curette in order to avoid contamination from gingival and apical parts. The explants are seeded in a Petri dish with cell culture medium and 10% fetal calf serum and then cultured at 37°C in an atmosphere of 5% CO_2. Cells were allowed to migrate from the explants, and after approximately two weeks the number of cells was high enough for passage.

In papers IV and V, HGEP and HUVEC primary cells were used. These were bought commercially and were seeded, grown, and harvested in accordance with the directions from the company.

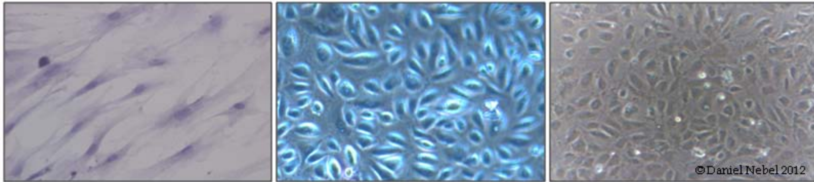

Figure V: Three different types of primary cells were used in the studies. Left: PDL cell fibroblasts stained with hematoxylin-eosin. The cells show characteristic spindle-shaped morphology and are aligned in parallel clusters. Middle: gingival epithelial cells with cuboidal or columnar shape. Right: human endothelial vein cells from the umbilical cord, HUVECs, which are cobble-stone shaped and grow in clusters.

The endothelial cells that we used were derived from the umbilical cord and were vein-derived endothelial cells (HUVEC). Endothelial cells from different organs have shown a wide range of heterogeneity (Garlanda *et al.* 1997). Thus, it cannot be completely ruled out that HUVECs have a different phenotype from gingival microvascular endothelial cells. In recent years, a technique has been developed for isolation of gingival microvascular endothelial cells (DeCarlo *et al.* 2008). Even so, it is challenging to isolate these cells. Contamination with other cell types in culture, limited sources, low yields, and slow proliferation are factors that make gingival endothelial cells difficult to isolate. Even so, irrespective of origin primary human endothelial cells are of interest in periodontal research, since periodontal pathogens (e.g. *P. gingivalis*) have been found in coronary arteries (Marcelino *et al.* 2010), suggesting a link between periodontitis and atherosclerosis.

All of the cell experiments were performed in cell culture medium without phenol red, to avoid the estrogen-like activity of phenol red, using dextran-coated charcoal-stripped fetal calf serum in order to remove the estrogens in serum.

mRNA expression

Affymetrix gene array

Knowing that periodontitis is an inflammatory disease caused by a complex immune response involving the release of hundreds of different proteins, it is difficult to choose any particular one to study. Previous studies have identified a number of proteins that are associated with periodontitis, but it is likely that a number of important pathways have not yet been identified. The human genome is completely mapped. Even though we do not know the functions of all genes, it is known which gene/genes code(s) for a specific protein. Prior to the study resulting in paper III, we carried out an Affymetrix whole-gene array on PDL cells. One group was treated with *E. coli* LPS and the other group was treated with LPS in combination with 500 nM E_2. The objective of the array procedure was to identify genes regulated by E_2 that might be interesting for further investigation. Total RNA was extracted and purified. A complete genome microarray on 28,869 genes was performed, comparing gene expression in the two groups. The cut-off limit was set to a twofold change. Estrogen caused an upregulation of 38 genes, while 28 genes were downregulated. Estrogen-regulated genes were associated with cell metabolism and cell signaling, but so were genes associated with an early inflammatory response. Some of the genes, like CCL3, were targeted with this technique prior to qRT-PCR and ELISA assays.

qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was the technique used for all gene data presented in the papers. One-step quantitative real-time PCR measurements were performed using QuantiFast SYBR Green RT-PCR. Each sample was analyzed in duplicate. Gene expression was calculated using glyceraldehyde-

3-phosphate dehydrogenase (GAPDH) as reference gene, as described by Pfaffl (Pfaffl 2001).

Determination of collagen and DNA synthesis and cell viability

Synthesis of collagen and DNA was assessed by measuring incorporation of radiolabeled proline and thymidine into newly synthesized collagen and DNA, respectively. Cell viability was determined by trypan-blue exclusion test.

Protein expression

Enzyme-linked immunosorbent assay (ELISA) (Engvall *et al.* 1971) was the method used to obtain data on cytokine/chemokine protein production and data on inflammation markers in mice. Levels of cytokines/chemokines were determined in cell supernatants, and these data were normalized to the amount of total protein in each sample using the method of Lowry (Lowry *et al.* 1951). Each sample was analyzed in duplicate.

Morphometry

In paper II, sagital tissue sections from mice mandible were used to assess tooth attachment. After decalcification, the tissue was embedded and cut into thin, 4-µm sections. These sections included teeth with surrounding periodontal tissues: gingiva, root cementum, PDL, and alveolar bone. Tooth attachment was assessed by measuring the alveolar bone height and the apical termination of the junctional epithelium, using image analysis software (Figure VI).

Figure VI: Alveolar bone height in mouse mandible was determined by measuring the distance between the cemento-enamel (C-E) junction of the distal surface of the first molar and the mesial surface of the second molar, respectively, and the highest point on the alveolar crest (A.C.) in longitudinal sections of mandibles stained with hematoxylin and eosin. Bar represents 100 μm.

Immunocytochemistry and immunohistochemistry

In paper IV, immunocytochemistry and immunohistochemistry were used to detect the estrogen receptors in HGEP cells and gingival biopsies. Gingival punch biopsies were used from tissue from the marginal gingival epithelium, including epithelium and connective tissue. The biopsies were obtained from both healthy sites (probing pocket depth < 4 mm and no bleeding on probing) and inflamed sites (probing pocket depth > 5 mm and bleeding on probing) from six individuals (three male subjects and three female subjects). Prior to experiments, different dilutions of the ERα and

ERβ primary antibodies were tested. The sections were stained with either a polyclonal ERα antibody or an ERβ antibody. Mouse (C57BL/6 strain) uterus was included as a positive control for both antibodies. In the negative controls, the primary antibodies were omitted. All scoring was done blind. For each biopsy and staining, at least three sections were analyzed.

Ovariectomy

In the second paper, ovariectomy in female mice was used as a way of studying the effect of estrogen. The method is a well-established experimental technique in rodents, inhibiting endogenous ovarian production of estrogen in order to obtain an experimental model with a hormonal status similar to that observed in post-menopausal women. During the surgical procedure, both ovaries were removed. This led to termination of all estrogen production and the mice therefore become estrogen-deficient.

Statistics

All values are presented as mean ± S.E.M. Statistical significance was calculated using ANOVA and Student's two-tailed t-test for unpaired comparisons with Bonferroni correction for post hoc analysis as appropriate. P-values of less than 0.05 were assumed to indicate statistical significance.

Ethical approval

The studies were approved by the Human Ethical Committee at Lund University, Lund, Sweden (Paper I, III, IV, V) and the Animal Ethics Committee at Lund University, Lund, Sweden (Paper II).

RESULTS AND DISCUSSION

Effects of LPS on PDL cells and HUVEC

We observe at both the mRNA level and the protein level that PDL cells are capable of producing IL-6 when exposed to *E. coli* LPS. IL-6 concentration increased by about 30 times (Figure VII) within 24–72 h (**Papers I** and **V**). Also, an increase in the cellular MCP-1 concentration of 2–3 times was observed when PDL cells were exposed to *E. coli* LPS (**Paper I**). PDL cells express factors that are important in the TLR4 signaling pathway e.g. TLR4, MD-2, and MyD88 (Hatakeyama *et al.* 2003). *E. coli* LPS is a known TRL4 agonist, suggesting that the production of IL-6 and MCP-1 observed in response to this stimulation was mediated though TLR4. By contrast, no stimulation of IL-6 production was seen using *P. gingivalis* LPS as an inducer (**Paper V**). Unlike *E. coli* LPS, *P. gingivalis* LPS acts as a TLR2 agonist and has also been shown to be a TLR4 antagonist (Yoshimura *et al.* 2002). Although TLR2 is expressed in PDL cells (Hatakeyama *et al.* 2003), it is clear that *P. gingivalis* is a weak inducer of IL-6 production. Several other studies have shown that PDL cells have the capacity to express other cytokines also, e.g. GRO-α, IL-1β, RANKL and TNF-α (Jönsson *et al.* 2009; Shu *et al.* 2008; Wada *et al.* 2004). Thus, PDL cells may promote periodontal inflammation.

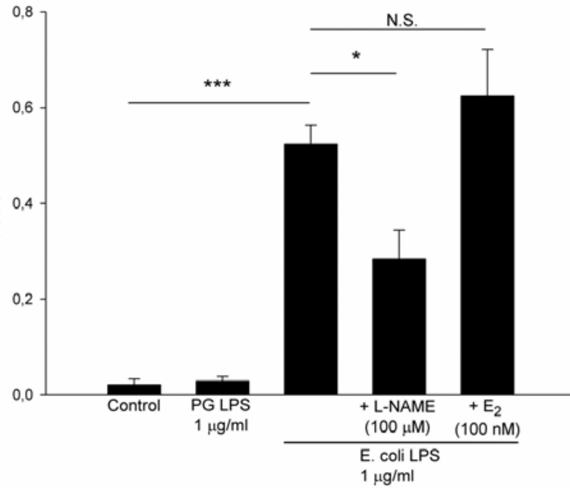

*Figure VII: Human PDL cells were stimulated for 24 h with E. coli LPS or P. gingivalis (PG) LPS (1 μg/ml) in the absence or presence of L-NAME (100 μM) or 17β-estradiol (E_2, 100 nM). IL-6 concentration increased by about 30 times after stimulation with E. coli LPS. The NO synthase blocker, L-NAME, reduced E. coli LPS-induced IL-6 protein production. Values are mean ± S.E.M. *$p < 0.05$; ***$p < 0.001$. N.S., not significant.*

The standardized culture conditions ensure reproducible experiments, and it follows that all the cells have similar phenotype and characteristics. On the other hand, it is likely that periodontal tissue from different patients responds differently in terms of cytokine production. We have shown that estrogen regulates cytokine expression differently in PDL cells depending on the genetic origin of the cells (**Paper III**). It is possible that inter-individual response in cytokine production may be an important factor determining susceptibility regarding development of periodontitis.

The expression of IL-6 in LPS-stimulated PDL cells is considerably lower than that observed in inflammatory cells, e.g. monocytes (**Paper V**). One can ask whether the secretion of pro-inflammatory cytokines by the periodontal fibroblast cells is of clinical relevance.

It is clear that an inflammatory cell has a greater capacity for initiating and driving inflammation, but also that the host tissue cells play a role in inflammation and that they should not be neglected. The number of periodontal host cells greatly exceeds that of inflammatory cells. Furthermore, PDL cells are always present at the location where the inflammation starts, and may have an important role in the initial recruitment of white blood cells to the site of inflammation. When the inflammatory cascade has started, it is likely that the cells of the periodontal tissue have a subordinate role in inflammation.

In contrast to PDL cells, LPS-stimulated HUVEC do not express significantly higher levels of IL-6 than control cells. TLR4 is normally localized in the plasma membrane. In contrast, TLR4 in HUVEC is mainly located in the Golgi apparatus (Makó *et al.* 2010). The cellular localization of TLR4 probably explains the poor stimulation of IL-6 in HUVECs. In summary, LPS-stimulated IL-6 production appears to depend on the cell type.

Effects of estrogen and *E.coli* LPS on the functional properties of PDL cells

E. coli LPS with or without estrogen has no effect on the primary function of PDL cells, i.e synthesis and secretion of collagen. Cell viability and DNA synthesis were also found to be unaffected by LPS and estrogen (**Paper I**), suggesting that estrogen works mainly as a promoter of PDL cell differentiation to a more specialized osteoblast-like cell type, as suggested by Liang. (Liang *et al.* 2008). This indicates that there are no off-target effects of LPS in PDL cells.

High levels of estrogen show an anti-proliferative effect on HGEP cells (**Paper V**). Estrogen causes a reduction in DNA synthesis, both at 500 nM and at 10 μM. This effect is not seen in PDL cells with a lower and more physiological dose of estrogen. It is not clear how the anti-proliferative effect is mediated. Any of the two classical ERs, ERα or ERβ, or the new membrane-bound putative ER, GPR30, may be involved. These high concentrations of estro-

gen are uncommon under physiological conditions. The highest peaks of estradiol are seen during the second and third trimester of pregnancy. There have been several studies showing an increase in gingivitis during pregnancy compared to non-pregnant women (Rakchanok *et al.* 2010). An anti-proliferative effect due to high levels of estrogen (e.g. during pregnancy) could reduce the capacity of gingival epithelial cells to resist the bacterial load in a gingivitis situation. Usually, gingivitis in pregnancy is explained by an increase in angiogenesis and vascular permeability, but based on the data presented in the present thesis an effect of estrogen on gingival epithelial cell proliferation cannot be ruled out.

Estrogen and NOS blocker inhibit cytokine and chemokine production in PDL cells

In **Paper III**, we showed that estrogen regulates chemokine expression in PDL cells. The pattern is complex and involves both a downregulation and an upregulation of chemokines. We showed estrogen-induced downregulation of CCL3 mRNA, while the expression of CCL2 mRNA was unaffected by estrogen. The effect of estrogen on CCL5 expression shows inter-individual variation, suggesting that the genetic origin of PDL cells might influence CCL5 expression (Figure VIII). As will be discussed later, PDL cells express predominantly ERβ. The effect of estrogen is therefore probably mediated through this receptor.

E. coli LPS-induced IL-6 production is inhibited by the NOS blocker L-NAME (100 µM), by about 30% (**Paper V**, Figure VII). The selective iNOS blocker aminoguanidine had no effect on the LPS-induced IL-6. Our results suggest that eNOS but not iNOS may be involved, and they are supported by the results of Kikuiri et al. who showed that human PDL cells express eNOS but not iNOS at both the mRNA level and the protein level (Kikuiri *et al.* 2000).

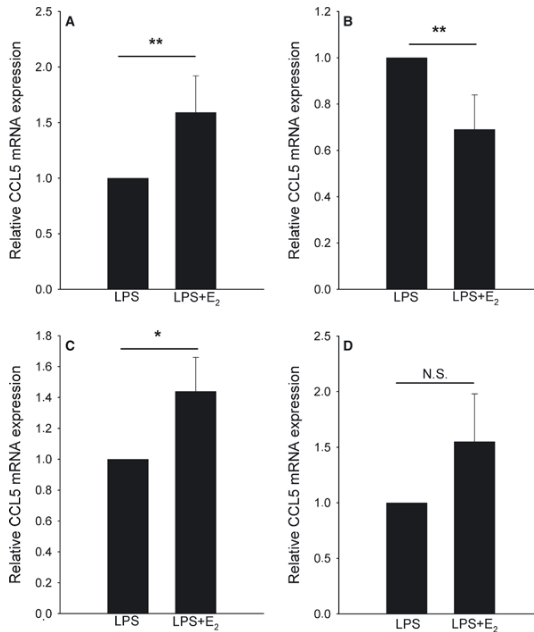

*Figure VIII: The effects of estrogen on PDL cell CCL5 mRNA levels depend on inter-individual variations. PDL cells were treated for 24 h with lipopolysaccharide (LPS) (0.5 lg/mL) in the absence or in the presence of 100 nM E_2. Panels A and B show data from cells derived from the two boys and panels C and D show data from the two girls. Values are means ± S.E.M. *p < 0.05; **p < 0.01. NS, not significant.*

Effects of ovariectomy and aging on mouse periodontium

In **Paper II**, we did not observe any attachment loss in female mice within the observation period of 26 weeks using a morphometric technique. The distance between the cemento-enamel junction and the alveolar crest was similar at all ages. The apical termination of the junctional epithelium was at the cemento-enamel junction at all ages. These findings show clearly that mice do not develop periodontitis spontaneously in the model. The hypothesis was that estrogen would exert a protective, anti-inflammatory effect. In this case, the ovariectomized (OvX) mice would develop more periodontitis than the animals with intact ovaries. However, no difference in tooth attachment was observed between OvX mice and

control mice (**Paper II**). Our results can not exclude a possible effect of estrogen in spite of the fact that we could not see any attachment loss in any of the groups.

There have been a few studies suggesting that OvX rats develop less periodontitis than sham-operated control animals (Duarte *et al.* 2004a; Duarte *et al.* 2004b). In a cotton ligature model, Anbinder and coworkers showed that there was no effect of OvX in rats, in contrast to Duarte (Anbinder *et al.* 2006). Estrogen replacement therapy has been shown to have a positive effect on periodontitis in OvX rats (Duarte *et al.* 2004b). There have been no previous studies showing effects of OvX on tooth attachment in mice. In conclusion, the effects of OvX on periodontium of rodents are still not clear.

Our study showed that young mice do not develop measurable periodontal bone loss. Recently, Liang and coworkers have shown that old mice (>18 months of age) show periodontal bone loss and elevated expression of pro-inflammatory cytokines (Liang *et al.* 2010). These studies thus indicate that older mice could be used to serve as controls in future studies on periodontitis.

There are positive aspects to using a periodontitis model in mice without applying ligatures or other methods for induction of inflammation. The aim when actively inducing periodontitis is to speed up the progression. Even though there is attachment loss, it is not desirable to provoke a fast progression that does not correspond to the typical, slowly developing periodontitis often observed in the clinical situation. Different effects of OvX in mice as compared to rats may also be due to a different bacterial microflora. Another study design with either induction of periodontitis or a longer observation period would better answer the question of whether OvX has any effect on tooth attachment.

Distribution of estrogen receptors in the gingiva

In biopsies from human gingiva, we found strong ERβ immunoreactivity but no or very weak ERα immunoreactivity in all six sub-

jects (Figure IX). The signal was found in the nuclei in cells of all epithelial cell layers and also in cells of the lamina propria (**Paper IV**). The number of subjects is limited, and it cannot totally be ruled out that there are subjects with another ER subtype distribution. These findings are consistent with those of Valimaa *et al.* (2004), who reported that ERβ is the predominant ER in the gingival epithelium. In cultured HGEP a weak signal for ERα was also observed beside a very strong ERβ signal. In tissues, the immunoreactivity is more difficult to interpret due to the complexity of the tissue structure. It is possible that there was also a weak ERα signal in the biopsies that was difficult to identify. Another possibility is that degradation of ERα occurs in the tissue but not in the cultured cells.

No differences in ERα/ERβ expression patterns were observed between male and female subjects. High ERβ expression, but no ERα expression, was observed in both healthy and diseased sites, suggesting that the effects of estrogen on gingival epithelial cells are mediated through ERβ. In a study carried out by Karthik and coworkers, ER expression was determined in four different groups. In the groups of post-menopausal women, there was a significant reduction in ER expression in the gingiva of women with chronic periodontitis compared to those with healthy periodontium (Karthik *et al.* 2009). Comparing Karthik's experiment with ours, there are obvious similarities in the study design. However, we used a "split-mouth-like design" where both the healthy biopsy and the diseased biopsy are obtained from the same donor. This has one big advantage: other factors that are not possible to control will exercise the same influence on both groups. On the other hand, one can only see differences between a healthy and a diseased site in the same patient. The patient's ER expression pattern may influence the susceptibility regarding development of periodontitis, and it was not possible to study this potential relationship using our study design.

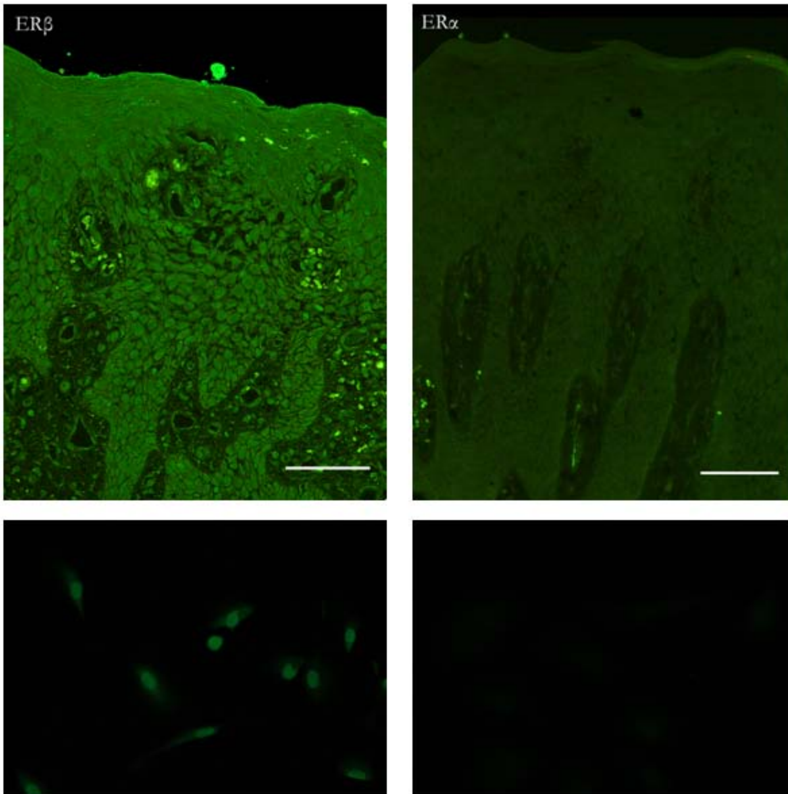

Figure IX: Gingival biopsies (upper row) and gingival epithelial cells (HGEP, lower row) stained for ERβ (left column) or ERα (right column). A stong ERβ signal was seen throughout all tissue preparations and cell samples. Not only epithelial cells but also the cells of the underlying connective tissue, i.e. the lamina propria, expressed ERβ. No or a very weak signal for ERα was seen in the HGEP. No difference in ER expression was seen in biopsies from healthy sites or sites of gingival inflammation (not shown). Bars represent 50 μm.

The same pattern of ER subtype distribution with a strong signal for ERβ has been seen in other cell types in the periodontium, e.g. PDL cells (Jonsson *et al.* 2004). To my knowledge, there have been no studies demonstrating ER in human cementoblasts, a cell type known to be difficult to culture since few cells are viable. In osteoblasts and osteoclasts however, both ERα and ERβ are expressed

(Vanderschueren *et al.* 2004). It is clear that ER expression is cell-type dependent, suggesting a differential effect of estrogen depending on ER subtype expression. If most periodontal cells show the same ER expression pattern, the periodontium could be a target for pharmacological treatment if the effects of estrogen prove to be beneficial. A selective ERβ drug affecting only periodontal cells would not have unwanted side effects in breast tissue, causing proliferation and increased risk of breast cancer, since the proliferative effects of estrogen in breast tissue are mediated by ERα.

CONCLUSIONS

- *E. coli* LPS enhances MCP-1 and IL-6 production in PDL cells, suggesting that PDL cells are capable of recruiting leukocytes to the inflammation site (**Paper I**).

- *E. coli* LPS with or without estrogen does not affect other properties of PDL cells such as proliferation and collagen synthesis. Estrogen does not reverse the LPS-mediated cytokine synthesis (**Paper I**).

- Removal of ovarian production of female sex hormones by ovariectomy for 6 weeks has no influence on tooth attachment, suggesting that estrogen does not have any influence on tooth attachment in mice, within 6 weeks post-ovariectomy (**Paper II**).

- Tooth attachment is preserved with age in mice within a period of six months. This indicates that mice hardly develop periodontitis without active induction within this time frame (**Paper II**).

- Estrogen regulates chemokine expression in PDL cells in a complex manner involving both downregulation and upregulation of gene activity. Estrogen exerts both anti-inflammatory and pro-inflammatory effects through these mechanisms (**Paper III**).

- HGEP cells show strong ERβ immunoreactivity but low ERα immunoreactivity both *in vivo* and in culture, suggesting that ERβ is the predominant ER subtype in HGEP (**Paper IV**).

- Estrogen attenuates DNA synthesis in gingival epithelial cells at high concentrations but not at low concentrations, indicating that there is a concentration-dependent mechanism (**Paper IV**).

- *E. coli* LPS (but not *P. gingivalis* LPS) stimulates IL-6 production in PDL cells through a mechanism probably involving nitric oxide formation via endothelial nitric oxide synthase (**Paper V**).

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LPS-induced MCP-1 and IL-6 production is not reversed by oestrogen in human periodontal ligament cells

Daniel Jönsson [a,b,*], Daniel Nebel [a,b], Gunilla Bratthall [a], Bengt-Olof Nilsson [b]

[a] Department of Periodontology, Faculty of Odontology, Malmö University, SE-205 06 Malmö, Sweden
[b] Department of Experimental Medical Science, Faculty of Medicine, Lund University, BMC D12, SE-221 84 Lund, Sweden

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ABSTRACT

Objective: Periodontal ligament (PDL) cells express oestrogen receptors but the functional importance of oestrogen in PDL cells exposed to bacterial endotoxins is not known. Here we investigate if the inflammation promoter lipopolysaccharide (LPS) affects PDL cell production of interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), C-reactive protein (CRP) and/or normal functional PDL cell characteristics such as collagen synthesis and cell proliferation and if oestrogen modulates the effects of LPS.

Methods: PDL cells were obtained from periodontal ligament of premolars. PDL cells were treated with *Escherichia coli* LPS in the absence or presence of oestrogen (17β-oestradiol, E_2). Cellular concentration of IL-6, MCP-1 and CRP was determined by enzyme-linked immunosorbent assay (ELISA). Collagen synthesis was determined by L-[^{3}H]proline incorporation. Cell proliferation was assessed by DNA synthesis measurement using [^{3}H]thymidine incorporation.

Results: Stimulation with LPS (500 ng/ml to 10 μg/ml) increased IL-6 production in a concentration-dependent manner. Lower concentration (100 ng/ml) of LPS had no effect. LPS-induced stimulation of IL-6 was not reversed by a physiologically high concentration (100 nM) of E_2. LPS increased also MCP-1 production which was unaffected by E_2. Treatment with E_2 alone had no effect on either IL-6 or MCP-1. Stimulation with LPS had no effect on CRP. LPS did not affect collagen synthesis and cell proliferation, reflecting normal physiological properties of PDL cells.

Conclusions: LPS stimulates PDL cell IL-6 and MCP-1 production but has no effect on the normal physiological properties of PDL cells. LPS-induced IL-6 and MCP-1 is not reversed by oestrogen suggesting that oestrogen exerts no anti-inflammatory effect via this mechanism.

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1. Introduction

The periodontal ligament (PDL) is a connective tissue connecting the root cementum of the tooth to the surrounding alveolar bone. The periodontal ligament is composed of PDL cells and connective tissue with high collagen content. The PDL cells are fibroblast-like cells producing collagen. The cells possess also some osteoblastic features, and data have been presented suggesting that PDL cells may change phenotype in response to the inflammation promoter lipopolysaccharide (LPS) and cytokines into a phenotype expressing cytokines and proteolytic enzymes.[1–3]

* *Corresponding author at*: Department of Periodontology, Faculty of Odontology, Malmö University, SE-205 06 Malmö, Sweden. Tel.: +46 40 6658553; fax: +46 40 6658405.
E-mail address: daniel.jonsson@od.mah.se (D. Jönsson).
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IL-6 is supposed to be involved in immune responses associated with pathogenesis of inflammatory diseases in many organs and tissues such as blood vessels, heart, intestine, brain, the musculoskeletal system and periodontium.[4–10] IL-6 induces C-reactive protein (CRP), which is a 115 kDa protein belonging to the acute phase protein group.[11] CRP is induced in response to different pathophysiological conditions including inflammation, infection and cell damage. CRP binds macromolecular ligands such as damaged cell membranes and phospholipids. When bound to ligand CRP may activate the complement pathway and/or function as an antibody.[11] Monocyte chemoattractant protein-1 (MCP-1) is a chemokine molecule stimulating chemotaxis of monocytes to the site of inflammation.[12] MCP-1 is supposed to be involved in the pathogenesis of inflammatory diseases such as atherosclerosis and periodontitis.[13–16]

Oestrogen acts as ligand for oestrogen receptor (ER) α and ERβ, which show a tissue specific distribution.[17–20] ERα and ERβ are not only expressed in classical target organs for oestrogen, such as breast, ovary and uterus, but they are also widely expressed in other tissues.[19–21] The endogenous oestrogen 17β-oestradiol (E_2) binds to both ERα and ERβ with high affinity.[22] Besides affecting development and growth of breast and uterus oestrogen has been reported to affect many other tissues and organs.[20,21] Oestrogen has been reported to possess anti-inflammatory properties in CNS and in the cardiovascular system involving, e.g. reduced monocyte recruitment.[23–26]

Previous observations suggest weak expression of ERα mRNA in human PDL cells.[27] We have previously shown that human PDL cells express preferentially ERβ protein, suggesting that the effects of oestrogen in human PDL cells are mediated mainly via this ER subtype.[28] Oestrogen has been reported to stimulate PDL cell mineralised nodule formation.[29] We have recently shown that proliferation of cultured human PDL cells is unaffected by oestrogen treatment under physiological conditions.[30] Treatment with oestrogen alone has no effect on collagen formation as shown both in rat periodontal tissues and in human PDL cells.[30,31] The functional importance of oestrogen in PDL cells exposed to bacterial endotoxins such as LPS has not been investigated. The aims of the present study were to investigate if LPS affects PDL cell production of IL-6, MCP-1, CRP and/or normal functional PDL cell characteristics such as collagen synthesis and cell proliferation and if oestrogen may modulate the effects of LPS.

2. Materials and methods

2.1. Cells

PDL cells were obtained from premolars extracted for orthodontic reasons from two female subjects 12 and 13 years of age. The patients and their parents were informed and the parents gave written consent. The study was approved by the Human Ethical Committee at Lund University, Lund, Sweden. The periodontal ligament was gently scraped off from the middle third of the root surface to avoid contamination from the gingival and apical tissues. Tissue explants from four premolars in each subject were seeded providing eight clones of PDL cells. These clones of cells responded identically to LPS and E_2. No differences in the responses to LPS and E_2 were observed between clones derived from the two female subjects. The tissue explants were transferred to cell culture flasks containing Dulbecco's modified Eagle's medium supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml), glutamine (1.16 g/l) and 10% fetal calf serum. The flasks were placed in a water-jacketed cell/tissue incubator with 5% CO_2 in air. The cells were allowed to migrate from the explants and after reaching confluence the cells were trypsinated (0.25%) and reseeded at a density of 80,000 cells/ml. Experiments were performed on subconfluent cells in passages 3–5. Stimulation of DNA synthesis in human breast cancer MCF-7 cells by E_2 was used as positive control. The MCF-7 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as the PDL cells.

2.2. Experimental procedure

Before experiments the normal culture medium was exchanged for phenol red-free culture medium containing dextran-coated charcoal stripped fetal calf serum to remove the oestrogen-like activity of phenol red and oestrogens derived from the serum. The phenol red-free culture medium with charcoal stripped fetal calf serum was used throughout the experiment. After 6 h pre-treatment with or without a physiological concentration of E_2 (100 nM, Sigma Chemicals, St. Louis, MO, USA) the cells were treated with or without *Escherichia coli* 0111:B4 LPS (100 ng/ml to 10 μg/ml, Sigma Chemicals, St. Louis, MO, USA) for 24 h, 72 h, 5 days or 21 days. In order to detect any possible effect of oestrogen we used a physiologically high concentration (100 nM) of E_2. The pre-ovulatory plasma concentration of E_2 is around 2 nM but increases in pregnancy several fold to about the same concentration as used by us in the present study.[32,33] For 3 weeks treatment medium and drugs were exchanged every week to ensure optimal experimental conditions.

At the end of the incubation cells were washed carefully in PBS and scraped off from the culture flasks using commercially available cell scrapers (Sarstedt, Newton, NC, USA). Then the cells were sonicated 2$\times$ 10 s on ice and centrifuged at $1700 \times g$ at 4 °C for 5 min. The supernatant was collected for the determination of total protein, IL-6, MCP-1 and CRP. Cell lysate was used for the determination of collagen and DNA synthesis.

2.3. Assessment of cell viability and cell morphology

Cell viability was determined by trypan-blue exclusion test. Cells were washed in 0.9% NaCl and then incubated for 2 min with 0.4% trypan-blue. The cells were washed three times to remove unspecific staining and the number of stained cells was determined using an Olympus CK40 microscope (Olympus Europa GmbH, Hamburg, Germany). Cell morphology was assessed by phase contrast microscopy using an Olympus CK40 microscope.

2.4. Measurement of IL-6, MCP-1 and CRP protein

IL-6, MCP-1 and CRP were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis,

MN, USA). Determination of protein concentration by ELISA was performed as recommended by the manufacturer. The concentration of IL-6, MCP-1 and CRP proteins was normalized to the total protein concentration. Total protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) based on the Lowry method.[34]

2.5. *Determination of collagen and DNA synthesis*

Collagen and DNA synthesis were determined by measuring the incorporation of L-[^{3}H]proline (10 μCi) and methyl-[^{3}H]thymidine (10 μCi), both radioisotopes from Amersham (Amersham Biosciences Europe, Uppsala, Sweden), into newly synthesized collagen and DNA, respectively, as described.[30,35,36] The radiolabelled proline and thymidine were present during the last 24 h of the incubation. Radioactivity was measured in a Beckman liquid scintillation counter (Beckman LS6500, Beckman Instruments Inc., Fullerton, CA, USA), expressed as disintegrations per minute (DPM) and normalized to total protein concentration.

2.6. *Statistics*

Values are presented as means ± S.E.M. Statistical significance was calculated using ANOVA and Student's two-tailed *t*-test for unpaired comparisons with Bonferroni test for post hoc analysis as appropriate. *P* values less than 0.05 were regarded to denote statistical significance.

3. Results

3.1. *Effects of LPS and oestrogen on PDL cell IL-6 concentration*

Stimulation with 500 ng/ml and 10 μg/ml LPS for 24 h increased the cellular IL-6 concentration by three and nine times, respectively (Fig. 1). A lower LPS concentration (100 ng/ml) had no effect on IL-6 (0.07 ± 0.06 pg/μg protein in LPS-treated cells vs. 0.08 ± 0.01 pg/μg protein in control cells, *n* = 3 observations in each group). Since no effect of 100 ng/ml LPS was observed on IL-6 production only higher concentrations of LPS were used in the subsequent experiments. Combined treatment with a physiologically high concentration of E_2 (100 nM) and LPS had no effect on LPS-induced IL-6 production (Fig. 1). Oestrogen-alone (100 nM E_2) treatment had no effect on IL-6 production (Fig. 1). After stimulation with LPS (500 ng/ml) for 72 h IL-6 was detected in three out of three cell samples (0.34 ± 0.03 pg/μg protein, *n* = 3), while no IL-6 was detected in four out of four control samples. Co-treatment with 500 ng/ml LPS and E_2 (100 nM) had no effect on LPS-induced IL-6 also at this longer time-point (0.34 ± 0.03 pg/μg protein in LPS-treated cells vs. 0.26 ± 0.08 pg/μg protein in LPS + E_2-treated cells, *n* = 3 in each group). Stimulation with 10 μg/ml LPS for 72 h increased IL-6 by about 10 times, i.e. with the same magnitude as observed at 24 h (0.88 ± 0.28 pg/μg protein in LPS-treated cells vs. 0.09 ± 0.05 pg/μg protein in control cells, *n* = 3 in each group, $P < 0.05$). These data show that LPS has similar effects at 24 and 72 h of stimulation suggesting that the responses to LPS reach a plateau within this time frame.

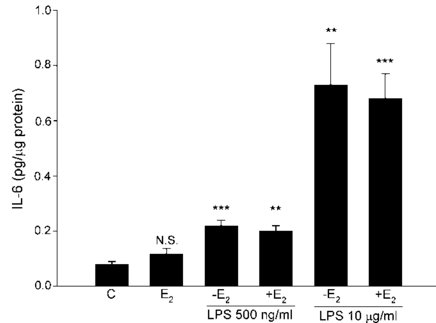

Fig. 1 – Cellular concentration of IL-6 after stimulation 24 h with E_2 alone (100 nM) and with LPS (500 ng/ml or 10 μg/ml) in the absence or in the presence of 100 nM E_2. LPS increases IL-6 in a concentration-dependent manner. Values are presented as means ± S.E.M. of three to six observations in each group. **P < 0.01 and ***P < 0.001 when compared with control samples (C). N.S. = not significant. No significant difference was observed between cells treated with LPS in the absence or presence of E_2. Statistical significance was calculated using ANOVA with Bonferroni test for post hoc analysis.

3.2. *Effects of LPS and oestrogen on PDL cell MCP-1 concentration*

Stimulation with LPS (500 ng/ml and 10 μg/ml) for 24 h increased the cellular MCP-1 concentration by two to three times (Fig. 2). As shown in Fig. 2, the increase in LPS-induced MCP-1 production was not reversed by E_2 treatment (100 nM). A 20 times increase in LPS concentration from 500 ng/ml to 10 μg/ml had a similar effect on MCP-1 production showing that the concentration–response curve reaches plateau-phase already at 500 ng/ml. Stimulation with LPS (10 μg/ml) for 72 h increased MCP-1 by about five times (3.14 ± 0.90 pg/μg protein in LPS-treated cells vs. 0.59 ± 0.09 pg/μg protein in control cells, *n* = 3 in each group, $P < 0.05$). Treatment with E_2 alone for 72 h had no effect on MCP-1 (0.83 ± 0.12 pg/μg protein in E_2-treated cells vs. 0.59 ± 0.09 pg/μg protein in control cells, *n* = 3 in each group). Long-term (21 days) treatment with LPS (500 ng/ml) increased MCP-1 by about four times (4.67 ± 0.36 pg/μg protein in LPS-treated cells vs. 1.23 ± 0.05 pg/μg protein in control cells, *n* = 4 in each group, $P < 0.001$). The LPS-induced increase in MCP-1 production was not reversed by 100 nM E_2 (4.77 ± 0.57 pg/μg protein, *n* = 4). LPS causes a similar increase in MCP-1 production at all time-points under study showing that maximal response is evoked within this time frame.

3.3. *Effects of LPS and oestrogen on PDL cell CRP concentration*

Stimulation with 500 ng/ml and 10 μg/ml LPS for 24 h had no effect on the cellular concentration of CRP as compared to

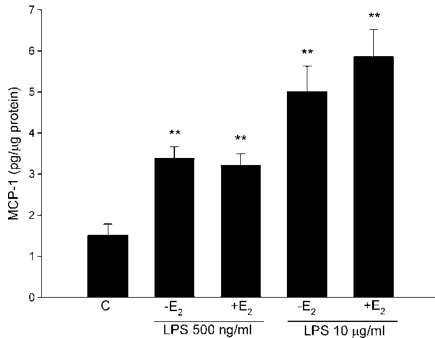

Fig. 2 – Stimulation with 500 ng/ml and 10 μg/ml LPS for 24 h increases the cellular MCP-1 concentration by two to three times. The LPS-induced increase in MCP-1 is not reversed by co-incubation with 100 nM E_2. Values are presented as means ± S.E.M. of three to six observations in each group. $^{**}P < 0.01$ when compared with control samples (C). No significant difference was observed between cells treated with LPS in the absence or presence of E_2. Statistical significance was calculated using ANOVA with Bonferroni test for post hoc analysis.

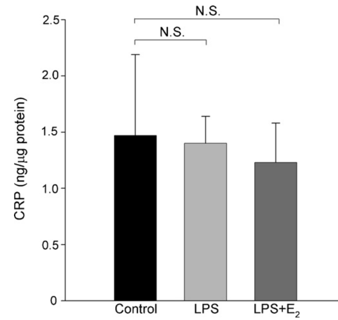

Fig. 3 – Stimulation with LPS (500 ng/ml) for 72 h in the absence or presence of 100 nM E_2 has no effect on cellular CRP concentration. Values are presented as means ± S.E.M. of four observations in each group. N.S. = not significant. No significant difference was observed between cells treated with LPS in the absence or presence of E_2. Statistical significance was calculated using ANOVA with Bonferroni test for post hoc analysis.

untreated control cells (0.25 ± 0.16 and 0.31 ± 0.19 ng/μg protein in response to 500 ng/ml and 10 μg/ml, respectively vs. 0.45 ± 0.39 ng/μg protein in control samples, $n = 3–6$). Combined treatment with LPS and 100 nM E_2 had no effect on CRP as compared to control cells and cells treated with LPS alone (data not shown). Also treatment with LPS (500 ng/ml) for a longer time period (72 h) in the absence or presence of 100 nM E_2 lacked effect on CRP concentration (Fig. 3).

3.4. *Assessment of cell viability*

The trypan-blue exclusion test showed very few trypan-blue stained PDL cells in control, LPS (500 ng/ml) and E_2 (100 nM)-treated culture flasks, suggesting high cell viability. Cell morphology, assessed by phase contrast microscopy, was not affected by treatment with 100 ng/ml to 10 μg/ml LPS or 100 nM E_2 (not shown).

3.5. *Effects of LPS on collagen and DNA synthesis*

Treatment with low (500 ng/ml) and high (10 μg/ml) concentration of LPS for 72 h had no effect on collagen synthesis assessed by [^{3}H]proline incorporation (Fig. 4). Also treatment with LPS (500 ng/ml) with or without 100 nM E_2 for a longer time period (5 days) had no effect on collagen synthesis as compared to untreated control cells (Fig. 5). Treatment with E_2 alone (100 nM) for 72 h had no effect on collagen synthesis (113,788 ± 9128 DPM/mg protein in E_2-treated cells vs. 92,006 ± 8149 DPM/mg protein in control cells, $n = 3$ in each group).

PDL cell proliferation, assessed by DNA synthesis measurement, was unaffected by stimulation for 72 h with high concentration (10 μg/ml) of LPS (Fig. 6). Also co-incubation with LPS (10 μg/ml) and E_2 (100 nM) lacked effect on DNA synthesis (Fig. 6). Long-term (21 days) stimulation with LPS (500 ng/ml) had no effect on DNA synthesis (390,138 ± 48,788 DPM/mg protein in LPS-treated cells vs. 348,266 ± 52,141 DPM/mg

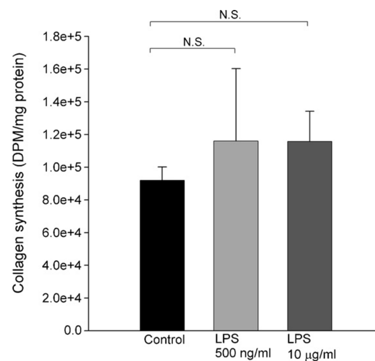

Fig. 4 – Stimulation with low (500 ng/ml) and high (10 μg/ml) concentrations of LPS for 72 h has no effect on collagen synthesis determined by incorporation of [^{3}H]proline into newly synthesized collagen. Values are means ± S.E.M. of three observations in each group. N.S. = not significant. Statistical significance was calculated using ANOVA with Bonferroni test for post hoc analysis.

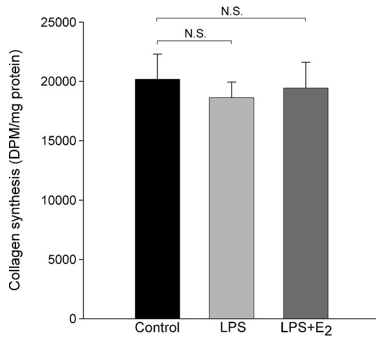

Fig. 5 – Treatment with LPS (500 ng/ml) for 5 days in the absence or presence of 100 nM E_2 has no effect on collagen synthesis. Values are means ± S.E.M. of four observations in each group. N.S. = not significant. No significant difference was observed between cells treated with LPS in the absence or presence of E_2. Statistical significance was calculated using ANOVA with Bonferroni test for post hoc analysis.

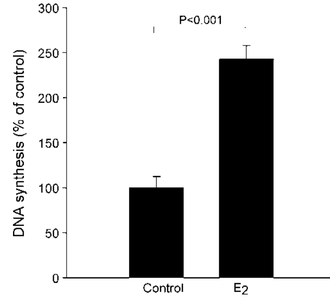

Fig. 7 – Treatment with 100 nM E_2 for 24 h stimulates DNA synthesis more than two times in human breast cancer MCF-7 cells used as positive control. DNA synthesis was determined by measuring incorporation of [^{3}H]thymidine into newly synthesized DNA and normalized to control values. Values are means ± S.E.M. of four observations in each group. Statistical significance was calculated using Student's two-tailed *t*-test for unpaired comparisons.

protein in control cells, $n = 4$ in each group). For positive control the effect of E_2 (100 nM) on DNA synthesis in human breast cancer MCF-7 cells was determined. As seen in Fig. 7 treatment with 100 nM E_2 for 24 h increased MCF-7 cell DNA synthesis by more than two times, showing that the physiologically high concentration of E_2 used in the present study is active.

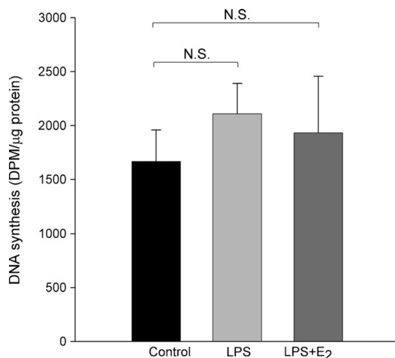

Fig. 6 – Treatment with LPS (10 μg/ml) for 72 h in the absence or presence of 100 nM E_2 has no effect on DNA synthesis. DNA synthesis was assessed by measuring incorporation of [^{3}H]thymidine into newly synthesized DNA. Values are means ± S.E.M. of three observations in each group. N.S. = not significant. No significant difference was observed between cells treated with LPS in the absence or presence of E_2. Statistical significance was calculated using ANOVA with Bonferroni test for post hoc analysis.

4. Discussion

In the present study we show that PDL cells, normally regarded as fibroblast-like cells producing collagen contributing to formation and regeneration of the periodontal ligament,[1] also produce IL-6 and MCP-1 when exposed to LPS. The time course reveals that maximal effect of LPS stimulation on IL-6 and MCP-1 production is achieved within 24–72 h. Our results on LPS-induced IL-6 production confirm findings by Ogura et al. [37] reporting that LPS from *Porphyromonas endodontalis*, isolated from infected root canals, induces IL-6 production in PDL cells and findings by Yamamoto et al. [3] reporting that *Porphyromonas gingivalis* induces mRNA for IL-6 in PDL cells. *E. coli* LPS is regarded to differ, both structurally and in its biological properties, from *P. gingivalis* LPS, but nevertheless the responses (e.g. cytokine mRNA expression) to *E. coli* LPS (0111:B4), the same LPS as we have used in the present study, have been shown to be similar as those to *P. gingivalis* LPS in human PDL cells.[38] MCP-1 stimulates leukocyte chemotaxis and is thought to be involved in the pathogenesis of various inflammatory diseases such as periodontitis.[12–16] The increase in PDL cell MCP-1 production by LPS suggests that the PDL cells, exposed to bacterial endotoxins, stimulate recruitment of leukocytes to the periodontal inflammation process by producing MCP-1. To our knowledge this is the first report showing that LPS increases MCP-1 production in human PDL cells. MCP-1 produced by PDL cells exposed to bacterial endotoxins may contribute significantly to the recruitment of leukocytes and thus be involved in the pathogenesis of periodontitis.

Results presented here show that the bacterial endotoxin LPS induces IL-6 and MCP-1 but not CRP production in human PDL cells. To unravel any time-dependence in the CRP response we tested the effects of LPS at both 24 and 72 h but at no time-point LPS had any effect on CRP production. The PDL cells expressed small but detectable amounts of CRP proposing that PDL cells produce constitutively CRP. CRP is produced mainly by the liver hepatocytes in response to cytokine stimulation, but other cell types, like PDL cells, may contribute to a local production.[11] Hepatocytes, predominantly via transcriptional control by IL-6, are responsible for the increase in systemic plasma CRP observed in response to inflammation, e.g. periodontitis, and tissue damage.[11,39–41]

The functional importance of oestrogen in PDL cells may involve stimulation of mineralised nodule formation but may also involve regulation of other cellular processes.[29] There are reports suggesting that oestrogen may exert an anti-inflammatory effect via reduction of leukocyte recruitment and their adhesion to the vascular endothelium via a mechanism involving suppression of MCP-1 and vascular cell adhesion molecule-1.[24–26,42–44] In the present study we show that the oestrogen E_2, in physiological concentrations (nM), has no effect on LPS-induced IL-6 and MCP-1 protein production in human PDL cells, suggesting that oestrogen exerts no anti-inflammatory effect via this mechanism. For standardization all experiments reported here were performed in PDL cells derived from female subjects. There is no reason to believe that PDL cells from male subjects would have responded differently to oestrogen since $ER\alpha$ and $ER\beta$ protein expression pattern is identical in male and female PDL cells.[28] Divergent effects of oestrogen on IL-6 have been reported in different experimental systems. Oestrogen reduces the IL-6 expression in injured arteries,[42] while it increases IL-6 in corneal epithelial cells.[45] MCP-1 been shown to be down-regulated by oestrogen in blood vessels, macrophages and in human breast cancer MCF-7 cells.[43,46,47] Our results show that neither LPS-induced IL-6 nor LPS-induced MCP-1 protein production is regulated by oestrogen in human PDL cells. These results suggest that oestrogen may affect IL-6 and MCP-1 protein expression differently depending on cell type, probably reflecting differences in cellular ER subtype expression pattern.

$ER\alpha$, $ER\beta$ and LPS regulate gene transcription via multiple mechanisms.[20,48] $ER\alpha$ and LPS have been reported to regulate gene transcription via the transcription factor $NF\kappa B$, suggesting that oestrogen, via $ER\alpha$, may influence LPS signalling at this level in the signalling pathway.[20,48] Here we show that oestrogen has no effect on LPS-induced IL-6 and MCP-1 protein production in human PDL cells, suggesting that oestrogen has no effect on LPS-induced $NF\kappa B$ in this experimental system. Human PDL cells show high expression of $ER\beta$ but low expression of $ER\alpha$,[28] and thus the lack of effect of oestrogen on $NF\kappa B$ signalling in PDL cells may be explained by the ER subtype expression pattern observed in these cells.

LPS stimulated IL-6 and MCP-1 protein production but it had no effect on normal PDL cell characteristics such as collagen formation and cell proliferation. The cells were stimulated with low and high concentrations of LPS for different times but under no experimental condition LPS affected normal cell characteristics. Treatment with LPS for as long as 3 weeks caused no alteration in cell proliferation. These data suggest that the bacterial endotoxin LPS affects specifically cytokine and chemokine production, while it has no effect on the normal physiological properties of PDL cells. Our data confirm findings by Yamamoto et al. [3] showing unaltered morphology in human PDL cells co-cultured with *P. gingivalis* or *Prevotella intermedia*. Thus, PDL cells seem to preserve their structural and physiological properties, i.e. collagen synthesis and proliferation, even when they are exposed to bacterial endotoxins such as LPS.

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ORIGINAL ARTICLE

Effects of ovariectomy and aging on tooth attachment in female mice assessed by morphometric analysis

DANIEL NEBEL[1,2], GUNILLA BRATTHALL[1], GUNNAR WARFVINGE[3] & BENGT-OLOF NILSSON[2]

[1]*Department of Periodontology, Faculty of Odontology, Malmö University, Malmö, Sweden,* [2]*Department of Experimental Medical Science, Lund University, BMC D12, Lund, Sweden and* [3]*Department of Oral Pathology, Faculty of Odontology, Malmö University, Malmö, Sweden*

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Abstract

Objective. Non-human primates, dogs, rats, hamsters and ferrets, have frequently been used as laboratory animals in periodontal biology and pathology. In the past, mice have been used less for this purpose, but nowadays attract a lot of interest because gene knockout and transgenic technologies utilize mice primarily. In this study, we investigate the effects of ovariectomy and aging on tooth attachment in female mice. *Material and methods.* Eight-week-old mice ($n = 15$) were divided into three experimental groups (control, $n = 5$; sham-operated, $n = 5$; ovariectomy, $n = 5$) and ovaries removed bilaterally. Attachment level, assessed by measuring alveolar bone height and apical termination of the junctional epithelium, was determined 6 weeks post-ovariectomy by digital morphometric analysis in sagittal sections of the mandible. The plasma level of the inflammation marker serum amyloid A (SAA) was determined by ELISA. In another series of experiments, tooth attachment was determined in female mice ($n = 7$) at 8–26 weeks of age. *Results.* Withdrawal of female sex hormone production by ovariectomy had no effect on alveolar bone height and apical termination of the junctional epithelium. The SAA level in plasma was unaffected by removal of the ovaries, suggesting that systemic inflammation is not induced by ovariectomy. Bone height was similar in mice sacrificed at 8–26 weeks of age and apical termination of the junctional epithelium was at the cemento-enamel junction at all ages. *Conclusions.* Removal of ovarian production of female sex hormones by ovariectomy has no influence on tooth attachment, and further tooth attachment is preserved with age in female mice.

Key Words: *Morphometry, mouse, ovariectomy, serum amyloid A, tooth attachment*

Introduction

Gene knockout technology developed in mice has attracted a great deal of interest and focus on the mouse as a laboratory animal. Knockout of genes in mice, important in periodontal biology and pathology, requires a phenotypic characterization of mouse periodontal tissues. In periodontology, animal studies have been performed mostly in non-human primates, dogs and rats, while fewer studies have been conducted in mice [1].

It has been suggested that female sex hormones influence periodontal tissues and the development of periodontal disease [2]. During pregnancy, which is associated with high levels of estrogen and progesterone in plasma, many women develop gingivitis and after menopause, which is associated with low levels of estrogen and progesterone, changes in periodontal tissues that may affect tooth attachment have been reported [3–6]. A higher frequency of gingival bleeding has been reported in estrogen-deficient women compared to women with normal levels of estrogens [5,7]. Post-menopausal women subjected to estrogen supplementation therapy have been reported to develop less clinical attachment loss over a 6 years period than those not on estrogen supplementation therapy [8], suggesting that estrogen may have a protecting effect. Ovariectomy is a well-established experimental technique in rodents inhibiting endogenous ovarian production of estrogens in order to obtain an experimental model with a hormonal status similar to that observed in post-menopausal women.

Correspondence: Bengt-Olof Nilsson, Department of Experimental Medical Science, Lund University, BMC D12, SE-221 84 Lund, Sweden. Tel: +46 46 2227767. Fax: +46 46 2224546. E-mail: bengt-olof.nilsson@med.lu.se

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Estrogen is a well-known regulator of bone metabolism and is assumed to act by inhibiting osteoclast formation and by reducing the bone-resorbing activity of terminally differentiated osteoclasts [9]. Estrogen deficiency thus causes elevated osteoclast activity and subsequently may lead to osteoporosis affecting tooth attachment. Elevated osteoclast formation and activity is mediated via increased receptor activator of nuclear factor κB ligand (RANKL)/osteoprotegerin (OPG) ratio and estrogen deficiency may either directly or indirectly, via elevated cytokine expression, increase this ratio [9].

Estrogen is assumed to exert both anti- and pro-inflammatory effects [10]. Autoimmune diseases such as rheumatoid arthritis are more common in women than in men suggesting that female sex hormones are pro-inflammatory, but, on the other hand, rheumatoid arthritis often improves during pregnancy, suggesting that the female sex hormones also have a beneficial anti-inflammatory effect [11]. Estrogen has been reported to reduce expression of cytokine and adhesion molecule expression in the brain and in the vasculature, thereby acting as an anti-inflammatory agent [12–14]. We and others have reported that human periodontal ligament cells express estrogen receptors, proposing that estrogen affects periodontal tissue function [15,16]. It can therefore be hypothesized that withdrawal of female sex hormone production by ovariectomy may affect the inflammatory and immunological responses, which in turn influence tooth attachment level. Changes in systemic inflammation response can be demonstrated by measuring the plasma level of the inflammation marker SAA.

The objectives of the present study were to determine the effects of withdrawal of sex hormone production by ovariectomy and aging on tooth attachment, i.e. alveolar bone height and connective tissue attachment, in female mice by using morphometric analysis, since it can be hypothesized that estrogen deficiency influences tooth attachment.

Material and methods

Animals and experimental procedures

Adult female mice of the NMRI strain (8-week-old littermates, 22 mice in total) were purchased from Taconic (Tornbjerg, Denmark) and kept under standardized conditions in a regular 12 h light/dark cycle. The mice were anesthetized with pentobarbital sodium (75 mg/kg, i.p.) and the ovaries were removed bilaterally in accordance with standard procedures. The ovaries were identified and carefully removed. In sham-operated animals, the abdominal wall was opened and the ovaries exposed. Ovariectomy was performed in 5 mice, and 5 mice were sham-operated. Five animals served as non-operated controls. Immediately after closing the abdominal wall, the mice were placed on a heated (37°C) pad to control body temperature and they recovered rapidly (within 6–8 h) from the operation. After a 6-week period with food (normal mouse chow) and water *ad libitum*, the mice were killed by cervical dislocation. Bodyweight was determined before operation and at the time of sacrifice. Blood samples were immediately collected in heparin pretreated tubes by aspiration from the heart. The mandible was removed and freed from muscle, fat and connective tissue, fixed in 4% paraformaldehyde (in phosphate buffered saline, PBS) for 2 days at 4°C, and then washed carefully in PBS. In another series of experiments, the effects of aging were determined. These mice ($n=7$) were littermates and kept together under standardized conditions. They were killed at 8, 14, 20, and 26 weeks of age. The mandibles were removed and processed as described above. The experiments were approved by the Animal Ethics Committee at Lund University (M210-06, October 2, 2006).

Determination of connective tissue attachment and alveolar bone height by morphometric analysis

The specimens were demineralized in citrate-buffered 22% formic acid for one week and subsequently dehydrated in graded series of ethanol and xylen. The mandibles were then divided between the incisors into right and left halves, embedded in paraffin and serially sectioned in 4 μm sagittal sections. The sections were stained with hematoxylin-eosin and analyzed using a light microscope (Nikon Eclipse 80i; Nikon Instr., Amstelveen, The Netherlands) equipped with a digital camera and image analysis software (Nikon DS-2Mv and DS-L1). Connective tissue attachment and alveolar bone height were determined morphometrically on digital images of the area between the 1st and 2nd molars. The connective tissue attachment level was determined by measuring the distance between the cemento-enamel junction and the apical termination of the junctional epithelium. The bone height was determined by measuring the distance between the cemento-enamel junctions at the distal surface of the 1st molar and the mesial surface on the 2nd molar, respectively, and the highest point on the alveolar crest (Figure 1). For every right and left half of the mandible, approximately 30 serial sections were analyzed and a mean value for each sample was computed. Morphometric analysis was performed in a blinded fashion.

Determination of serum amyloid A

Blood plasma levels of serum amyloid A were determined by ELISA using a kit from Tridelta Development Ltd., Kildane, Ireland. The samples were analysed in duplicate and serum amyloid A

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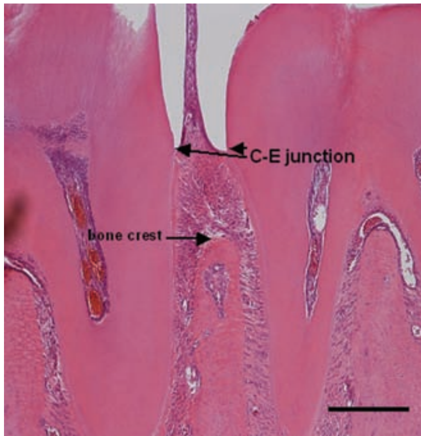

Figure 1. Alveolar bone height of the mouse mandible was determined by measuring the distance between the cemento-enamel (C-E) junction of the distal surface of the 1st molar and the mesial surface of the 2nd molar, respectively, and the highest point on the alveolar crest in longitudinal sections of mandibles stained with hematoxylin-eosin. This is a representative section from an unoperated control mouse 8 weeks of age. Bar represents 100 μm.

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determined according to the instructions of the manufacturer.

Statistics

Summarized data are presented as means and SD. Statistical significance was calculated using Student's two-tailed *t*-test for unpaired comparisons. *P*-values <0.05 were regarded as denoting statistical significance. Bonferroni analysis was used for multiple comparisons as appropriate.

Results

Bodyweights and plasma SAA levels

The mice recovered within 6–8 h from the anesthesia and operation. As shown in Table I, no differences in bodyweights were observed between operated and control mice, i.e. neither before ovariectomy nor at the end of the 6-week post-ovariectomy observation period. In both ovariectomized (OVX) and control mice, bodyweights were increased by about 7% during the observation period. The plasma level of SAA was determined 6 weeks post-ovariectomy. No difference in SAA was observed between OVX and control mice (19.4 (SD 1.6) μg/mL in OVX mice vs. 23.1 (SD 11.4) μg/mL in control mice; $n=3$–5), showing that ovariectomy caused no chronic systemic inflammatory response within the observation period.

Table I. Bodyweights (g) in ovariectomized (OVX) and control mice before ovariectomy (B) and at the end of the 6-week observation period (E). Each mouse was weighed before operation and 6 weeks post-ovariectomy. Values are presented as means and SD of 5 observations in each group

	B	E
Control	31.2 (1.0)	33.5 (0.6)**
OVX	30.2 (2.2)	33.1 (2.0)*

*$P<0.05$ and **$p<0.01$ compared to bodyweights before ovariectomy.

Alveolar bone height and connective tissue attachment level

The alveolar bone height, computed as described in Figure 1, was similar in OVX, sham-operated and control mice killed 6 weeks post-ovariectomy. Alveolar bone heights in OVX, sham-operated and control mice were 94.0 (SD 23.6) μm, 95.2 (SD 36.1) μm, and 115.3 (SD 47.1) μm; $n=8$–12. The apical termination of the junctional epithelium was at the cemento-enamel junction in every individual animal in all three experimental groups, showing that the connective tissue attachment level was unaffected by the ovariectomy (Figure 2). Bone morphology, assessed in the hematoxylin-eosin stained sections, was similar in OVX, sham-operated and control animals (Figure 2). Lacunae with osteocytes were observed in both OVX and control mice.

In one set of experiments, we assessed the effects of aging on alveolar bone height and apical termination of the junctional epithelium in non-ovariectomized female littermate mice. Between 8, 14, 20, and 26 weeks of age, the bodyweights increased as the mice became older. The mice weighed about 60% more at 26 weeks of age than at 8 weeks of age (31.2 (SD 0.9) g at 8 weeks vs. 51.2 (SD 9.0) g at 26 weeks; $p<0.01$, $n=3$–5). The distance between the cemento-enamel junction and the alveolar crest was similar at all ages. The mean distance between the cemento-enamel junction and the alveolar crest at 8, 14, 20, and 26 weeks of age was 132.7 (SD 29.2) μm ($n=8$). The apical termination of the junctional epithelium was at the cemento-enamel junction in every individual animal at all ages (not shown).

Discussion

Here, we demonstrate morphometrically that tooth attachment in mice, assessed by measuring alveolar bone height and apical termination of the junctional epithelium, is not affected by removal of ovaries and aging between 8 and 26 weeks, suggesting that mouse periodontium is unaffected by periodontal pathogenic factors and systemic factors such as ovarian sex hormones within this time-frame. In the senescence-accelerated SAM mouse, changes in periodontal tissues, i.e. apical migration of the

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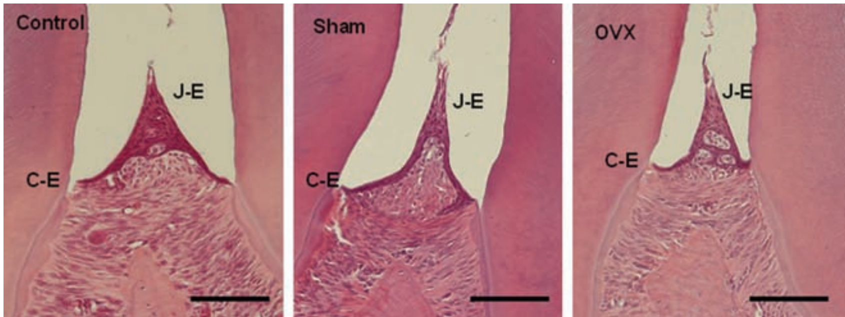

Figure 2. Assessment of the apical termination of the junctional epithelium in control, sham-operated (sham) and OVX mice. In each individual mouse, the apical termination of the junctional epithelium (J-E) was localized to the C-E junction in control, sham-operated, and in OVX mice. Bars represent 100 μm.

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junctional epithelium and a decrease in bone height, have been reported [17,18], suggesting that periodontal tissues are affected in pre-term senescence but not normal mice. Laboratory mice are normally used for biomedical investigations and experiments between 2 and 6 months of age [19], i.e. at the same age as used by us in the present study. Our data show that tooth attachment is unaltered by age and lack of ovaries within this time-frame, leading to the conclusion that tooth attachment is stable and robust and that changes in attachment do not occur without provocation in the female mouse. Our conclusions are based on robust and conclusive data, although we have to admit that the animal number in each experimental group is small.

The SAA protein is a major acute-phase reactant in all vertebrates produced mainly by the liver in response to inflammation promoters and cytokines/chemokines [20]. During inflammation, the *in vivo* concentration of SAA may increase by as much as 1000 times. The SAA protein shows the same response speed and sensitivity as C-reactive protein (CRP) [20,21]. In the present study, we show that the ovariectomy induces no change in the basal production of inflammation markers as demonstrated by unaltered plasma SAA level in OVX compared to control mice. These data suggest that withdrawal of endogenous female sex hormone production in mice has no impact on the systemic inflammatory response. In humans divergent effects of female sex hormones on CRP have been reported. In post-menopausal women receiving hormone replacement therapy both increased and unaltered level of CRP have been reported [22,23].

In the present study, we report that removal of endogenous sex hormone production by ovariectomy in mice has no effect on connective tissue attachment level and alveolar bone height determined 6 weeks after ovariectomy. It might be argued that 6 weeks is too short an observation time. However, in rats, a decrease in mandibular alveolar bone height 6 weeks post-ovariectomy, i.e. at the same time-point after ovariectomy at which we observed no effect in mice, has been reported by Cao et al. [24], suggesting that there is an important difference between rats and mice in their alveolar bone response to removal of the ovaries. Osteoclastogenesis has been reported to be induced in the OVX rat periodontium, suggesting that alveolar bone decomposition observed in the OVX rat is caused by elevated activity of osteoclasts [25]. We cannot rule out the possibility that ovariectomy in mice would affect periodontal tissues after, for example, ligation of teeth or treatment with inflammation promoters, but nevertheless rats seem to be more sensitive than mice to removal of the ovaries.

Characterization of mouse periodontal tissues and tooth attachment at different ages and hormonal status, as performed by us in the present study, has an impact on the future evaluation of periodontal tissue phenotype in transgenic and gene knockout mouse models. The numbers of transgenic and gene knockout mouse models are increasing successively and thus more and more attention will be drawn to the mouse as laboratory animal, which will undoubtedly have an impact on future periodontal research.

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Declaration of interest: The authors reports no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Differential regulation of chemokine expression by estrogen in human periodontal ligament cells

D. Nebel[1,2], D. Jönsson[1,2], O. Norderyd[2], G. Bratthall[2], B.-O. Nilsson[1]

[1]Department of Experimental Medical Science, Lund University, Lund, Sweden and [2]Department of Periodontology, Faculty of Odontology, Malmö University, Malmö, Sweden

Nebel D, Jönsson D, Norderyd O, Bratthall G, Nilsson B-O. Differential regulation of chemokine expression by estrogen in human periodontal ligament cells. J Periodont Res 2010; 45: 796–802. © 2010 John Wiley & Sons A/S

Background and Objective: Estrogen modulates inflammatory responses, but the mechanisms involved have not yet been identified. Periodontal ligament (PDL) cells produce chemokines (a group of chemoattractant molecules that recruit leukocytes) and it has been suggested that estrogen modulates periodontal inflammation by regulating the expression of chemokines by PDL cells. Therefore, the objectives of this study were to investigate the regulation of chemokine ligand 2 [CCL2/monocyte chemoattractant protein 1 (MCP-1)], chemokine ligand 3 [CCL3/macrophage inflammatory protein-1α (MIP-1α)] and chemokine ligand 5 (CCL5/RANTES) by estrogen in human PDL cells.

Material and Methods: PDL cells were obtained from the PDL of premolars, extracted for orthodontic reasons, from two boys and two girls (16 and 17 years of age). PDL cell *CCL2*, *CCL3* and *CCL5* mRNA transcripts were determined by quantitative real-time PCR. The concentrations of CCL2, CCL3 and CCL5 proteins were determined by ELISAs.

Results: Treatment with 0.5 μg/mL of lipopolysaccharide (LPS, from *Escherichia coli*) + 100 nM 17β-estradiol (E_2) for 24 h reduced the expression of *CCL3* mRNA by about 40% compared to PDL cells treated with LPS alone. Attenuation of *CCL3* mRNA was not associated with a decrease in CCL3 protein within 48 h, suggesting a slow turnover of the CCL3 protein. Interindividual differences in the effects of E_2 on *CCL5* mRNA expression were observed. E_2 (100 nM) increased the expression of *CCL5* by 40–60% in PDL cells derived from two subjects but reduced the expression of *CCL5* by about 30% in cells from another subject. *CCL2* mRNA and CCL2 protein were highly expressed, but not regulated by E_2. Similar data were observed in cells obtained from both boys and girls.

Conclusion: Regulation, by estrogen, of chemokine expression in PDL cells shows a complex pattern involving the down-regulation as well as the up-regulation of chemokines, suggesting that estrogen exerts both anti-inflammatory and proinflammatory effects through these mechanisms.

Dr Bengt-Olof Nilsson, DDS, PhD, Department of Experimental Medical Science, Lund University, BMC D12, SE-221 84 Lund, Sweden
Tel: +46 46 2227767
Fax: +46 46 2224546
e-mail: bengt-olof.nilsson@med.lu.se

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The female sex hormone, estrogen, regulates gene transcription via estrogen receptor (ER)α and ERβ (1). Both ER subtypes are widely expressed in different cells and tissues and they show distinct and specific patterns of expression (1,2). Human periodontal ligament (PDL) cells have been shown to possess binding sites for radio-

labelled 17β-estradiol (E_2) and to express mRNA for ERs (3,4). We have shown that human PDL cells express preferentially ERβ immunoreactivity, while the signal for ERα is much lower, suggesting that PDL cells express predominantly ERβ protein (5,6). Taken together, these data show that PDL cells express ERs, but the functional importance of PDL cell ERs remains to be clarified. Stimulation of ERα and ERβ with the most important endogenous estrogen, E_2, has no effect on the functional properties of human PDL cells, such as collagen synthesis and cell proliferation (7). PDL cells are fibroblast-like cells that produce collagen, but data have been presented showing that these cells may be transformed into a more inflammatory-like cell phenotype that produces cytokines and chemokines (8–11), suggesting that PDL cells play a role as producers of cytokines and chemokines responsible for the recruitment of white blood cells in periodontal inflammation.

Estrogen has been suggested to exert both proinflammatory and anti-inflammatory effects (12–14). Stimulation of recruitment and adhesion of white blood cells to the vascular endothelium is an initial step in the inflammatory reaction, which is attenuated by estrogen (15–17). A possible mechanism behind estrogen-induced reduction of white blood cell recruitment to the endothelium is down-regulation of the vascular cell adhesion molecule-1, as shown previously by Caulin-Glaser *et al.* (18), Simoncini *et al.* (19) and Mukherjee *et al.* (20). Lowered chemokine production is another possible mechanism of action explaining estrogen-induced attenuation of white blood cell recruitment (21,22). Chemokine ligand 2 [CCL2/monocyte chemoattractant protein 1 (MCP-1)], chemokine ligand 3 [CCL3/macrophage inflammatory protein-1α (MIP-1α)] and chemokine ligand 5 (CCL5/RANTES) are three important chemokines produced by many different cell types stimulating the recruitment of white blood cells to the site of inflammation (23–25). The expression of CCL2 and CCL3 has been reported to be low in healthy periodontal tissue but to increase with severity of periodontal disease (26–28). Human PDL cells have been reported to express mRNA for *CCL2* and *CCL5* upon stimulation with viable *Porphyromonas gingivalis* (29).

Here, we investigated the effects of estrogen on the production of chemokines from PDL cells, and found that a physiological concentration of the endogenous estrogen, E_2, differentially regulates chemokine expression in human PDL cells.

Material and methods

Cells and cell culture

The PDL cells were collected from four subjects – two boys, 16 and 17 years of age, and two girls, 16 and 17 years of age – who were referred for extraction of premolars on orthodontic indications. The patients and their parents were informed orally, and in writing, of the purpose of the study and the parents gave written approval for the PDL cells to be used. The study design and the experiments were approved by the Human Ethical Committee at Lund University (Lund, Sweden). Immediately after extraction, the teeth were washed in phosphate-buffered saline (PBS) and the middle third of the periodontal ligament was scraped off using a sterile curette. The apical and gingival parts of the periodontal ligament were not used in order to avoid contamination with cell types other than PDL fibroblasts. PDL explants from each subject were seeded in cell-culture Petri dishes containing Dulbecco's modified Eagle's medium supplemented with antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin), glutamine (1.16 g/L) and 10% fetal calf serum, and the dishes were then placed in a water-jacketed cell/tissue incubator with 5% CO_2 in air. The cells migrating from the explants were trypsinized (0.25%) after reaching confluence and were then reseeded at a density of 600,000 cells/mL. Experiments were performed on cells reaching 80% confluence in passages three to five. At these passages the PDL cells show fibroblast morphology, with a spindle-like cell shape, which is characteristic of fibroblasts (6). Before the start of the experiments cell density was evaluated carefully using a phase-contrast microscope (Olympus CK40; Olympus Europa GmbH, Hamburg, Germany).

Experimental procedure

Twenty-four hours before starting the experiments, standard cell-culture medium was replaced with fetal calf serum-free and phenol red-free medium to achieve standardized conditions with quiescent cells and to remove the estrogen-like activity of phenol red. E_2 (Sigma Chemicals, St Louis, MO, USA) was included 2 h before lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4 LPS; Sigma) and was then present during the 24 or 48 h incubation with LPS. LPS was dissolved in PBS and E_2 was dissolved in ethanol. Controls received ethanol ($< 0.1\%$) as vehicle. Each cell-culture dish (52 mm in diameter; Nunc, Roskilde, Denmark) containing PDL cells at 80% confluence represents one sample/observation for either quantitative real-time PCR or ELISA. Each sample was analyzed in duplicate both for PCR assays and for ELISAs.

Quantitative real-time PCR

The PDL cells were washed carefully in PBS and then total RNA was extracted and purified using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The concentration and purity of RNA was measured at 260/280 nm in a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The RNA concentration in each sample was about 75 ng/μL. The RNA samples were then subjected to one-step quantitative real-time PCR measurements using QuantiFast SYBR Green RT-PCR kits (Qiagen) and QuantiTect primer assays (Qiagen) on a Roche real-time thermal cycler (Roche, Basel, Switzerland). Each sample was analyzed in duplicate. The expression of *CCL2*, *CCL3* and *CCL5* genes was calculated using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the reference gene, as described by Pfaffl (30). The expression of *GAPDH*

mRNA was not affected by E_2 treatment. The PCR primers (QuantiTect Primer Assays) for CCL2 (HS_CCL2_1_SG), CCL3 (HS_CCL3_2_SG), CCL5 (HS_CCL5_1_SG) and GAPDH (HS_GAPDH_2_SG) were purchased from Qiagen. The CCL2, CCL3 and CCL5 primers showed similar efficiencies.

Measurement of chemokine proteins

The PDL cells were washed carefully in PBS and scraped off the culture dishes using cell scrapers (Sarstedt, Newton, NC, USA). Then the cells were sonicated 2 × 10 s on ice and centrifuged at 1700 *g* and 4°C for 5 min. The concentrations of CCL2 and CCL3 proteins were determined in the cell supernatant using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the instructions supplied by the manufacturer. Each sample was analyzed in duplicate. The concentrations of CCL2 and CCL3 were normalized to the total protein concentration determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

Statistics

Values are presented as means ± standard error of the mean. Statistical significance was calculated using the Student's two-tailed *t*-test, and *p*-values of < 0.05 were regarded as denoting statistical significance.

Results

Effects of E_2 on the *CCL3* mRNA level

Stimulation with E_2 (100 nM) in the presence of LPS (0.5 μg/mL) for 24 h reduced the *CCL3* mRNA level by about 35% vs. stimulation with LPS alone in PDL cells derived from a 16-year-old boy, suggesting that E_2 reduces *CCL3* expression (Fig. 1). Analysis of the *CCL3* mRNA level was repeated in PDL cells derived from another subject (a 17-year-old girl). In these cells, combined treatment with LPS (0.5 μg/mL) and E_2 (100 nM) also reduced (by about 40%) the *CCL3* mRNA level vs. treatment with LPS alone (1.00 in LPS-treated cells vs. 0.57 ± 0.12 in LPS + E_2-treated cells; $n = 6$ observations in each group, $p < 0.001$). Down-regulation of the *CCL3* mRNA transcript by E_2 was confirmed in PDL cells from a third subject, a 17-year-old boy (1.0 in cells treated with LPS alone vs. 0.55 ± 0.13 in cells treated with LPS + E_2; $n = 6$ observations in each group, $p < 0.001$). Down-regulation of *CCL3* mRNA by E_2 was thus observed in PDL cells originating from three different subjects.

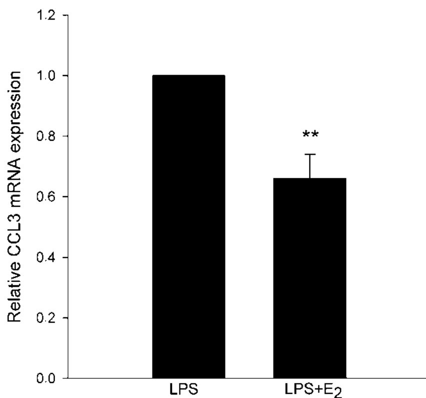

Fig. 1. Quantitative PCR shows that treatment with lipopolysaccharide (LPS) (0.5 μg/mL) + 17β-estradiol (E_2) (100 nM) reduces the level of *CCL3* mRNA transcript by about 35% compared to treatment with LPS alone in periodontal ligament (PDL) cells derived from a 16-year-old boy. Similar results were observed in cells derived from two other subjects included in the study. The cells were stimulated with LPS, with or without E_2, for 24 h. Values are presented as means ± standard error of the mean of six observations in each group. $^{**}p < 0.01$ compared with LPS alone.

Effects of E_2 on the PDL cell CCL3 protein concentration

The concentration of CCL3 protein in PDL cells was very low (at, or even below, the lowest standard). Treatment of PDL cells derived from three donors (the same donors analyzed for *CCL3* mRNA expression presented above) with LPS (0.5 μg/mL) + E_2 (100 nM) for 24 h tended, but not significantly, to decrease the CCL3 protein concentration vs. treatment with LPS alone (0.29 ± 0.11 pg/μg of protein in LPS-treated cells vs. 0.14 ± 0.03 pg/μg of protein in LPS + E_2-treated cells; $n = 8$ in each group). Treatment with LPS (0.5 μg/mL) + E_2 (100 nM) for a longer period of time (48 h) had no effect on the concentration of CCL3 protein vs. treatment with LPS alone (Fig. 2).

Effects of E_2 on the PDL cell *CCL2* mRNA level and the CCL2 protein concentration

The relative mRNA expression level for *CCL2*, normalized to that of the housekeeping gene *GAPDH*, was about 55% higher than the mRNA expression level of *CCL3* in LPS-stimulated (24 h of stimulation with 0.5 μg/mL of LPS) PDL cells derived from the 17-year-old girl (Fig. 3). Higher expression of *CCL2* mRNA vs. *CCL3* was observed also in cells derived from two other subjects. The PCR data showing high expression of *CCL2* was confirmed at the protein level. The CCL2 protein level was about three times higher than that of CCL3 in PDL cells treated with 0.5 μg/mL of LPS for 24 h (0.89 ± 0.07 pg/μg of protein for *CCL2* vs. 0.29 ± 0.11 pg/μg of protein for *CCL3*; $n = 3$ and 8 observations in each group, respectively, $p < 0.05$).

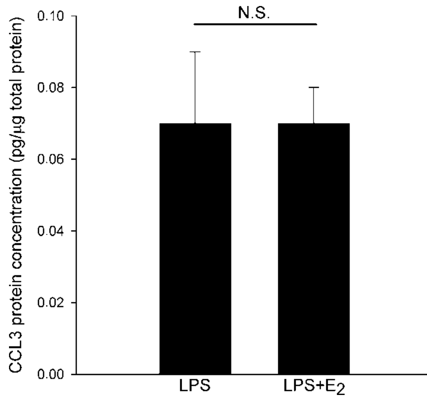

Fig. 2. 17β-Estradiol (E_2) (100 nM) has no effect on the CCL3 protein concentration, determined by ELISA, in periodontal ligament (PDL) cells derived from a 16-year-old boy. Cells derived from this subject were also used for real-time PCR and these data are presented in Fig. 1. The cells were stimulated with lipopolysaccharide (LPS) (0.5 µg/mL), with or without E_2, for 48 h. Values are presented as means ± standard error of the mean of four observations in each group. NS, not significant.

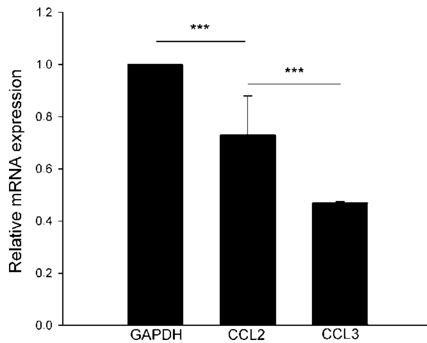

Fig. 3. Relative expression level of *CCL2* and *CCL3* mRNA transcripts normalized to that of *GAPDH* in lipopolysaccharide (LPS)-stimulated (0.5 µg/mL for 24 h) periodontal ligament (PDL) cells derived from a 17-year-old girl. The *CCL2* gene shows 55% higher expression compared with the *CCL3* gene. *CCL2* mRNA expression was higher than that of *CCL3* also in PDL cells derived from two other subjects included in this study. Values are means ± standard error of the mean of six observations in each group. ***$p < 0.001$.

Treatment with LPS (0.5 µg/mL) + E_2 (100 nM) for 24 h had no effect on the *CCL2* mRNA transcript level and CCL2 protein concentration compared to stimulation with LPS alone in PDL cells derived from the 17-year-old girl (Fig. 4). Moreover, chronic treatment (21 d) with LPS (0.5 µg/mL) + E_2 (100 nM) had no effect on the concentration of CCL2 protein compared to treatment with LPS alone (data not shown). Similar data for CCL2 were observed in cells derived from the other three subjects included in this study.

Effects of E_2 on the mRNA level of *CCL5* in PDL cells

Treatment with 100 nM E_2 in the presence of LPS (0.5 µg/mL) for 24 h increased the *CCL5* mRNA level by about 60% compared to treatment with LPS alone in PDL cells derived from the 17-year-old boy (Fig. 5A). In PDL cells derived from the 16-year-old boy, costimulation with LPS and E_2 caused a 30% decrease in *CCL5* mRNA vs. stimulation with LPS alone (Fig. 5B). LPS + E_2 increased the *CCL5* transcript level by 40% vs. LPS alone in PDL cells obtained from the 17-year-old girl, whereas E_2 had no effect in cells derived from the 16-year-old girl (Fig. 5C,D). Taken together, the effects of E_2 on CCL5 vary between PDL cells originating from different subjects, suggesting that the response to E_2 is dependent on interindividual differences.

Discussion

In the present study we demonstrated a differential regulation of chemokine genes by E_2 in human PDL cells, suggesting that estrogen exerts both pro-inflammatory and anti-inflammatory effects through these mechanisms. We showed estrogen-induced down-regulation of *CCL3* mRNA, while the expression of *CCL2* mRNAwas unaffected by estrogen in PDL cells derived from three individual subjects. Interindividual variations in E_2-induced effects on PDL cell *CCL5* expression were demonstrated, suggesting that the effects of estrogen on CCL5 depend on the genetic origin of the PDL cells. The E_2-evoked effects on chemokine expression were observed in cells derived from boys as well as girls, suggesting that these mechanisms are independent of gender. We used cells derived from subjects of similar age to minimize interindividual differences. PDL cells derived from male and female subjects express ERα and ERβ similarly (5), supporting the fact that PDL cells from male and female

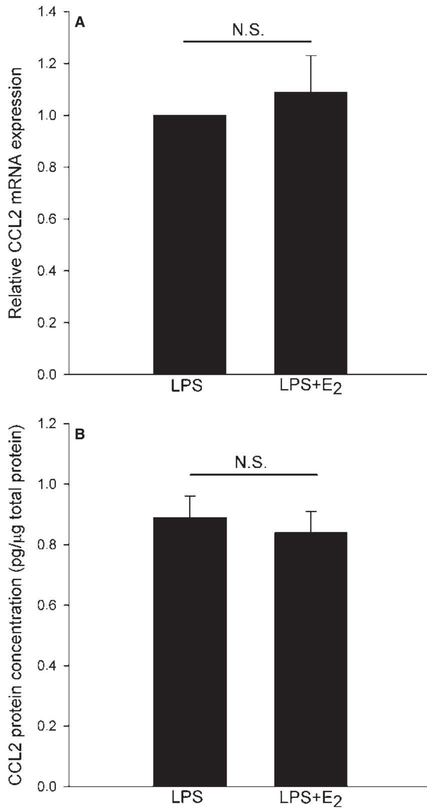

Fig. 4. Expression of (A) *CCL2* mRNA and (B) CCL2 protein in periodontal ligament (PDL) cells treated for 24 h with lipopolysaccharide (LPS) (0.5 μg/mL) in the absence or in the presence of 100 nM 17β-estradiol (E_2). The PDL cells were derived from a 17-year-old girl. Values are means ± standard error of the mean of three to six observations in each group. NS, not significant.

subjects respond similarly to estrogen. We used a high, but still physiological, concentration (100 nM) of E_2. This is about the same concentration of E_2 observed in plasma during pregnancy (31). Regulation of PDL cell chemokine expression by estrogen, as demonstrated here, is probably more important in situations with high plasma concentrations of estrogen (e.g. in premenopausal women and during pregnancy), than in situations with low estrogen concentrations (such as after the menopause).

Treatment with estrogen decreased the expression of mRNA for *CCL3* but had no significant effect on the cellular concentration of CCL3 protein, suggesting that the reduction in *CCL3* mRNA induced by E_2 (−40%) was not sufficient to cause a reduction in the protein level. Another possible explanation for the lack of detectable E_2-induced reduction of CCL3 protein may be the combination of low CCL3 protein levels and a too low sensitivity of the CCL3 ELISA. We investigated the effects of estrogen on CCL3 protein at 24 h (i.e. the same time-point at which estrogen down-regulates the *CCL3* transcript) and at 48 h, but estrogen had no effect at either time-point, suggesting that the cellular CCL3 protein concentration is maintained for at least 48 h, although expression of the *CCL3* mRNA transcript is reduced by about 40%. These data suggest a slow turnover of the CCL3 protein.

In this study we identified the *CCL3* and the *CCL5* genes to be regulated by estrogen in human PDL cells subjected to stimulation with the *E. coli* promoter of inflammation, LPS. In human PDL cells, *E. coli* LPS and LPS from the well-known periodontal disease pathogen *P. gingivalis* have been shown to induce similar levels of cytokine expression (9), and thus it is reasonable to suggest that our data are representative for the *in vivo* situation. We used a concentration of LPS (0.5 μg/mL) that has been shown previously to induce cytokine and chemokine production without affecting collagen synthesis and cell proliferation in human PDL cells (11,32). *CCL3* mRNA has been reported to be expressed in human gingival epithelial cells, but not in human gingival fibroblasts (33). We demonstrated that human PDL cells express *CCL3* mRNA, suggesting cell-type-specific expression of this chemokine among different types of oral fibroblasts. Interestingly, the PDL cell expression level of the CCL2 chemokine was higher at both mRNA and protein levels than that of the CCL3 chemokine, suggesting that PDL cells are able to produce high amounts of CCL2. Thus, because the PDL cells show a high expression of CCL2, and this chemokine promotes recruitment of monocytes/macrophages (25), we suggest that PDL cells play an important role in attracting monocytes to the periodontal inflammation but that estrogen has no effect on this process.

We demonstrated that estrogen reduces *CCL3* gene expression in human PDL cells, suggesting that estrogen

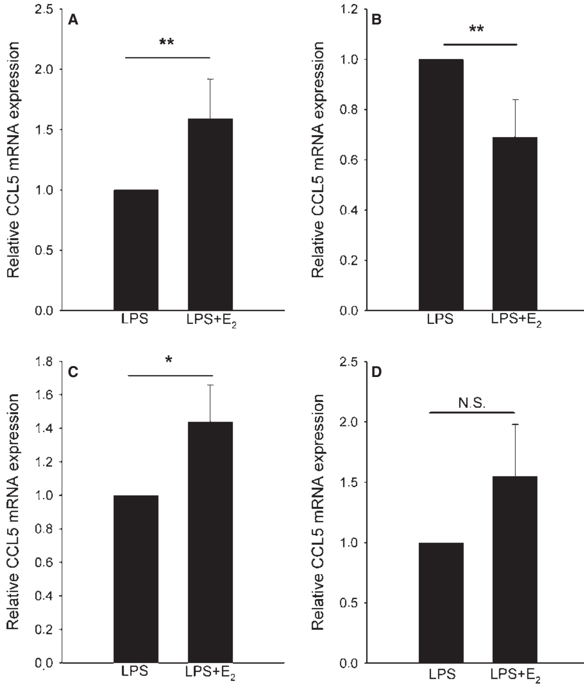

Fig. 5. The effects of 17β-estradiol (E_2) on periodontal ligament (PDL) cell *CCL5* mRNA levels depend on interindividual variations. The PDL cells were treated for 24 h with lipopolysaccharide (LPS) (0.5 μg/mL) in the absence or in the presence of 100 nM E_2. Panels A and B show data from cells derived from the two boys, 17 and 16 years of age, respectively. Panels C and D show data from the two girls, 17 and 16 years of age, respectively. Values are means ± standard error of the mean of five to seven observations in each group. $*p < 0.05$; $**p < 0.01$. NS, not significant.

attenuates the recruitment of white blood cells to the inflammatory reaction via this mechanism. By contrast, estrogen up-regulated *CCL5* gene activity in PDL cells from two out of four subjects, suggesting that estrogen stimulates recruitment of T cells to the inflammatory reaction via this mechanism. Taken together, estrogen exerts both anti-inflammatory and pro-inflammatory effects via these mechanisms. We have previously shown that the chemokine GROα, which is another important chemoattractant for neutrophils, is also, like CCL2, not regulated by estrogen (34). In this study we showed that both CCL3 and CCL5 are regulated by estrogen, while CCL2 is not. Thus, estrogen seems to differentially regulate chemokine expression in human PDL cells.

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Estrogen regulates DNA synthesis in human gingival epithelial cells displaying strong estrogen receptor β immunoreactivity

D. Nebel[1,2], G. Bratthall[2], E. Ekblad[1], O. Norderyd[2,3], B.-O. Nilsson[1]

[1]Department of Experimental Medical Science, Lund University, Lund, Sweden, [2]Department of Periodontology, Faculty of Odontology, Malmö University, Malmö, Sweden and [3]Department of Periodontology, The Institute for Postgraduate Dental Education, Jönköping, Sweden

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Background and Objective: Estrogen acts via estrogen receptor (ER) α and β. The expression pattern of ERs and their importance in gingival tissues are not fully understood. In this study, we investigate gingival ER expression and effects of estrogen on gingival epithelial cell proliferation.

Material and Methods: Gingival biopsies were obtained from both healthy and diseased sites in three male and three female subjects. Expression of ERα and β was determined by immunohistochemistry. Effects of 17β-estradiol (E_2) on cell proliferation, monitored by measuring DNA synthesis, were studied in cultured human gingival epithelial HGEPp.05 cells.

Results: Estrogen receptor β, but not ERα, immunoreactivity was demonstrated in nuclei of epithelial cells in all layers of the gingival epithelium, but also in cells of the lamina propria. No differences were observed between male and female subjects. The same pattern, i.e. high ERβ expression but no ERα expression, was observed in both healthy and diseased sites within each individual. No differences in the intensity of the ERβ immunoreactive signal and the number of ERβ-positive nuclei were observed between healthy and diseased gingiva. Treatment with a physiological concentration of E_2 (10 nM) had no effect on DNA synthesis in ERβ- and ERα-expressing HGEPp.05 cells. In contrast, E_2 at high concentrations (500 nM and 10 μM) reduced DNA synthesis by 60–70%.

Conclusion: Human gingival epithelial cells display strong ERβ but low ERα immunoreactivity both *in vivo* and in culture. Estrogen attenuates gingival epithelial cell DNA synthesis at high but not low concentrations, suggesting a concentration-dependent mechanism.

Dr Bengt-Olof Nilsson, DDS, PhD, Department of Experimental Medical Science, Lund University, BMC D12, SE-221 84 Lund, Sweden
Tel: +46 46 2227767
Fax: +46 46 2224546
e-mail: bengt-olof.nilsson@med.lu.se

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Two estrogen receptors (ERs) have been identified and named ERα and ERβ (1–3). Estrogen receptor α and ERβ are transcription factors which, in the presence of ligand, bind to unique sequences of DNA to regulate gene activity. These two ER subtypes are expressed in various organs and tissues and show a distinct and specific expression pattern (4). Cultured human periodontal ligament cells have been reported to express ERα mRNA,

but the signal was very weak (5). In another study, human periodontal ligament cells and gingival fibroblasts were shown to lack ERα mRNA expression (6). Cultured human periodontal ligament cells express ERβ protein, as demonstrated by immunocytochemistry, while no ERα immunoreactivity was detected (7,8). Human buccal mucosa has been shown to express ERα mRNA but not ERα protein (9). Epithelial cells within healthy human gingiva have been reported to express ERβ but not ERα protein (10). Taken together, these reports suggest that cells within oral tissues express predominantly ERβ protein in physiological conditions.

The functional importance of oral tissue ER is not fully understood. Data have been presented showing that estrogen stimulates the production of periodontal ligament cell mineralized nodules, suggesting that estrogen promotes periodontal ligament cell bone formation (11). Estrogen has also been reported to reduce *Escherichia coli* lipopolysaccharide-induced periodontal ligament cell cytokine expression and to regulate (both upregulate and downregulate) *E. coli* lipopolysaccharide S-stimulated periodontal ligament cell chemokine expression, suggesting that estrogen is involved in the regulation of periodontal inflammation via these mechanisms (12,13). Both Norderyd *et al.* (14) and Reinhardt *et al.* (15) have reported a higher frequency of gingival bleeding in postmenopausal women with low serum estrogen levels compared with those having high levels of estrogen, suggesting that estrogen exerts a protective effect by reducing gingival inflammation. Plasma estrogen levels rise dramatically in pregnancy owing to massive production of estrogens by the growing placenta (16). In pregnant women gingivitis often develops, suggesting a proinflammatory estrogen-induced effect (17). Estrogen thus seems to execute both anti- and proinflammatory effects on gingival tissues, but the mechanisms have not been identified.

Välimaa *et al.* (10) reported that healthy human gingiva expresses ERβ but not ERα, as studied by immunohistochemistry. The expression pattern of ER subtypes in diseased gingival tissue is not known. Furthermore, no data have been presented on the functional importance of gingival epithelial cell ERs. The objectives of the present study were to determine ER subtype expression in gingival biopsies from both healthy and diseased sites and also to address the physiological importance of gingival ERs by investigating the effects of estrogen on gingival epithelial cell proliferation.

Material and methods

Subjects and biopsies

Punch biopsies (4 mm in diameter) were obtained from both healthy and diseased gingival tissues from six individuals (three male and three female subjects). The biopsies included tissue from the marginal gingival epithelium, i.e. gingiva in direct connection to the gingival margin. Healthy gingival tissue was selected by clinical periodontal status, i.e. probing pocket depth < 4 mm and no bleeding on probing. The teeth adjacent to the site where healthy gingiva was collected did not suffer from periodontitis. The criteria probing pocket depth > 5 mm and bleeding on probing identified diseased sites. The patients included in the study were suffering from generalized severe chronic periodontitis. They were referred to the periodontal clinic for treatment, which included flap surgery at teeth not responding with pocket closure (< 6 mm) after initial cause-related therapy and scaling. They were over 40 years of age and they were nonsmokers (see Table 1). We excluded premenopausal women because of the fluctuations in plasma estrogen levels occurring with phases of the menstrual cycle (16), by having > 50 years of age as an inclusion criterion for female subjects. The women included in the study were not on hormone replacement therapy. The biopsies were fixed in 4% paraformaldehyde (in phosphate-buffered saline) and then kept in 70% ethanol until further processing. The experiments were undertaken with written consent of each subject and in accordance with the World Medical Association Declaration of Helsinki and with the approval of the Regional Ethical Committee at Lund, Sweden.

Immunohistochemistry

The fixed biopsies were embedded in paraffin and cut into 4 μm sections. The sections were dewaxed, rehydrated with descending concentrations of ethanol and rinsed in distilled water. Antigen retrieval was performed by microwaving for 15 min in citrate buffer (pH 6.0). Sections were stained with either a polyclonal ERα antibody (NeoMarkers code Ab-16; Thermo Scientific, Waltham, MA, USA) raised in

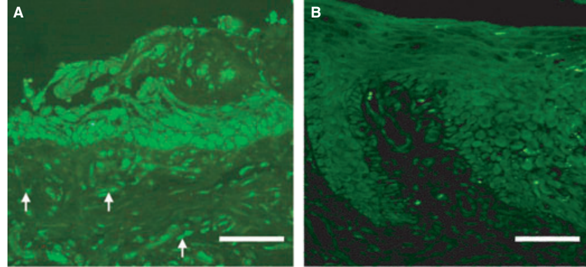
Table 1. Age, sex and medication of the six patients (three male and three female subjects) included in the present study

Identification	Sex	Age (years)	Diseases and medication
Patient 1	Male	64	High blood pressure, Trombyl®; Pfizer AB, Sollentuna, Sweden (anti-thrombotic)
Patient 2	Female	79	None
Patient 3	Male	44	High blood pressure, Enalapril®; Sandoz Novartis Sverige AB, Täby, Sweden (angiotensin-converting enzyme inhibitor)
Patient 4	Female	51	Abnormal thyroid gland function, Levaxin®; Nycomed AB, Stockholm, Sweden (synthetic thyroid hormones), Metoprolol®; Hexal AB, Helsingborg, Sweden (β-adrenergic blocker) and Simvastatin®; Merck NM AB, Stockholm, Sweden (cholesterol synthesis inhibitor)
Patient 5	Male	57	None
Patient 6	Female	56	None

rabbits at a dilution of 1:100 or an ERβ antibody raised in chickens (ERβ 503; kindly provided by the Professor Jan-Åke Gustafsson's laboratory at the Karolinska Institute, Stockholm, Sweden) at a dilution of 1:500. In preliminary experiments, different dilutions of both primary antibodies were tested, and 1:100 for the ERα antibody and 1:500 for the ERβ antibody resulted in high-intensity fluorescence with low background staining. The ERβ 503 antibody has been extensively characterized in different cell systems before use in immunohistochemistry (18,19). After preabsorption of the ERβ 503 antibody with ERβ protein (protein in 50-fold excess), no ERβ immunoreactivitiy was observed in rat aorta and rat prostate, tissues known to be ERβ positive. In preliminary experiments, we confirmed the nuclear staining of gingival epithelial cells observed with the ERβ 503 antibody by using a commercially available rabbit polyclonal ERβ antibody (Affinity Bioreagents code PA1-311; Thermo Scientific). Data on ERβ immunoreactivity presented in Figs 1–4 and in Table 2 are on the chicken ERβ 503 antibody. The site of antigen–antibody reaction was visualized with fluorescein isothiocyanate-conjugated secondary anti-rabbit or anti-chicken antibodies (both from Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Mouse (C57BL/6 strain) uterus was included as a positive control for both ERα and ERβ antibodies. The animal experiments were approved by the Animal Ethics Committee at Lund University, Lund, Sweden. It is well known that uterus expresses ERα as well as ERβ, as shown in both humans and rodents, making uterus from these species a most suitable positive control (19–21). For negative controls, the primary ERα and ERβ antibodies were omitted.

The ERα and ERβ immunoreactivities in healthy and diseased gingival tissue were compared by scoring the nuclear immunoreactive signal according to the following four criteria: + + +, intense staining, all or almost all nuclei positive; + +, intermediate staining, many positive nuclei; +, weak staining, few positive nuclei: and 0, no staining. The immunostaining was scored by three independent observers in a blinded fashion, and a summarized verdict was reached. The three observers obtained identical or almost identical results. For each biopsy and staining, at least three sections were analysed.

Male, 64 years of age



Female, 79 years of age

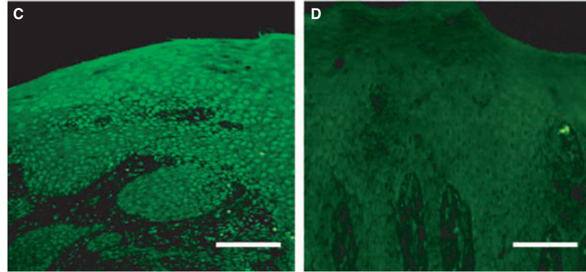


Fig. 1. Nuclear estrogen receptor (ER) β but not nuclear ERα immunoreactivity is shown in epithelial cells of healthy gingival tissues from a male subject, 64 years of age (A and B), and a female subject, 79 years of age (C and D). The figure shows staining with ERβ antibody (A and C) and staining with ERα antibody (B and D). Not only epithelial cells but also the cells of the underlying connective tissue, i.e. the lamina propria, express ERβ (arrows). No nuclear ERα immunoreactivity is observed in cells within the lamina propria. For each biopsy, three sections were analysed. Identical results were observed in biopsies obtained from three male and three female subjects. Scale bars represent 50 μm.

Cells and cell culture

Human primary gingival epithelial HGEPp.05 cells were purchased from CellnTec (CellnTec Advanced Cell Systems AG, Bern, Switzerland) and cultured in CnT-24 cell culture medium (CellnTec) supplemented with antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin) and fetal calf serum (10%). After reaching confluence, cells were trypsinized (0.25% trypsin) and reseeded at a density of 100,000 cells/mL. The cells were cultured in air containing 5% CO_2 and used at passages 2–4, when they had reached about 80% confluence. For ERα and ERβ immunocytochemistry, cells were seeded on glass coverslips and fixed in a mixture of 2% formaldehyde and 0.2% picric acid in phosphate buffer (pH 7.2). The same antibodies and dilutions as used for tissue sections (please see previous subsection) were applied also for the HGEPp.05 cells. Before experiments, standard cell culture medium was replaced with serum-free and phenol red-free medium to obtain standardized conditions with quiescent cells and to remove the estrogen-like activity of phenol red.

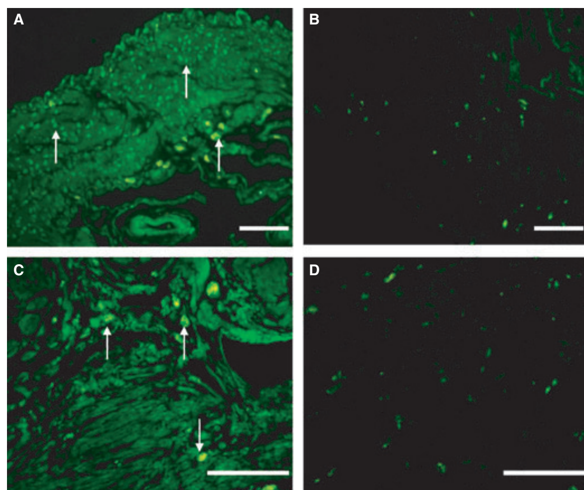


Fig. 2. Mouse uterus, used as a positive control, shows nuclear immunoreactivity for ERβ (A) and ERα (C) in myometrial smooth muscle cells and in the stroma cells of the endometrium. Arrows represent examples of ERβ- and ERα-positive nuclei. For each tissue specimen, at least three sections were analysed. No immunoreactivity was observed after omission of ERβ (B) and ERα antibodies (D). Scale bars represent 50 μm.

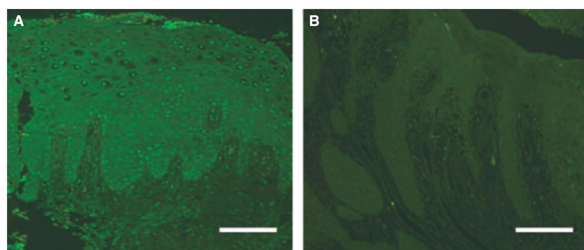


Fig. 3. Nuclear ERβ (A) but not nuclear ERα immunoreactivity (B) is observed in epithelial cells of diseased gingival tissue from a female subject, 79 years of age. Not only epithelial cells but also the cells of the underlying connective tissue, lamina propria, express ERβ (A). No nuclear ERα immunoreactivity is observed in cells within the lamina propria (B). The ERα and ERβ expression pattern in healthy gingival tissue obtained from this subject is shown in Fig. 1C,D. Identical results were observed in biopsies obtained from three male and three female subjects. For each biopsy, three sections were analysed. Scale bars represent 50 μm.

Measurements of DNA synthesis were performed with 5% dextran-coated charcoal-stripped fetal calf serum as a growth promoter representing sub-maximal growth stimulation. Stripped fetal calf serum was used to remove steroids from the serum. The 17β-estradiol (E_2) was purchased from Sigma (Sigma Chemicals, St Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). Control cultures received DMSO as vehicle.

Measurement of DNA synthesis

The DNA synthesis was determined by measuring the incorporation of methyl-[^{3}H]-thymidine (PerkinElmer Inc., Boston, MA, USA) into newly synthesized DNA as described by Jönsson *et al.* (22). The radiolabelled thymidine (5 μCi) was present during the last hour of the 24 h incubation with E_2. Radioactivity was measured in a Beckman liquid scintillation counter (Beckman LS6500; Beckman Instruments Inc., Fullerton, CA, USA). The radioactivity was expressed as disintegrations per minute and normalized to total protein concentration determined by using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

Statistics

Values are presented as means ± SEM. Statistical significance was calculated using ANOVA and Student's two-tailed *t*-test for unpaired comparisons with Bonferroni correction for *post hoc* analysis as appropriate. Values of $p < 0.05$ were regarded as denoting statistical significance.

Results

Expression of ERα and ERβ in healthy gingiva

In biopsies collected from healthy sites, nuclear ERβ but not ERα immunoreactivity was observed in all layers of the gingival epithelium (Fig. 1). Nuclear ERβ but not ERα immunoreactivity was observed also in cells residing in the underlying connective tissue, i.e in the lamina propria (Fig. 1). Almost all of the cell nuclei within all layers of the epithelium and the lamina propria were ERβ positive. In the lamina propria, both round cells with small nuclei, probably representing lymphocytes, and cells with large elongated nuclei, probably representing fibroblasts, were ERβ positive. Identical results were observed in biopsies collected from three male and three female subjects (Fig. 1). In Table 1 age, sex, diseases and medication of the patients included in this study are presented. In mouse uterus, serving as positive control,

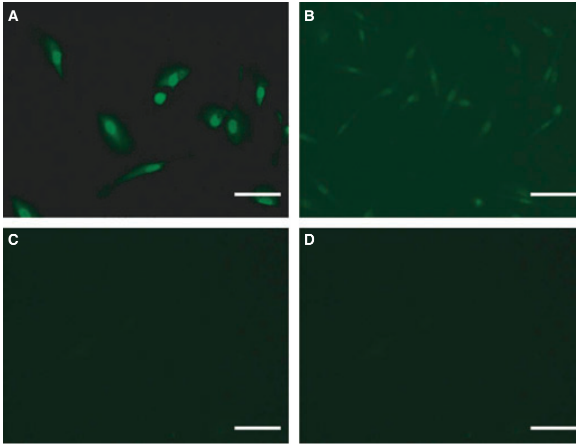


Fig. 4. Cultured human gingival epithelial HGEPp.05 cells express strong ERβ (A) but weak ERα immunoreactivity (B). Both ERβ and ERα immunoreactivities were almost exclusively localized to nuclei. No immunoreactivity was observed after omission of ERβ (C) and ERα antibodies (D). Determination of ERα and ERβ immunoreactivity in HGEPp.05 cells was repeated in cells at passages 2–4, showing identical results. At least two glass coverslips were analysed at each passage for each staining. Scale bars represent 10 μm.

Table 2. Estrogen receptor (ER) α and ERβ nuclear immunoreactivities in biopsies from healthy and diseased (periodontitis) gingiva obtained from six different patients

Identification	ERα epithelium	ERα lamina propria	ERβ epithelium	ERβ lamina propria
Patient 1, healthy gingiva	0	0	+ + +	+ + +
Patient 1, diseased gingiva	0	0	+ + +	+ + +
Patient 2, healthy gingiva	0	0	+ + +	+ + +
Patient 2, diseased gingiva	0	0	+ + +	+ + +
Patient 3, healthy gingiva	0	0	+ + +	+ + +
Patient 3, diseased gingiva	0	0	+ + +	+ + +
Patient 4, healthy gingiva	0	0	+ + +	+ +
Patient 4, diseased gingiva	0	0	+ + +	+
Patient 5, healthy gingiva	0	0	+ + +	+ + +
Patient 5, diseased gingiva	0	0	+ + +	+ + +
Patient 6, healthy gingiva	0	0	+ + +	+ + +
Patient 6, diseased gingiva	0	0	+ + +	+ + +

Immunoreactivities in biopsies were classified as follows: + + +, intense staining, all or almost all nuclei positive; + +, intermediate staining, many positive nuclei; +, weak staining, few positive nuclei; and 0, no staining. At least three sections were analysed for each biopsy and ER staining. The immunostaining was scored by three independent observers in a blinded fashion. The summarized scores of the three observers are presented. Age, sex and medication for the individual patients are presented in Table 1.

nuclear immunoreactivity for both ERα and ERβ was observed in the smooth muscle cells of the myometrium and in the stromal cells of the endometrium (Fig. 2). No ERα and ERβ immunoreactivity was observed after omission of the respective primary antibody (Fig. 2).

Expression of ERα and ERβ in diseased gingiva

Expression of ERα and ERβ was analysed by immunocytochemistry in healthy (probing pocket depth < 4 mm and no bleeding on probing) and diseased gingival tissues (probing pocket depth > 5 mm and bleeding on probing) obtained from six patients (three male and three female subjects). Age, sex and medication of the six patients included in the present study are presented in Table 1. Gingival biopsies were collected from both healthy and diseased sites within each of the six patients. Nuclear ERβ but not ERα immunoreactivity was demonstrated within both epithelial cells and connective tissue cells of the lamina propria in diseased gingival tissue (Fig. 3). Epithelial cells as well as connective tissue cells in diseased gingival tissues showed high nuclear ERβ but no nuclear ERα immunoreactive signal in all six patients. The nuclear ERα and ERβ immunoreactivity in both the gingival epithelium and the lamina propria were scored according to predetermined criteria as described in the Material and methods. Almost all the cells in both healthy and diseased gingival epithelium and lamina propria were ERβ positive (Table 2). The ERβ expression was identical in healthy and diseased gingiva. No detectable nuclear ERα immunoreactive signal was observed in either healthy or diseased gingival epithelium and lamina propria (Table 2). The gingival ERα and ERβ expression pattern was almost identical in all six patients under study (Table 2).

Expression of ERα and ERβ and effects of E_2 on DNA synthesis in cultured human primary gingival epithelial cells

Cultured human primary gingival epithelial HGEPp.05 cells expressed strong nuclear ERβ and weak nuclear ERα immunoreactivity (Fig. 4). As demonstrated in Fig. 4, the ERβ immunoreactive signal was much stronger than that for ERα. No ERα and ERβ immunoreactivity was observed after omission of the respective primary antibody (Fig. 4). Stimulation with E_2 at 500 nM for 24 h reduced growth-promoter-induced (5% fetal calf serum) DNA synthesis by about 60% (Fig. 5). Treatment with 10 μM E_2 attenuated DNA synthesis by about 70% (Fig. 5). A low physiological concentration of E_2 (10 nM) had no effect on DNA synthesis (Fig. 5).

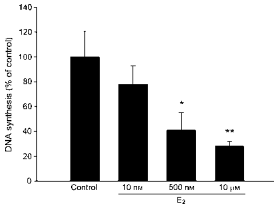


Fig. 5. Effects of stimulation with 17β-estradiol (E_2) for 24 h on DNA synthesis in cultured human gingival epithelial HGEPp.05 cells. DNA synthesis was determined by measuring incorporation of radiolabelled thymidine into newly synthesized DNA during the last hour of the 24 h incubation. Values are presented as means ± SEM of five to 10 observations in each group. *$p < 0.05$ and **$p < 0.01$, compared with control.

Discussion

Here, we show that ERβ is the predominant estrogen receptor in healthy as well as diseased human gingiva, implying that the effects of estrogen on gingival tissues in both health and disease are mediated via this receptor. Gingival epithelial cells in healthy gingiva have been reported previously to express the ERβ protein (10). Our data confirm the findings reported by Välimaa *et al.* (10), but in addition to their report, we show now that diseased gingiva has a similar ER subtype expression pattern to healthy gingiva, i.e. strong ERβ immunoreactivity but no ERα immunoreactivity. Semi-quantitative analysis of ERβ expression in healthy and diseased gingiva within individual subjects showed no difference in ERβ immunoreactive signal between healthy and diseased sites, suggesting that periodontal/gingival inflammation does not affect the expression level of ERβ observed in healthy tissue. Thus, ERβ seems to be responsible for estrogen signalling in both healthy and diseased gingiva. Our data demonstrating high ERβ expression in gingival tissues are in accordance with previous reports showing high ERβ concentrations in other nonreproductive tissues (4).

We report no difference in gingival ER subtype expression between male and female subjects, suggesting that gingival ERβ, which seems to be the dominating gingival ER subtype, plays an important role in male as well as female gingiva. Three of six subjects included in this study took medication because of various diagnoses, e.g. high blood pressure. We have no reason to believe that the patients' medication affected their ER subtype expression pattern, because the pattern of ER subtype expression was similar in all patients whether or not they were on medication and similar in all patients independent of their type of medication. Furthermore, the drugs used by the three patients on medication were not steroids or other possible ER ligands, which might interfere with ER expression level. Thus, we suggest that the patients' medication had no influence on gingival ERα and ERβ expression, although we cannot completely rule out the possibility that the patients' medication and their medical condition affected expression of ERs.

In the present study, we show that cultured human gingival epithelial HGEPp.05 cells express strong immunoreactivity for ERβ but also a weak ERα immunoreactive signal. In gingival biopsies, no immunoreactivity for ERα was detected, suggesting plasticity in gingival epithelial cell ERα expression depending on the milieu where the cells reside, i.e. if they are in culture or localized in their natural *in vivo* context with blood vessels, nerves and extracellular matrix. High concentrations (500 nM and 10 μM) of E_2 reduced DNA synthesis in cultured human gingival epithelial HGEPp.05 cells, but in contrast, a low concentration of E_2 (10 nM) had no effect, suggesting differential effects of estrogen on gingival epithelial cell proliferation depending on concentration. The preovulatory plasma concentration of E_2 is about 2 nM but increases severalfold in pregnancy (16). Thus, the low concentration (10 nM) of E_2 that we used is within the physiological range. In contrast, 500 nM and 10 μM are regarded as high concentrations, probably reflecting a pharmacological rather than a physiological situation (16). In the cultured human skin keratinocyte cell line NCTC 2544 cells, E_2 (10 nM) produces a slight but significant increase in proliferation (23). Estrogen has been reported to accelerate cutaneous wound healing in female mice independent of its anti-inflammatory activities (24,25). Estrogen-induced improvement of cutaneous wound healing in mice is proposed to be mediated via ERβ (25). Thus, previous studies in mice and in a cultured human skin keratinocyte cell line suggest that estrogen promotes skin epithelial cell proliferation. We demonstrate in the present study that estrogen has no effect on proliferation of cultured human gingival epithelial cells at a physiologically relevant concentration, but attenuates proliferation at a pharmacological dose. Estrogen seems thus to have different effects on skin and gingival epithelial cell proliferation. These differences might reflect differences in ER subtype expression pattern between these two epithelial cell types.

Redundant, nonredundant and sometimes opposite effects have been reported for ERα and ERβ (4). Differential cellular expression of ER subtypes makes it possible for estrogen to have selective effects in different cell types (4). Our data suggest that estrogen, acting via ERβ, regulates gingival epithelial cell proliferation, causing anti-proliferation at high but not low concentrations. Gingival epithelial ERβ may, however, also mediate estrogenic effects other than proliferation, e.g. on the metabolic system. Estrogen has been shown to regulate enzymes involved in oxidative phosphorylation in different tissues and cells, such as blood vessels and periodontal ligament cells (26,27), suggesting that estrogen regulates the cellular production of ATP, thereby influencing many cellular processes. Phytoestrogens, such as the isoflavonoid genistein, have been shown to activate ERβ with high specificity (28), implying that this type of ERβ ligand also influences gingival epithelial estrogenic signalling.

In summary, we conclude that ERβ seems to be the predominant ER subtype in human gingival tissue and that a pharmacological concentration of estrogen attenuates gingival epithelial cell proliferation.

Acknowledgements

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V

DIFFERENTIAL EFFECTS OF LPS FROM *ESCHERICHIA COLI* AND *PORPHYROMONAS GINGIVALIS* ON IL-6 PRODUCTION IN HUMAN PERIODONTAL LIGAMENT CELLS

Daniel Nebel[1,2*], Joel Arvidsson[1,2], Johan Lillqvist[1,2], Anders Holm[1] and Bengt-Olof Nilsson[1]
[1]Department of Experimental Medical Science, Lund University, BMC D12, SE-221 84 Lund, Sweden
[2]Department of Periodontology, Faculty of Odontology, Malmö University, SE-20506 Malmö, Sweden

Running title: Differential regulation of IL-6

Keywords: IL-6; LPS; nitric oxide; PDL cells

***Correspondence:** Dr. Daniel Nebel
Department of Periodontology
Faculty of Odontology
Malmö University
SE-20506 Malmö
Sweden
Phone: +46-40-6658453
E-mail: Daniel.Nebel@mah.se

Abstract

Background and Objective: Periodontal ligament (PDL) cells have been shown to produce IL-6 upon stimulation with inflammation promoters but the mechanisms involved are not fully understood. Here we determine IL-6 production in human PDL cells, endothelial cells and monocytes stimulated by *E. coli* or *P. gingivalis* LPS. Furthermore, we investigate signaling pathways regulating IL-6 expression. *Material and Methods*: Human PDL cells were from explants of periodontal ligament of teeth extracted for orthodontic reasons. Human endothelial cells and monocytes were from Lonza and ATCC, respectively. IL-6 mRNA and protein were determined by quantitative real-time PCR and ELISA, respectively. *Results*: Stimulation with LPS from *E. coli* (1 μg/ml) for 24 h enhanced PDL cell IL-6 expression several fold demonstrated both on transcript and protein levels but *P. gingivalis* LPS (1-5 μg/ml) had no effect. On the other hand, neither *E. coli* LPS nor *P. gingivalis* LPS promoted IL-6 production in endothelial cells. Treatment with the non-selective nitric oxide synthase inhibitor L-NAME (100 μM) reduced *E. coli* LPS-induced PDL cell IL-6 by 30%, while neither aminoguanidine (10 μM), an inhibitor of inducible nitric oxide synthase, nor estrogen (17β-estradiol, 100 nM) influenced IL-6. Treatment with the glucocorticoid dexamethasone (1 μM) totally prevented the *E. coli* LPS-induced PDL cell IL-6. In monocytes, serving as positive control, both *E. coli* LPS and *P. gingivalis* LPS stimulated IL-6. *Conclusions*: *E. coli* LPS but not *P. gingivalis* LPS stimulates PDL cell IL-6 production partially through a mechanism probably involving nitric oxide formation via endothelial nitric oxide synthase.

Introduction

The periodontopathogen *Porphyromonas gingivalis* (*P. gingivalis*) is regarded as a major etiologic factor in periodontitis leading to production of cytokines/chemokines, such as IL-6, associated with the progression of the disease (1,2). In the periodontium, not only classical inflammatory cells, such as white blood cells and macrophages, but also periodontal ligament (PDL) cells and gingival fibroblasts respond to bacterial lipopolysaccharide (LPS) by producing cytokines and chemokines (3), suggesting that also PDL cells and gingival fibroblasts may promote periodontal inflammation. Also endothe-

lial cells and gingival epithelial cells have been reported to produce cytokines in response to LPS stimulation (4-6), suggesting that also these cell types may contribute to the cytokine/chemokine load in periodontal inflammation. LPS is thought to promote cytokine/chemokine expression by binding to Toll-like receptors (TLRs), which through either a myeloid differentiation factor (MyD88)-dependent or a MyD88-independent pathway activates the transcription factor nuclear factor-κB (NF-κB) causing transcription of cytokine/chemokine genes, but still the mechanisms are not fully understood (7). LPS primarily acts through TLR4 but LPS from some bacterial species also activates cells via TLR2 (7). Interestingly, *P. gingivalis* LPS has been reported to act as an agonist for TLR2 but as an antagonist for TLR4 (8).

Estrogen has been shown to influence mechanisms involved in the inflammatory reaction, however its role in inflammation is complex involving both anti- and pro-inflammatory actions (9). The biologically most active estrogen, E_2, has been reported to modulate LPS-induced cytokine and chemokine expression in human PDL cells (10,11), suggesting that estrogen modulates periodontal inflammation by controlling PDL cell production of cytokines and chemokines. Estrogen-induced regulation of PDL cell cytokine/chemokine production is probably mediated via estrogen receptor β (ERβ), since this ER subtype is highly expressed in PDL cells (12,13), but we cannot completely rule out that also ERα is involved. Inhibition of nitric oxide synthase (NOS) has been shown to prevent alveolar bone loss in experimental periodontitis, suggesting that nitric oxide (NO) modulates inflammation and bone resorption in periodontitis (14, 15). NO is formed in an enzymatic reaction using the amino acid arginine as substrate (16). This reaction is catalyzed by three isoforms of nitric oxide synthase (NOS) named endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (16).

In the present study we compare effects of LPS from *P. gingivalis* with those of LPS from *Escherichia coli* (*E. coli*) on IL-6 production in human PDL cells and human endothelial cells showing that LPS from *E. coli* is a much more powerful stimulator of IL-6 production than LPS from *P. gingivalis*. The involvement of estrogen, corticos-

teroids and NO in LPS-induced IL-6 production is assessed using a pharmacological approach. We disclose that inhibition of NO production reduces *E. coli* LPS-induced IL-6 production. Furthermore, we show that *E. coli* LPS enhances IL-6 production several fold in PDL cells, while it has no significant effect on IL-6 in endothelial cells, suggesting a cell typr specific effect.

Materials and methods

Cells and cell culture

PDL cells were obtained from premolars extracted for orthodontic reasons. The patients and their parents were informed and the parents gave written consent. The study was approved by the Human Ethical Committee at Lund University, Lund, Sweden. The periodontal ligament was gently scraped off from the middle third of the root surface and then washed in phosphate buffered saline (PBS) in order to avoid contamination from the gingival and apical tissues. Tissue explants from four premolars in two subjects (one boy and one girl 14 years of age) were seeded providing eight clones of PDL cells. These clones of cells responded identically to treatment with the different drugs specified below. The tissue explants were transferred to cell culture flasks containing Dulbecco's modified Eagle's medium supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml), glutamine (1.16 g/l) and 10% fetal calf serum. The flasks were placed in a water-jacketed cell/tissue incubator with 5% CO_2 in air. The cells were allowed to migrate from the explants and after reaching confluence the cells were trypsinized (0.25% trypsin) and reseeded at a density of 80,000 cells/ml. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Lonza, Walkersville, MD, USA) and cultured in endothelial cell culture medium as recommended by the manufacturer. Human THP-1 monocytes (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium as recommended. Experiments were performed in subconfluent cells in passages 2–5.

Experimental procedure

Before experiments the normal culture medium was exchanged for phenol red-free culture medium containing dextran-coated charcoal

stripped fetal calf serum to remove the estrogen-like activity of phenol red and estrogens derived from the serum. The phenol red-free culture medium with charcoal stripped fetal calf serum was used throughout the experiment. After 2 h pre-treatment with or without E_2 (100 nM, Sigma Chemicals, St. Louis, MO, USA), dexamethasone (1 µM, Sigma), N^G-nitro-L-arginine methyl ester (L-NAME, 100 µM, Sigma) or aminoguanidine (10 µM, Sigma), the cells were treated with or without *E. coli* 0111:B4 LPS (1 µg/ml, Sigma) or *P. gingivalis* LPS (1-5 µg/ml, InvivoGen, San Diego, CA, USA) for 24-72 h in the continuous presence of E_2, L-NAME, dexamethasone or aminoguanidine. In order to detect any possible effect of estrogen we used a physiologically high concentration (100 nM) of E_2. The pre-ovulatory plasma concentration of E_2 is around 2 nM but increases in pregnancy several fold to about the same concentration as used by us in the present study (17). L-NAME, at 100 µM, inhibits all three isoforms of nitric oxide synthase (NOS), i.e. eNOS, iNOS and nNOS, while aminoguanidine selectively reduces iNOS with an IC_{50} of about 10 µM (18). E_2 and dexamethasone were dissolved in dimethylsulfoxide (DMSO), while L-NAME and aminoguanidine were dissolved in PBS. Controls received vehicle as appropriate.

Quantitative real-time PCR

Cells were washed carefully in PBS and then total RNA was extracted and purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Concentration and purity of RNA was measured at 260/280 nm in a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). One-step quantitative real-time PCR measurements were performed using QuantiFast SYBR Green RT-PCR kit (Qiagen) and QuantiTect primer assays (Qiagen) on a Step One Plus Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Each sample was analyzed in duplicate. IL-6 and MCP-1 gene expression was calculated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference gene as described by Pfaffl (19). The PCR primers (QuantiTect Primer Assays) for IL-6 (Hs_IL6_1_SG), MCP-1 (Hs_CCL2_1_SG) and GAPDH (Hs_GAPDH_2_SG) were purchased from Qiagen.

ELISA

The cells were washed carefully in PBS and scraped off the culture dishes using cell scrapers (Sarstedt, Newton, NC, USA). Then the cells were sonicated 2 x 10 s on ice and centrifuged at 1700 x *g* at 4 °C for 5 min. The IL-6 protein was determined in the cell supernatant using enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN, USA). IL-6 protein level was determined according to instructions by the manufacturer. Each sample was analyzed in duplicate. The IL-6 level was normalized to the total protein concentration determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

Statistics

Values are presented as means ± S.E.M. Statistical significance was calculated using ANOVA and Student's two-tailed t-test for unpaired comparisons with Bonferroni correction for post hoc analysis as appropriate. P values less than 0.05 were regarded to denote statistical significance.

Results

Effects of *E. coli* and *P. gingivalis* LPS on IL-6 production in PDL cells

Stimulation with *E. coli* LPS (1 µg/ml) for 24 h increased mRNA for IL-6 by 4 to 5 times, while, on the other hand, *P. gingivalis* LPS, administered at the same concentration (1 µg/ml), had no effect on PDL cell IL-6 mRNA expression (Fig. 1). A similar pattern was observed for monocyte chemoattractant protein-1 (MCP-1) transcript as well, i.e. stimulation with *E. coli* LPS (1 µg/ml) for 24 h increased MCP-1 mRNA by about 4 times, while *P. gingivalis* LPS (1 µg/ml) had no effect (Fig. 2). Treatment with 1 µg/ml *E. coli* LPS for 24 h increased IL-6 protein concentration by about 30 times in PDL cells (Fig. 3), while, on the contrary, *P. gingivalis* LPS (1 µg/ml) had no effect on PDL cell IL-6 production confirming the mRNA data (Fig. 3).

The *E. coli* LPS-induced (1 µg/ml) increase in PDL cell IL-6 protein production was attenuated by about 30% by the NOS blocker L-NAME (100 µM), while, on the other hand, E_2 (100 nM) had no effect (Fig. 3). The selective iNOS blocker aminoguanidine (10µM) had no effect on *E. coli* LPS-induced IL-6 (0.81±0.06 pg/µg protein in PDL cells treated with LPS vs. 0.76±0.13 pg/µg protein in PDL cells treated with LPS+aminoguanidine, n=5 in each group). The glucocorticoid dexamethasone (1 µM) completely abolished the PDL cell IL-6 production induced by 1 µg/ml *E. coli* LPS (Fig. 4).

Effects of *E. coli* and P. *gingivalis* LPS on IL-6 production in HUVEC endothelial cells

Treatment with *E. coli* LPS (1 µg/ml) for 24 h had no effect on IL-6 concentration in human HUVEC umbilical vein endothelial cells (0.0187±0.0025 pg/µg protein in *E. coli* LPS treated cells vs. 0.0122±0.0015 pg/µg protein in control cells, n=4 in each group). Stimulation with *P. gingivalis* LPS at both 1 and 5 µg/ml for 24 h as well as a longer time point (72 h) had no effect on HUVEC IL-6 protein concentration (data not shown).

Effects of *E. coli* and *P. gingivalis* LPS on IL-6 production in THP-1 monocytes

Effects of *E. coli* and *P. gingivalis* LPS on IL-6 production in human THP-1 monocytes were determined for positive control. Treatment with 1 µg/ml *E. coli* LPS and 1 µg/ml *P. gingivalis* LPS for 24 h increased IL-6 protein by about 80 and 6 times, respectively (Fig. 5), showing that monocytes produce IL-6 in response to both *E. coli* and *P. gingivalis* LPS.

Fig. 1

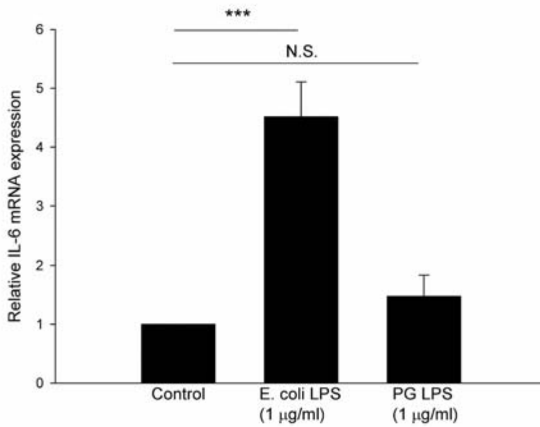

Fig. 1. Stimulation with *E. coli* LPS (1 μg/ml) but not *P. gingivalis* (PG) LPS (1 μg/ml) for 24 h increases mRNA for IL-6 in human PDL cells. Values are means ± S.E.M. of 5-6 observations in each group. *** represents $P<0.001$. N.S.= not significant.

Fig. 2

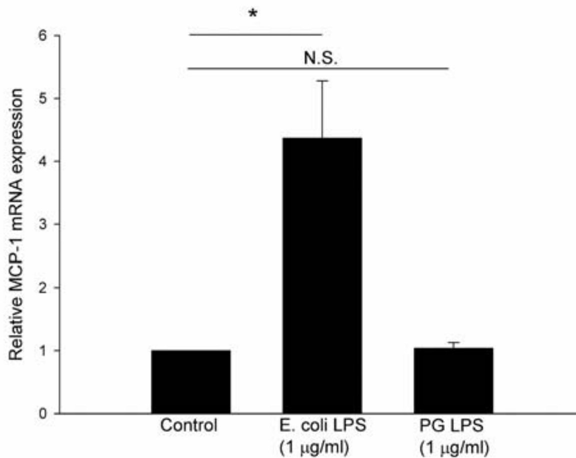

Fig. 2. Stimulation with *E. coli* LPS (1 μg/ml) but not *P. gingivalis* (PG) LPS (1 μg/ml) for 24 h increases mRNA for MCP-1 in human PDL cells. Values are means ± S.E.M. of 6 observations in each group. * represents $P<0.05$. N.S.= not significant.

Fig. 3

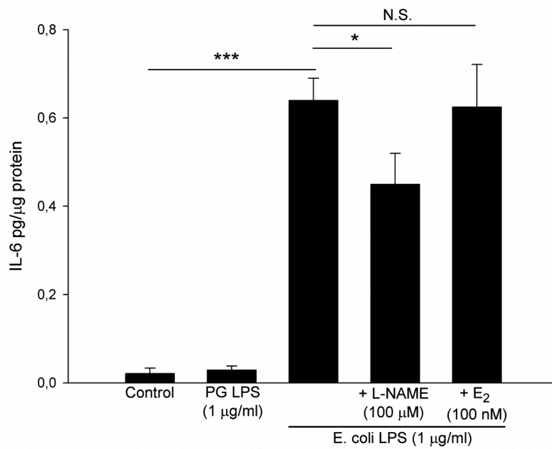

Fig. 3. *E. coli LPS*-induced PDL cell IL-6 protein production is reduced by the NO synthase blocker L-NAME. Human PDL cells were stimulated for 24 h with *E. coli* or *P. gingivalis* (PG) LPS (1 µg/ml) in the absence or presence of L-NAME (100µM) or 17β-estradiol (E_2, 100 nM). IL-6 content was normalized to total protein concentration in each sample. Values are means ± S.E.M. of 4-12 observations in each group. * and *** represent P<0.05 and 0.001, respectively. N.S.= not significant.

Fig. 4

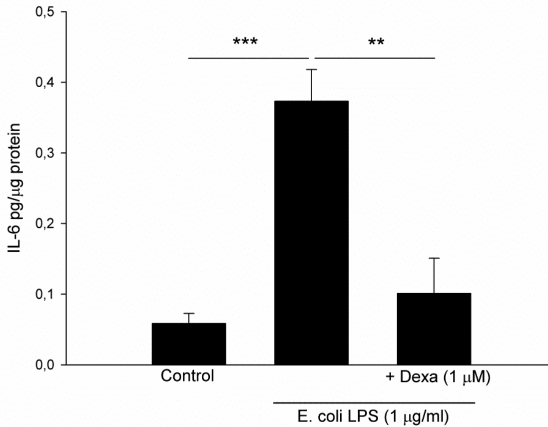

Fig. 4. The glucocorticoid dexamethasone (Dexa, 1 µM) abolishes completely PDL cell IL-6 protein production stimulated by 1 µg/ml *E. coli* LPS at 24 h. IL-6 content was normalized to total protein concentration in each sample. Values are means ± S.E.M. of 5 observations in each group. ** and *** represent P<0.01 and P<0.001, respectively.

Fig. 5

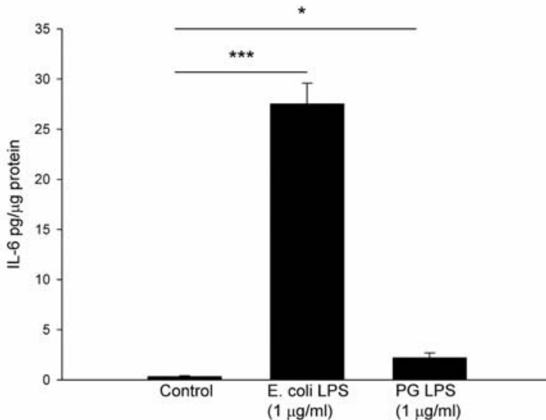

Fig. 5. Both *E. coli* and *P. gingivalis* (PG) LPS (1 µg/ml for both *E. coli* and *P. gingivalis* LPS) stimulate IL-6 protein production in human THP-1 monocytes used for positive control. IL-6 content was normalized to total protein concentration in each sample. Values are means ± S.E.M. of 5 observations in each group. * and *** represent P<0.05 and P<0.001, respectively.

Discussion

Here we show that *E. coli* LPS stimulates IL-6 production several fold in human PDL cells. On the other hand, LPS from *P. gingivalis* has no effect on PDL cell IL-6 production, suggesting that LPS derived from *E. coli* is a more powerful stimulator of PDL cell IL-6 production than LPS from *P. gingivalis*. Our data confirm those by Jones *et al.* (20) showing that *E. coli LPS* causes a much more powerful stimulation of IL-6 than *P. gingivalis* LPS in mouse gingival fibroblast cell line ESK-1 cells. Interestingly, Morandini *et al.* (21) show recently that stimulation with *P. gingivalis* LPS (the same LPS, from InvivoGen, as the one used by us in the present study), administered in the same concentration (1 µg/ml) and for the same time (1-24 h) as in our study, has no effect on IL-6 expression, neither on mRNA nor on protein levels, in PDL cells and gingival fibroblasts from the same donors. Taken together these data strongly suggest that *P. gingivalis* LPS is a weak stimulator of IL-6 production both in PDL cells and gingival fibroblasts.

Additionally, our data show that *E. coli* LPS stimulates IL-6 production in PDL cells but not in HUVEC endothelial cells, suggesting that *E. coli* LPS acts in a cell type specific manner. HUVEC endothelial cells are widely used studying human endothelial cell physiology and pathophysiology. Indeed, Makó *et al.* (22) have recently shown that LPS is a poor stimulator of IL-6 in HUVECs probably due to the cellular localization of TLR4. These authors show that endothelial cell TLR4 is mainly localized to the Golgi apparatus. We used human THP-1 monocytes for positive control showing that both *E. coli* LPS and *P. gingivalis* LPS stimulate IL-6 production in these cells, suggesting that LPS from *P. gingivalis* promotes IL-6 production from monocytes. However, although *P. gingivalis* LPS clearly stimulates monocyte IL-6 production (6-fold increase) it is much less potent than *E. coli* LPS (80-fold increase).

We show in the present study that the NOS blocker L-NAME, blocking all three isoforms of NOS, but not the selective iNOS blocker aminoguanidine, reduces partially *E. coli* LPS-induced IL-6 production in PDL cells, suggesting that the signaling pathway involves NO formed via eNOS and/or nNOS rather than via iNOS. L-NAME was

used in a concentration (100 µM) that inhibits all NOS isoforms and aminoguanidine in a concentration (10 µM) that is equivalent to its IC_{50} concentration for iNOS (18). Our results suggesting that eNOS but not iNOS may be involved in *E. coli* LPS-induced PDL cell IL-6 production are supported by findings by Kikuiri *et al.* (23) showing that human PDL cells express eNOS but not iNOS as demonstrated both on mRNA and protein levels. The nNOS isoform is primarily expressed in neuronal tissues making it less likely that this NOS isoform is involved (16). The attenuation of PDL cell IL-6 production by L-NAME is small compared to that of the glucocorticoid dexamethasone which totally prevents *E. coli* LPS-induced IL-6. Indeed, Jönsson *et al.* (24) have previously observed that *E. coli* LPS-induced GROα chemokine production is abolished by dexamethasone in PDL cells, suggesting that both IL- 6 and GROα chemokine expression in PDL cells are sensitive to glucocorticoid treatment. Glucocorticoids are supposed to exert their anti-inflammatory effects by inhibiting NF-κB transcriptional effects (25). Thus, since dexamethasone fully prevents *E. coli* LPS-induced IL-6 in PDL cells it is reasonable to conclude that transcription of the IL-6 gene in PDL cells stimulated by *E. coli* LPS involves NF-κB.

Estrogen (E_2) has been reported to modulate inflammation and immune responses (9). In the present study E_2 failed to prevent *E. coli* LPS-induced IL-6 in PDL cells. In order to detect any possible effect of E_2 we used a high but still physiological concentration of E_2, i.e. 100 nM (17). The lack of effect of E_2 on E. coli-induced IL-6 observed in the present study confirms data reported by Jönsson *et al.* (26) showing that both acute (24 h) and chronic (72 h) treatment with 100 nM E_2 has no effect on IL-6 production in human PDL cells stimulated by *E. coli* LPS.

In summary, we show that *E. coli* LPS, but not *P. gingivalis* LPS, is a powerful stimulator of PDL cell IL-6 production and that combined treatment with *E. coli* LPS and the non-selective NOS blocker L-NAME reduces this response, suggesting that *E. coli* LPS acts, at least partially, through stimulation of NO formation. Furthermore, we show that *E. coli* LPS enhances IL-6 production several fold in PDL cells, while it has no significant effect on IL-6 in endothelial cells, suggesting a cell type specific effect.

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APPENDIX

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Review Article

The human periodontal ligament cell: a fibroblast-like cell acting as an immune cell

D. Jönsson[1,2], D. Nebel[1,2], G. Bratthall[2], B.-O. Nilsson[1]
[1]Department of Experimental Medical Science, Lund University, Lund, Sweden and [2]Department of Periodontology, Faculty of Odontology, Malmö University, Malmö, Sweden

Jönsson D, Nebel D, Bratthall G, Nilsson B-O. The human periodontal ligament cell: a fibroblast-like cell acting as an immune cell. J Periodont Res 2011; 46: 153–157. © 2010 John Wiley & Sons A/S

Background: Periodontal ligament cells are fibroblast-like cells characterized by collagen production but also possessing some osteoblastic features. In the light of numerous studies presented during recent times, which show that human periodontal ligament cells also produce cytokines and chemokines in response to inflammation promoters, it is reasonable to suggest that periodontal ligament cells play a role as promoters of periodontal inflammation through these mechanisms.

Material and Methods: The periodontal ligament, which harbours the periodontal ligament cells, is a part of the attachment apparatus comprised of periodontal ligament cells, extracellular matrix and fibres, attaching the root cement to the alveolar bone. Periodontal ligament cells are in close proximity to bacteria within the plaque and the pocket, and thus these cells are readily accessible to bacterial endotoxins and other promoters of inflammation.

Results: Cytokines and chemokines, released by periodontal ligament cells upon stimulation with inflammation promoters, reach the blood vessels easily thanks to rich vascularization of the periodontium stimulating recruitment of white blood cells to the site of inflammation. In addition to classical inflammatory cells, such as leucocytes, macrophages and mast cells, the periodontal ligament cells also contribute to periodontal inflammation via their production and release of cytokines and chemokines.

Conclusion: Therefore, pharmacological treatment of periodontitis should aim to reduce the release of proinflammatory agents not only from classical inflammatory cells but also from periodontal ligament cells.

Dr Bengt-Olof Nilsson, DDS, PhD, Department of Experimental Medical Science, Lund University, BMC D12, SE-221 84 Lund, Sweden
Tel: +46 46 2227767
Fax: +46 46 2224546
e-mail: bengt-olof.nilsson@med.lu.se

Key words: chemokine; cytokine; glucocorticoid; inflammation; periodontal ligament cell

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The process of generating human periodontal ligament cells

The human periodontal ligament cells used in experimental studies are obtained by allowing cells to grow out of periodontal ligament tissue explants kept under growth-stimulating conditions (cell culture medium containing 10% foetal calf serum as growth promoter). This protocol was established by Somerman *et al.* (1,2), and it has been used in numerous studies. The explants are scraped off the middle third of the root surface to avoid contamination with marginal or apical tissues. The teeth used for collecting explants are normally extracted for orthodontic reasons from boys and girls between 12 and 18 years of age. The periodontal ligament cells start to grow out from the explants within about 10 d. The viability of the cells is high, probably due to the fact that the cells originate from young and healthy individuals. After passage (confluent cells trypsinized in 0.25% trypsin), the cells are re-seeded and then used for experiments in passages two to eight. In passages three to

five, the cells respond in a similar manner to stimulation with hormones and growth promoters (3), suggesting that their phenotype is maintained, although some osteogenic markers, e.g. alkaline phosphatase activity, have been shown to be decreased at later (passage 6) compared with earlier passages (4). The periodontal ligament cells in passages three to five show a fibroblast-like morphology, with a spindle-shaped cell form (5). Cell viability decreases at later passages (beyond passage 7), suggesting that the periodontal ligament cells then go into senescence (2).

Periodontal ligament cells produce collagen and show some osteoblast-like features

The periodontal ligament cells produce and secrete extracellular matrix components, such as collagen, building up the periodontal ligament and its fibres, to secure attachment of root cement to alveolar bone and to allow regeneration of the periodontal ligament to occur upon injury. Indeed, periodontal ligament cells have a high production of collagen (1,6). Besides producing collagen, periodontal ligament cells may also produce mineralized tissue. Human periodontal ligament cells have been shown to express high levels of alkaline phosphatase activity and bone-associated proteins, such as osteonectin, and to form mineralized nodules, suggesting that periodontal ligament cells are osteogenic cells (2,7). The periodontal ligament cells do not, however, respond to calcitonin or parathyroid hormone and they do not express other bone marker proteins, such as bone sialoprotein 1, in contrast to osteoblasts (2). Piche *et al.* (8) showed that periodontal ligament cell clones derived from periodontal ligament explants obtained from two different individuals exhibited high and low basal alkaline phosphatase activity, respectively, suggesting a functional heterogeneity between periodontal ligament cell clones originating from different individuals. Taken together, these data show that the periodontal ligament cells do not fully behave as classical osteoblasts. Human periodontal ligament cells and gingival fibroblasts show many functional similarities, but they also possess different functional characteristics, e.g. high alkaline phosphatase activity is detected in periodontal ligament cells but not in gingival fibroblasts (9).

Heterogeneity of periodontal ligament cells in culture

Periodontal ligament cells in culture possess many diverse cell-phenotype characteristics, e.g. those typical for fibroblasts, such as fibroblast morphology and collagen production (1,3,6), and those typical for osteocytes and osteoblasts, such as alkaline phosphatase activity and expression of bone-associated proteins (2,7). It is possible that a subpopulation of periodontal ligament cells showing osteogenic properties is derived from bone cells dislodged into the periodontal ligament during extraction. Although cultured periodontal ligament cells respond in a similar manner to hormones and growth factors in passages three to five (3), suggesting that they represent a rather homogeneous and stable population of cells, we cannot rule out the possibility that periodontal ligament cells in culture represent multiple cell types. The stimulation of periodontal ligament cell production of cytokines and chemokines by inflammation promoters described below is a consistent and highly reproducible periodontal ligament cell response to this type of stimulation, probably representing a global property of periodontal ligament cells in culture, although we cannot completely rule out the possibility that this response is associated only with a subpopulation of cultured periodontal ligament cells.

Inflammation promoters stimulate periodontal ligament cell cytokine and chemokine mRNA and protein production

Recent studies suggest that human periodontal ligament cells, in addition to their fibroblastic and osteoblastic properties, also possess functional characteristics similar to those of leucocytes and leucocyte-derived cells (e.g. macrophages) involved in classical innate immunity. The periodontal ligament cells have been shown to express and produce cytokines and chemokines in response to inflammation promoter stimulation, as shown both at the mRNA and at the protein level (see Table 1 for references). In unstimulated human periodontal ligament cells, cytokine and chemokine transcript and protein levels are low or below the limit of detection, but they increase severalfold upon stimulation with inflammation promoters, such as bacterial lipopolysaccharide (LPS). Lipopolysaccharide also induces cytokine production in human gingival fibroblasts (10,11), suggesting that periodontal ligament cells and gingival fibroblasts act together to promote proinflammatory actions. Cytokine and chemokine production by periodontal ligament cells is observed in response to stimulation with low, intermediate and high concentrations (1 ng/mL to 10 μg/mL) of LPS (12,13). Lipopolysaccharide-induced periodontal ligament cell cytokine/chemokine expression is observed within hours but also after several days (3–21 d) of treatment, showing that both acute and long-term stimulation with inflammation promoters activate cell-signalling pathways leading to cytokine/chemokine production (12).

Inflammation promoter-induced cytokine and chemokine production is observed in experiments performed in periodontal ligament cells in passages three to five, i.e. in cells which have been trypsinized several times and cultured for many weeks from the time point at which the cells start to grow out of the periodontal ligament tissue explants (about 10 d after tooth extraction and seeding of explants). Lipopolysaccharide-induced cytokine and chemokine production in primary periodontal ligament tissue explants and in cells derived directly from the explants (i.e. at short time points) may be associated with ordinary inflammatory cells that happen to be scraped off the tooth, but the stimulation of cytokine/chemokine production by LPS observed in cells at passages three to five is most probably due to stimulation of long-lived and persistent cells, such as classical periodontal ligament fibroblasts. It is also important to conclude that both acute

Table 1. A review of papers presenting data on proinflammatory stimulus-induced cytokine/chemokine expression in human periodontal ligament cells.

Reference	Proinflammatory stimulus	Cytokine/chemokine	Transcript/protein level
Jönsson *et al.* (12)	*Escherichia coli* LPS	IL-6, MCP-1	Protein
Jönsson *et al.* (13)	*E. coli* LPS	GROα	mRNA and protein
Okada *et al.* (19)	TNF-α	IL-6	Protein
Yamamoto *et al.* (29)	*Porphyromonas gingivalis, P. intermedia*	IL-1β, IL-6, IL-8	mRNA
Ogura *et al.* (30)	*P. endodontalis* LPS	IL-6	mRNA and protein
Agarwal *et al.* (31)	*Actinobacillus actionomycetem-comitans* LPS, *E. coli* LPS	IL-1β, IL-6, IL-8	mRNA and protein
Shu *et al.* (32)	*E. coli* LPS	TNFα, IL-1β, IL-6, RANKL	mRNA and protein
Yamaji *et al.* (33)	*P. gingivalis* LPS, *E. coli* LPS	IL-6, IL-8	mRNA and protein
Engels-Deutsch *et al.* (34)	*Streptococcus mutans*	IL-6, IL-8	Protein
Ozaki *et al.* (35)	TNFα, IL-1β	MCP-1	mRNA and protein
Lee *et al.* (36)	Reactive oxygen species (H_2O_2)	IL-8	mRNA and protein
Morandini *et al.* (37)	*P. gingivalis* LPS	IL-6	Protein
Wada *et al.* (38)	*E. coli* LPS	IL-1β, TNFα	mRNA

(24 h) and chronic (3–21 d) LPS stimulation of periodontal ligament cells seeded at passages three to five enhances cytokine and chemokine production several-fold (12).

Mechanisms behind inflammation-promoter-induced periodontal ligament cell cytokine and chemokine expression

Lipopolysaccharide binds to its receptor, the Toll-like receptor 4 (TLR4), and this complex regulates gene transcription of cytokine and chemokine genes via different adaptor proteins and transcription factors (14,15). Toll-like receptor 4 signalling involves a MyD88-dependent as well as a MyD88-independent pathway causing activation of nuclear factor-κB (NF-κB). The MyD88-independent signalling involves activation of the adaptor molecule TRAM, which forms a complex with another adaptor molecule named TRIF. This complex then binds the adaptor protein tumor necrosis factor receptor associated factor 6 (TRAF6), leading to activation of NF-κB. Both the MyD88-dependent and the MyD88-independent activation of NF-κB induce expression of inflammatory genes. Thus, NF-κB is a possible drug target for anti-inflammatory treatment. In a murine periodontal ligament cell line, Patil *et al.* (16) have shown that *Actinobacillus actinomycetemcomitans* and *Escherichia coli* LPS-induced IL-6 expression is dependent on multiple MAPK pathways, including ERK and c-jun N-terminal kinase (JNK), demonstrating that many intracellular signalling pathways regulate periodontal ligament cell cytokine gene activity.

Glucocorticoids and human periodontal ligament cells

The glucocorticoids bind to the nuclear glucocorticoid receptor to form a ligand–receptor complex. This complex is supposed to attenuate inflammation by inhibition of NF-κB-dependent transcription of proinflammatory genes (17). Dexamethasone is the most widely used glucocorticoid in biological experiments. Human periodontal ligament cells accumulate radioactive ([^{3}H]-labelled) dexamethasone, suggesting that periodontal ligament cells express the glucocorticoid receptor (18). Treatment with dexamethasone has been shown to reduce tumour necrosis factor-α-induced interleukin-6 and interleukin-8 production (19,20) and to reduce LPS-induced chemokine ligand 1 (GROα) chemokine expression (13) in human periodontal ligament cells. Besides suppressing cytokine/chemokine production via inhibition of NF-κB, dexamethasone drives the periodontal ligament cells towards an osteoblastic phenotype (21). However, a selective response to dexamethasone, promoting the osteoblastic features of a subpopulation of osteoblast-like periodontal ligament cells, may represent an alternative mechanism by which dexamethasone affects periodontal ligament cells in culture. Although dexamethasone is supposed to reduce inflammation via a direct mechanism involving inhibition of NF-κB, the transition of periodontal ligament cells from a fibroblast-like to an osteoblast-like cell phenotype and/or the stimulation of a subpopulation of osteoblast-like cells by dexamethasone may indirectly reduce the periodontal ligament cell production of proinflammatory factors. It is reasonable to suggest that glucocorticoids may be used to block the unwanted production of periodontal ligament cell cytokines and chemokines, but the indirect effects of glucocorticoid treatment are many. Therefore, other more specific pharmacological approaches are probably necessary, e.g. through interactions with periodontal ligament cell MyD88 and/or MAPK signalling, to achieve inhibition of periodontal ligament cell cytokine/chemokine expression.

Proinflammatory activity of fibroblast-like cells other than periodontal ligament cells

The periodontal ligament cell is not the only fibroblast-like cell type that produces cytokines/chemokines upon

stimulation with inflammation promoters. Bronchial and nasal fibroblasts and airway smooth muscle cells respond to interleukin-1β, tumour necrosis factor-α, viral elements and mechanical strain by increased production of both cytokines and chemokines, a mechanism believed to be involved in the pathophysiology of allergic inflammation, airway inflammation and pulmonary fibrosis (22–25). Fibroblast-like cells within synovial membranes, such as synovial fibroblasts, produce collagen and other connective tissue molecules to form the joint capsule, but in rheumatoid arthritis the synovial fibroblasts are transformed into an invasive cell type that produces a wide range of cytokines and chemokines (26,27). Renal fibroblasts produce cytokines/chemokines in response to proinflammatory stimuli, such as LPS and tumour necrosis factor-α, a mechanism contributing to the process of renal fibrosis (28). In conclusion, proinflammatory stimuli induce cytokine and chemokine production in different populations of fibroblasts within many tissues and organs, representing a mechanism involved in the pathophysiology of inflammatory diseases.

Concluding remarks

The human periodontal ligament cell produces not only periodontal ligament extracellular matrix but also proinflammatory cytokines and chemokines upon stimulation with inflammation promoters. Therefore, the periodontal ligament cell most probably plays a significant role to initiate recruitment of leucocytes in periodontal inflammation. Thus, pharmacological anti-inflammatory treatment of periodontal disease should aim at reducing the periodontal ligament cell production of cytokines and chemokines. It is an interesting and thrilling proposal that periodontal ligament cells themselves are responsible for initiating periodontal inflammation via these mechanisms.

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MALMÖ UNIVERSITY
205 06 MALMÖ, SWEDEN
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