Original Article

Gene Expression Analysis of Chronically Inflamed and Healthy Human Periodontal Ligament Cells in vivo

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ABSTRACT

Background: In the periodontium, the functions of the cell populations regarding the host-mediated tissue destruction in health and disease are not well understood. The purpose of this study was to measure the expression of genes differentially expressed in chronically inflamed periodontal ligament (PDL) cells compared to healthy PDL cells.

Methods: We compared the genome-wide gene expressions of chronically inflamed and healthy PDL cells by microarray analysis, and validated the data by real-time RT-PCR to identify the genes that might play distinct roles in chronic periodontal disease *in vivo*.

Results: The expression rates of 14,239 genes were investigated and 3,165 of them were found differentially expressed by at least two-fold; the expression rates of 1,515 genes were significantly upregulated and the expression rates of 1,650 genes were significantly downregulated in inflamed PDL cells.

Conclusion: We focused on mainly structural components, for example, laminins and integrins, as well as degrading enzymes, for example, MMPs and cathepsins. The molecular composition of the laminin network varies in chronically inflamed compared to healthy PDL cells *in vivo*. Furthermore, integrin alpha6beta4, together with laminin-332, might be involved in chronic periodontal inflammation. Diverse keratins were upregulated, indicating that the epithelial cell rests of Malassez might also be involved in chronic periodontitis. The microarray analysis has identified a profile of genes potentially involved in chronic periodontal inflammation *in vivo*.

Keywords: Extracellular matrix, inflammation, microarray analysis, periodontal ligament.

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Introduction

The periodontium consists of the periodontal ligament (PDL), the gingiva, the root cementum, and the alveolar bone. The gingiva attaches to the tooth surface through the junctional epithelium and the alveolar bone through the PDL.[1,2] The PDL is a connective tissue that occupies the periodontal space between the root of the tooth and the alveolus, with an average width of 0.25 mm.[3] The PDL also consists of a collagen- and proteoglycan-rich extracellular matrix (ECM). Although the predominant connective tissue cell within the PDL is the fibroblast, the tissue presents a heterogeneous cell population.[4] Furthermore, other PDL cell types, like the epithelial cell rests of Malassez (ERM), seem to make up an important part of the normal structure within the PDL, and can be seen not as isolated groups of cells, but as a network of epithelial cells surrounding the root. The ERM are thought to be crucial for successful and predictable periodontal regeneration.[5]

In periodontal diseases, microorganisms initiate and maintain the degenerative processes in the periodontium.[6] In response to the bacterial invasion, host-mediated tissue destruction occurs, for exam-

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ple, by complement activation and the release of lysosomal enzymes and connective tissue matrix metalloproteinases (MMPs).[6] At the inflammatory site, inflammatory cells are mobilized, and MMPs are secreted as proenzymes and activated by various stimulatory factors.[7] However, the significance and relative contributions of the host factors to the disease process remain largely unresolved.

The purpose of this study was to measure the expression of genes differentially expressed in chronically inflamed PDL cells compared to healthy PDL cells, to further characterize biological processes occurring in the chronic degeneration of the periodontium *in vivo*. For this purpose, we used microarray technology to compare gene expression profiles in PDL cells from patients with healthy periodontiums and those with chronic periodontitis, and validated the data by using real-time RT-PCR.

Materials and Methods

Patient selection

Periodontal tissue was collected from 32 patients at the Dental Medical School of the University of Goettingen (12 men, 20 women, aged between 18 and 72 years). The tissue probes were taken from teeth, either extracted for orthodontic reasons or due to carious lesions (healthy periodontium) or from teeth with generalized chronic periodontitis.[8] The differentiation between both groups was performed using clinical and radiological parameters, i.e., clinical attachment loss, increase in probing depth and radiographic bone loss. Teeth with healthy PDL had no clinical signs of inflammation (no bleeding on probing), exhibited a probing depth of ≤ 3.5 mm and had no radiographic detectable bone loss. In contrast, teeth with generalized chronic inflammation exhibited obvious clinical signals of inflammation (bleeding on probing),

Table 1. Differentially expressed genes between chronically inflamed and healthy PDL cells by DNA microarray.

Downregulated in inflamed PDL cells			Upregulated in inflamed PDL cells		
Name	**old change**	**Description**	**Name**	**Fold change**	**Description**
LAMA2	-2.8	laminin alpha2	LAMB3	2	laminin beta3
LAMA4	-2.2	laminin alpha4	SDC4	3.2	syndecan 4
LAMB1	-2.8	laminin beta1	ITGA6	1.7	integrin alpha6
LAMB2	-2	laminin beta2	ITGB4	2.6	integrin beta4
LAMC3	-10.1	laminin gamma3	IL6	2.4	interleukin 6
NID1	-2.2	nidogen-1	LTF	22.5	lactoferrin
NID2	-3.8	nidogen-2	NFκB	1.9	nuclear factor κB
COL1A1	-4	collagen type I, alpha 1	VEGF	3	vascular endothelial growth factor
COL5A1	-7.8	collagen type V, alpha 1	CASP3	2.4	caspase 3
COL11A1	-5.3	collagen type XI, alpha 1	CASP9	2	caspase 9
LTBP1	-7.5	latent transforming growth factor beta protein 1	MMP7	12.6	matrix metalloproteinase 7
FN1	-13.3	fibronectin-1	CTSB	3.4	cathepsin B
FBLN2	-4.6	fibulin 2	CTSC	1.4	cathepsin C
VIM	-3.7	vimentin	KRT4	119.4	keratin 4
SOX9	-3.6	SRY-box 9	KRT5	4.4	keratin 5
BGN	-6.3	biglycan	KRT6B	14.9	keratin 6B
CD14	-3.2	CD14 antigen	KRT7	2.3	keratin 7
IL1B	-3.7	interleukin 1, beta	KRT13	6	keratin 13
IL6R	-4.5	interleukin 6 receptor	KRT14	2.8	keratin 14
IL8	-6.8	interleukin 8	KRT15	6.3	keratin 15
MMP9	-27.7	matrix metalloproteinase 9	KRT16	4.7	keratin 16
MMP13	-19.2	matrix metalloproteinase 13	KRT17	4.5	keratin 17
TIMP1	-4.1	tissue inhibitor of metalloproteinase 1	KRT18	3	keratin 18
TIMP2	-4	tissue inhibitor of metalloproteinase 2	KRT19	2.9	keratin 19
TIMP3	-9.2	tissue inhibitor of metalloproteinase 3	KRT24	10.2	keratin 24

exhibited a probing depth of > 4 mm and had radiographic evidence of bone loss (1 to 5 mm). A detailed anamnesis of each patient was explored. All patients were without a medical history, i.e., heart diseases or diabetes, and were not on medication. In addition, the tissue samples used in our study were only taken from non-smokers. All patients gave their written informed consent according to the regulations of the Ethics Committee of the Medical Faculty of the University of Goettingen.

Labelling of cRNA for gene expression analysis arrays

The periodontal tissue was scraped off the roots of the teeth and total RNA from chronically inflamed and healthy PDL cells was isolated (Qiagen, Hilden, Germany).

Hybridization and scanning of gene expression analysis arrays

To monitor the relative abundance of mRNA for full-length human genes, the Affymetrix GeneChip HG_U133A was used, representing 22,283 probe sets (14,239 unique human genes). Labelled cRNA, fragmented to an average size of 100–150 bases, was hybridized to the GeneChips. For each pool of cRNA, two GeneChips were hybridized. A complete list of genes present on HG_U133A can be found in the Affymetrix database at http://www.affymetrix.com/analysis/index.affx.
Using the Affymetrix Data Mining Tool 3.0, gene expression was evaluated, and genes with at least a significant change in signal intensity of two-fold or greater were considered regulated (Table 1).

Real-time RT-PCR

For verification of the microarray data, genes of interest were chosen (Table 2) and primers (forward and reverse) were designed with the help of the primer[3] shareware (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Quantification was achieved with a Mastercycler ep Realplex S (Eppendorf, Hamburg, Germany). The RNA was reverse-transcribed with the help of the Advantage® RT-for-PCR kit (BD Biosciences, San Diego, CA, USA).

Experiments were performed two times in triplicate. The inter-test variation was ≤ 2%, and the intra-test variation was ≤ 1%. The PCR products were sequenced (Sequlab, Goettingen, Germany) to confirm their identity and melting curves were performed for each PCR. The relative ratios were calculated according to the algorithm of Pfaffl.[9]

Results

Before performing the microarray, we tested each total RNA extracted from healthy and chronically inflamed PDL cells to validate the amount of NFκB by real-time RT-PCR. An increase in fluorescence for the amplification of NFκB indicated the presence of mRNA for NFκB in inflamed periodontal tissue. In contrast, no NFκB mRNA

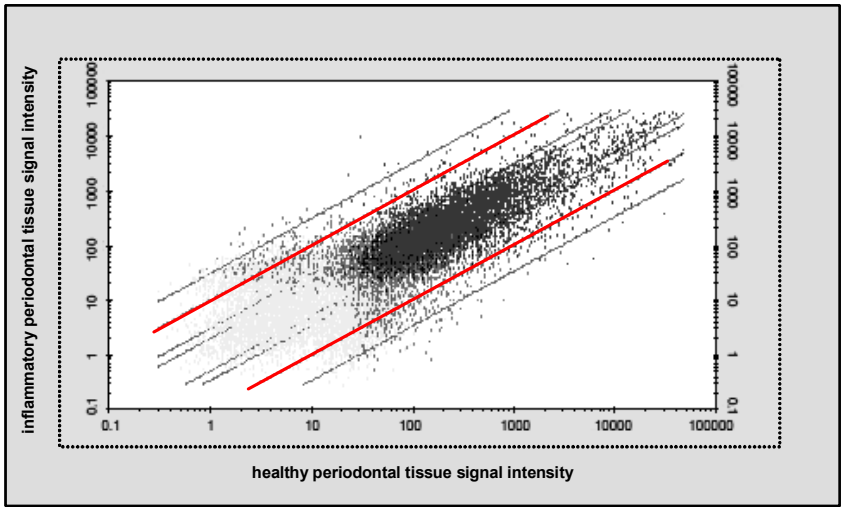

Figure 1. Differential expression between inflammatory and healthy periodontal tissue. Software used was GCOS1.2. Forward gridlines indicate expression ratios between the two probes. Each dot represents signal intensity measurement for a gene. The red lines present the thresholds of two-fold differential expression.

Table 2. Primers for real-time RT-PCR analysis.

Transcript	Forward primer	Reverse primer		Accession number
Integrin alpha6	gcgtcccattcccataact	tcgtctccacatccctctt	156 bp	NM_000210
Integrin beta4	gcttcacacctatttccctg	gacccagtcctcgtcttctg	186 bp	NM_000213
Laminin alpha2	gaggtttattccctgctgtc	ggtttggattgctgctgttt	174 bp	NM_000426
Laminin beta3	tgaagacagaggcagagg	aggtggcatagtagagca	193 bp	L25541
Cathepsin B	tactccccttcccaactg	cttgtcatcctcccacct	246 bp	NM_001908
Cathepsin C	gagacccttttcaacccctt	gctgccactgctatgctct	190 bp	NM_001814
Nuclear factor kappaB	tcaatggctacacaggacc	tcccgcttcttcacacact	206 bp	NM_021975
Vascular endothelial growth factor	tccaacttctgggctgttct	cccctctcctcttccttctc	151 bp	NM001025368

was detectable in healthy periodontal tissue (data not shown).

With the help of microarray technology, gene expression levels in chronically inflamed human PDL cells were compared to the gene expression profile in healthy human PDL cells (Figure 1). We selected genes of interest and clustered them according to their biological functions. A selection of the genes of interest is summarized in Table 1.

The expression rates of 14,239 genes were investigated (Figure 1) and 3,165 of them were found to be differentially expressed by at least two-fold. The expression rates of 1,515 genes were significantly upregulated, and the expression rates of 1,650 genes were significantly downregulated in inflamed PDL cells compared to healthy PDL cells.

Gene expression observed in the microarray analysis was further studied by real-time RT-PCR for eight genes of interest (Table 2, Table 3).

Discussion

Plaque-induced periodontitis has been defined as the presence of inflammation accompanied by loss of connective tissue and alveolar bone.[8] It is initially caused by specific periodontopathogenic bacteria.[10,11] They initiate the host activation in the periodontal tissues that destroys the ECM of the PDL and the bone that support the teeth.

After a classification of the 14,239 investigated genes, we further studied the different groups of ECM components (integrins, keratins, MMPs and TIMPs), as well as cathepsins. We are aware of the fact that the detection of mRNA does not automatically mean that the corresponding protein is translated or secreted. In the following, we discuss possible roles of the investigated genes, just assuming that the mRNA amounts correlate with the protein amounts in the PDL.

In the group of the ECM components, our results showed that the genes of both nidogens are

Table 3. Comparison of the fold changes by microarray and real-time RT-PCR analysis.

Accession number	Denotation	Transcript	Change	Fold change by microarray	Fold change by real-time RT-PCR
NM_000210	ITGA6	integrin alpha6	Increased	1.7	16
NM_000213	ITGB4	integrin beta4	Increased	2.6	2
NM_000426	LAMA2	laminin alpha2	Decreased	-2.75	-21.2
L25541	LAMB3	laminin beta3	Increased	2	68.6
NM_001908	CTSB	Cathepsin B	Increased	3.4	14.9
NM_001814	CTSC	Cathepsin C	Increased	1.4	5.4
NM_021975	NF-κB	Nuclear factor kappaB	Increased	1.9	2.6
NM001025368	VEGF	Vascular endothelial growth factor	Increased	3	17

significantly downregulated in chronically inflamed PDL cells compared with healthy PDL cells *in vivo*. Thus, as already hypothesized in colon and stomach tumorigenesis,[12] this may cause altered cell-ECM interactions and reduce the strength of basement membranes of blood vessels and ERM itself. We suggest that the significantly decreased expression of both nidogen genes may have a role in chronic periodontal disease *in vivo*.

Laminins are a family of multifunctional proteins representing the major noncollagenous protein constituents of basement membranes.[13] In adult human tissue, the expression of the laminin alpha3 chain mRNA is upregulated in healthy PDL fibroblasts compared to healthy gingival fibroblasts *in vitro*.[14] It was already shown that PDL fibroblasts express laminin alpha1 to alpha5, beta1 to beta3, gamma1, and gamma2 chain mRNAs in vitro, and that the predominant laminin chains detected in PDL fibroblast cultures were alpha2, alpha4, beta1, beta2, and gamma1.15 This suggests that healthy PDL cells secrete mainly laminin-411/421 (alpha4beta1gamma1/alpha4beta2gamma1) and laminin-211/221 (alpha2beta1gamma1/ alpha2beta2gamma1). Our *in vivo* data showed that the gene expression of only the laminin alpha2, alpha4, beta1, beta2, and gamma3 chains were significantly downregulated in inflamed PDL cells. These results lead to the assumption that laminin-213 [16,17] and laminin-423 [18] proteins may be significantly decreased in chronically inflamed PDL. In addition, we hypothesize that the laminin gamma3 chain mRNA may also be expressed in human PDL cells *in vivo* (personal observations, N. Gersdorff) and we also showed that chronically inflamed PDL cells may secrete mainly laminin-332, because this is the only laminin isoform that contains the beta3 chain. We therefore assume that the molecular composition of the laminin networks may vary in chronically inflamed PDL cells *in vivo*, and, as already stated,[19] the increase in the laminin beta3 chain may suggest a role of laminin-332 in chronic periodontal inflammation.

Integrins are a family of heterodimeric cell surface receptors.[20] Integrin alpha6beta4 heterodimers form adherens junctions, i.e., hemidesmosomes, which are localized in the internal basement membrane of the human gingival epithelium.[21,22] These junctions, together with laminin-332 anchoring filaments, attach the gingival epithelium to the underlying internal basement membrane.[23,24] Our results show that the expression of integrin alpha6beta4 mRNA is significantly increased in chronically inflamed PDL cells *in vivo*. As already hypothesized,[25,26] our results indicate that integrin alpha6beta4, in addition to laminin-332, might be involved in chronic periodontal inflammation by mediating epithelial cell migration during periodontitis.

In human periodontal disease, MMPs are detected in the connective tissue, and enzyme activities generally increase with the severity of the disease.[27] Tissue destruction during periodontitis results from an imbalance of MMPs over their tissue inhibitors (TIMPs).[7] Our results revealed significant differences in the expression of mRNAs encoding for MMP7, which were upregulated, and MMP9 and MMP13, which were both downregulated in chronically inflamed PDL cells. In the group of TIMPs, we showed that TIMP1, TIMP2 and TIMP3 mRNAs were significantly downregulated, and TIMP4 gene expression was only marginally increased *in vivo*. These results indicate that PDL cells might be an active source of MMP7 mRNA production in response to chronic inflammation and, hence, the reduction in TIMP1, TIMP2, and TIMP3 concentrations in chronic periodontal disease suggests a breakdown of the balance between the amount of MMPs and their inhibitors.

Cathepsins are thought to contribute to periodontal destruction during periodontitis.[28] Increases in the level and activity of several cathepsins have been shown in periodontitis.[29] Cathepsin B and cathepsin C are lysosomal proteases that play an essential role in immune and inflammatory processes.[7] A recent immunocytochemical study demonstrated that cathepsin B was localized in the PDL of the rat molar, and it was expressed in compressed sites during experimental tooth movement.[30] Further, the levels of cathepsin B in gingival crevicular fluid increased during orthodontic tooth movement.[30] Therefore, cathepsin B may play an important role in the process of collagen degradation during orthodontic tooth movement.[31] We showed that cathepsin B and cathepsin C mRNAs were increased during chronic periodontal inflammation, so they might also participate in connective tissue destruction in chronic periodontitis *in vivo*.

In summary, the present *in vivo* study is the first to compare the gene expression profiles of chronically inflamed and healthy PDL cells by means of DNA microarray as well as real-time RT-PCR. The

broad information gained was used to get a first idea of which genes might play distinct roles in chronic periodontal disease. Our results suggested that a specific gene expression profile is present in chronically inflamed PDL cells *in vivo*. Further studies are needed to entirely understand cellular activities during chronic periodontal inflammation *in vivo*.

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