The effects of extracts from periodontopathic bacteria on human periodontal fibroblasts stimulated with mineralization supplements

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Abstract: Bacterial effects on in vitro mineralization of human periodontal fibroblasts (HPF) have not yet been examined in great detail. In our study, we investigated the effects of soluble extracts of the periodontopathic bacteria Porphyromonas gingivalis, Bacteroides forsythus and, Treponema denticola on cell proliferation, mineralization, as well as on osteoblastic markers present in HPF cultured in vitro, such as alkaline phosphatase (ALP) activity and collagen content. Periodontal fibroblasts stimulated by β-glycerophosphate, ascorbic acid and dexamethasone (BAD) or by dexamethasone and ascorbic acid (DA) were compared to unstimulated cells. During the cultivation period, the stimulation of HPF by combined dexamethasone and ascorbic acid (DA) had a strong inductive effect on proliferation, ALP activity and collagen formation. The extracts obtained from the periodontal pathogens had a suppressing effect on the proliferation rate of HPF. The extracts from P. gingivalis, B. forsythus and T. denticola caused a decrease in ALP activity within 24 h of application. While extracts obtained from P. gingivalis and B. forsythus induced a reduction in collagen content in BAD- and DA-stimulated HPF cells, T. denticola extracts led to an increase in collagen. Our data suggest that specific periodontopathic bacteria may suppress tissue regeneration in vivo not only by activating host defense mechanisms but also directly via a suppression of growth and differentiation of HPF and a reduction in the extracellular collagen matrix. For the process of pocket formation, not even the direct influence of viable bacteria seems to be necessary. Additionally, long-distance effects of bacteria harboured in periodontal pockets or in root canals may be of importance. (J Oral Sci. 45, 127-137, 2003)

Introduction

Periodontopathic pathogens are considered important etiologic agents in the initiation and progression of marginal periodontitis. These microorganisms may mediate periodontal tissue destruction either directly through their multiple virulence factors or indirectly by host-mediated inflammatory responses (1,2). The removal of bacterial biofilms by scaling and root planing forms the basic concept of primary periodontal therapy (3). The main goals of periodontal regeneration are the initiation of reattachment of periodontal ligament fibers and restoration of the lost alveolar bone. Therapeutical measures are intended to support physiological mechanisms of tissue regeneration. In this context periodontal fibroblasts play a key role in the maintenance and renewal of the periodontium. They are the predominant cells found in the periodontium and have the ability to undergo mineralization as manifested through the action of various stimulating agents on in vitro culture systems (4-6). Different types of vitamins, hormones and growth factors have been shown to play an
important role in the stimulation and regulation of cellular processes involved in repair and regeneration such as chemotaxis, metabolism, growth and differentiation in the periodontium (7-8).

β-Glycerophosphate (β-GP) is known to promote mineral deposition when added to culture media of cells with osteogenic potential such as chick limb-bud mesenchymal cells (9), human and rat bone marrow cells (10-11), and human odontoblastic cells (12). It has been shown that 10 mM β-GP in a rat calvarial cell culture system resulted in the mineralization of osteoid nodules in two phases: an initiation phase that is dependent on alkaline phosphatase activity, and a progression phase that proceeds independently of the activity of alkaline phosphatase and does not require β-GP (13).

Dexamethasone is a member of the glucocorticoid class of hormones that has also been shown to increase the protein expression of Gq/11 in rat osteosarcoma cell line UMR 106-01 (14). Previous studies have revealed the biological roles of dexamethasone in inducing cell proliferation, chemotaxis, collagen I formation, and fibronectin-metabolism of human fetal lung fibroblasts (15), bone stromal cells (16), chick embryo cells (17-18), mouse calvarial cells (19), neuroepithelial cell lines (20) and periodontal ligament cells (21). The ability of dexamethasone to induce the sodium-dependant vitamin C transporter has been reported in a mouse osteoblastic cell line MC3T3-E1 (22).

Ascorbic acid plays an active role in the formation of collagen in the periodontal ligament (23). The stromal cell line ST2, derived from mouse bone marrow, differentiates into osteoblast-like cells in response to ascorbic acid. Ascorbic acid induces alkaline phosphatase (ALP) activity, the expression of mRNAs for proteins that are markers of osteoblastic differentiation, the deposition of calcium, and the formation of mineralized nodules by ST2 cells (24). Ascorbic acid has become a crucial component of alpha-MEM that stimulates differentiation of murine osteoclasts (24).

Therefore, bacterial effects on dexamethasone-ascorbic acid (DA) or β-glycerophosphate-ascorbic acid-dexamethasone (BAD) induced ALP activity and collagen formation in human periodontal fibroblasts have not been examined in as much detail. Our goal was to examine the combinations of β-glycerophosphate, ascorbic acid and dexamethasone (BAD) on the functional changes which might take place at different stages of cultivation. Additionally, we investigated the influence of extracts of the periodontopathic bacteria *Porphyromonas gingivalis*, *Bacteroides forsythus*, and *Treponema denticola* on HPF proliferation and differentiation. These selected bacterial species are closely associated with the severity of the disease process and the level of attachment loss (34-38).

**Materials and Methods**

Collection of human periodontal fibroblasts

Human periodontal fibroblasts (HPF) were obtained from tissues which remained attached to the roots of surgically extracted third molar teeth. Patients involved in this study were in good general health. The experiments were confirmed by the ethical board of the University of Leipzig (No. 75/2002). The middle third of the periodontal ligament was scraped off, rinsed with phosphate buffered solution (PBS: Gibco BRL, Grand Island, NY, USA), transferred to culture dishes and overlayed by coverglasses. D-minimum essential medium (D-MEM: Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin sulfate and 2.5 µg/ml amphotericin B were added. Cultures were incubated at 37°C in a humidified atmosphere of air plus 5% CO₂. The medium was changed twice a week until confluency was achieved. Confluent cells were subcultured using 0.05% trypsin and 0.53 mM EDTA. Cells between the 3rd and 6th passages were subjected to the experiments.

Stimulation with β-glycerophosphate, ascorbic acid and dexamethasone

Confluent periodontal fibroblasts were seeded at 10⁴ cells/cm² in 24-well plates using D-MEM supplemented with 10% FBS, and antibiotics. The concentrations of mineralizing supplements were: 10 mM β-glycerophosphate (B), 50 µg/ml ascorbic acid (A), and 10⁻⁷ M dexamethasone (D). They were applied in the combinations of DA or BAD. HPF without mineralization supplements and human skin fibroblasts were used as controls. All cell cultures were subjected to 24 days of incubation in order to observe nodule formation.

Preparation of soluble bacterial extracts

Working stocks for the bacteria used (*Bacteroides forsythus*, ATCC 367; *Porphyromonas gingivalis*, ATCC 33277; *Treponema denticola*; ATCC 35308) were kindly
supplied by Dr. S. Kneist, University of Jena. Bacterial cells were harvested by centrifugation at 12,000 \( \times g \) for 20 min at 4°C and washed three times with sterile phosphate buffered saline (PBS) at pH 7.2. The bacteria were resuspended in PBS and subjected to ultrasonic disruption (Sonopuls HD 70, Bandelin, Germany) at maximum power for 30 s in an ice bath. Sonicated samples were centrifuged again at 20,000 \( \times g \) for 20 min at 4°C. Supernatants were filter-sterilized (0.2 µm) and designated as soluble extracts. Protein concentrations were determined according to Bradford (39) using bovine serum albumin as a standard.

Testing the effects of soluble bacterial extracts

The effects of bacterial extracts were tested on the different stages of cell confluence and differentiation. Cultures were incubated for 24 h with bacterial extracts (10 µg protein) of each of the three pathogenic species as well as Escherichia coli LPS in 1 ml of the culture medium on the 24th day. In addition to the above-described experiments, control and mineralized HPF cultures were incubated with higher concentrations of soluble bacterial extracts (25 µg/ml and 250 µg/ml). The cultures were checked for apoptosis and necrosis using fluorescence-microscopy by staining the nuclei with Hoechst dye 33258 and propidium iodide. Another set of control cultures was incubated with the soluble bacterial extracts and left untouched without changing the cultural media for a period of 10 days.

Cell proliferation assay

The cells were harvested at days 0, 8, 16, and 24 or 24 h after addition of the soluble bacterial extracts in duplicate wells by incubating in 0.05% trypsin-EDTA. Cell number was determined using a hemocytometer counter. The cell viability was determined by using the Trypan blue exclusion test. Experiments were repeated three times.

Alkaline phosphatase activity

The cell cultures were washed with 500 µl of 10 mM Tris-buffered saline (TBS, pH 7.4, containing 0.9% sodium chloride). After cell washing, 500 µl of 10 mM TBS containing 1 mM phenylmethyl sulfonil fluoride solutions were added into the wells. The mixture was homogenized by a Polytron homogenizer (Kinematica, Switzerland). Fifty microliters of 10% Triton X-100 was added to each solution, stirred for 15 min and centrifuged at 12,000 \( \times g \) for 10 min. The supernatant was taken out for enzyme assay. One milliliter of 10 mM p-nitrophenyl phosphate (p-NPP) substrate in 100 mM 2-amino-2-methyl-1,3-propanediol-HCl buffer (pH 10.0), containing 5 mM MgCl\(_2\) was transferred into each tube, followed by the addition of 100 µl of supernatant. The samples were incubated at 37°C for 30 min. The reaction was stopped by adding 0.3 ml of 1 N NaOH. Absorbance was measured at 405 nm with a microtiter plate reader (Anthos HTIII, Anthos Lab. Instr., Austria).

Protein assay

Protein concentrations were determined according to Bradford (39) using bovine serum albumin as a standard.

Intra-situ measurement of collagen content

A slight modification of a previously described method (40) was used to measure the collagen content of the HPF cells. Cell layers were extensively washed with PBS before they were fixed with 1 ml of Bouin’s fluid for 1 h. The fixation fluid was removed by suction and culture plates were washed by immersion in running tap water for 15 min. The culture dishes were stained for 1 h under mild shaking on a shaker. The dye solution was removed by suction. Stained cell layers were extensively washed with 0.01 N HCl to remove all unbound dye. Cell morphology was photodocumented before dissolving the stained cells. The stained cells were dissolved in 0.1 N NaOH for 30 min at room temperature. The solution was transferred to a 1-ml cuvette and the optical density measured at 550 nm by a spectrophotometer (Uvikon 931 Kontron Instr., Italy).

Histology

Alizarin red staining was used to confirm the mineralization of the extracellular matrix. HPF cell morphology was observed at the different stages of cell confluence and differentiation using phase contrast microscopy (Axiovert 135, Zeiss, Germany).

Statistical Analysis

Data were analyzed using the Wilcoxon signed rank test for paired data. differences at \( P < 0.05 \) were considered significant.

Results

Cell proliferation of HPF cells was analysed after 0, 8, 16 and 24 days of cultivation. During this period, HPF stimulated with ß-glycerophosphate, ascorbic acid, and dexamethasone (BAD) were less proliferative than control HPF. However, DA-stimulated HPF cells showed higher proliferation rates (Fig. 1). Subsequently, the effects of 10 µg of soluble bacterial extracts on control cultures and HPF stimulated with BAD and DA were examined. The extracts obtained from these periodontal pathogens decreased the number of viable HPF cells. Cell loss in control HPF treated with P. gingivalis, B. forsythus and T. denticola was within
15%, except for those treated with *E.coli* LPS, which lost about 30% of the cells (Fig. 2a). In BAD cultures the soluble bacterial extracts had comparable effects on cell numbers, causing a decrease of 16% (Fig. 2b). In the DA-stimulated cells, *P. gingivalis* extracts and *E. coli* LPS reduced cell numbers by 25-30% respectively (Fig. 2c).

The induction of cell differentiation by mineralization supplements was monitored by determining the activity of ALP. A time-dependent increase of ALP activity from day 0 to day 24 among the BAD- and DA-stimulated HPF cells was found. BAD-stimulated cells showed a 2-fold increase, and the DA-stimulated HPF cells showed a 3-fold increase over the control group (Fig. 3). The level of ALP activity decreased after application of soluble bacterial extracts to the controls and both the BAD- and DA-stimulated cells. The results are shown in Figures 4a-c. The relative inhibition of ALP was 45% in the control cells, and 30% in both the BAD and DA stimulated HPF cells.

A time-dependent increase in collagen content was observed from day 0 to day 24 on the BAD- and DA-stimulated HPF cells. There was a 2-fold increase in collagen in the cultures of BAD-stimulated cells as compared with the control group (Fig. 5). The DA-stimulated cells showed a 3-fold collagen increase over the control cells. It was found that the collagen content in the cells treated with *B. forsythus* and *P. gingivalis* showed at least 20-30% inhibition in the control group, 25-35% in the BAD group, and 40-45% in the DA group (Fig. 6a-c). In contrast, the control and BAD-stimulated cells treated with *T. denticola* showed a slight increase in collagen content.

When the cultures containing extracts from *B. forsythus* or *P. gingivalis* were further cultivated for 10 days, the HPF cells as well as the collagen network completely disappeared and only some remnants of cellular products were visible microscopically. In control cultures without
bacterial extracts the HPF disappeared as well, however, the collagen network was still present. Under these extremely rough conditions no differences were found between BAD-and DA-stimulated or control HPF.

The control experiments investigating the induction of apoptosis or necrosis showed that the number of apoptotic or necrotic cell nuclei was less than 1% after 24 h. Apoptosis or necrosis were found just after the application of higher concentrations of bacterial extracts. When soluble bacterial extracts were added at 25 µg/ml, an increase in apoptotic or necrotic nuclei of more than 10% was found after an incubation period of 72 h. After adding bacterial extracts at 250 µg/ml, more than 10% of apoptotic or necrotic nuclei were found after 48 h followed by rapidly increasing rates of cell death.

**Discussion**

The objective of this study was to determine the effects of dexamethasone and ascorbic acid (DA) in comparison to β-glycerophosphate, ascorbic acid and dexamethasone (BAD) and to study the influence of extracts from *B. forsythus*, *P. gingivalis* and, *T. denticola* on HPF long-term cultures with emphasis on cell proliferation, alkaline phosphatase activity and collagen content.

In the cell proliferation assay we found that the DA-stimulated cells showed the highest proliferation rates. In contrast, HPF stimulated with BAD were less proliferative than control cells. The latter finding is in accordance with studies in which fibroblastic cells with osteogenic properties were used (41-42). The principle behind the downregulation of HPF proliferation might be similar to what happens in the osteoblastic cells. The findings of previous studies revealed that the functional relationships between the proliferation of osteoblastic cells and the synthesis of a bone extracellular matrix, its

![Fig. 3](image-url) Time course of the specific alkaline phosphatase activity of human periodontal fibroblasts stimulated with BAD or DA. * P < 0.05, when compared with the control group. Each value represents mean ± S.E. from triplicate experiments.

![Fig. 4a](image-url) Effects of Bacterial Extracts

![Fig. 4b](image-url) Effects of Bacterial Extracts

![Fig. 4c](image-url) Effects of Bacterial Extracts

**Fig. 4** Effects of soluble bacterial extracts (*P. g.*, *B.f.*, *T.d.*) and *E.coli* LPS on ALP activity of control HPF (Fig. 4a), and HPF incubated with BAD (Fig. 4b) and DA (Fig. 4c) for 24 h at day 24. * P < 0.05, when compared with the control, BAD and DA groups without extracts at day 24. Each value represents mean ± S.E. from triplicate experiments.
accumulation and maturation are essentially necessary for the mineralization process, thereby lessening the capacity of the cells to proliferate (10,43-46). An experimental model has shown that the presence of a source of phosphate ions leads to cell mineralization, which is largely dependent on the amount of the extracellular matrix and cell maturation; finally, cell proliferation ceases to function as the osteoblasts become trapped and embedded in the mineralizing matrix (10,47).

In comparison to BAD cultures, the control cultures as well as the DA-stimulated HPF showed a lower occurrence of mineralized nodules after the cultivation period. In this context, the role of β-glycerophosphate (β-GP) as an agent that can promote mineral deposition appears to be important. Some earlier studies investigated the development of bone-like tissue in vitro by periodontal ligament cells (48-49). They revealed that nodules were formed in the cultures upon addition of 10 mM Na-beta-glycerophosphate, resulting in their mineralization. These nodules were identified as bone-like elements based on the presence of osteoblast-like and osteocyte-like cells, collagenous matrix, a mineral composed of hydroxyapatite, and intense alkaline phosphatase activity (48). Other studies have also noted the osteoblastic potential of human periodontal ligament fibroblasts when induced by BAD to form a mineralized matrix which shares features with the mineralized matrix of bone, but this most likely represents a more immature type of in vitro mineralization (50).

The results of our study further confirmed that in vitro cultured HPF cells stimulated by BAD and DA showed increased ALP activity and collagen content in a time-dependent manner. The BAD-stimulated cells may have contained fibroblastic cell populations which are ALP-positive and capable of undergoing mineralization upon induction. In the case of DA-stimulated cells, these agents

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**Fig. 5** Time course of the collagen content of human periodontal fibroblasts stimulated with BAD and DA. * P < 0.05, when compared with the control group. Each value represents mean ± S.E. from triplicate experiments.

**Fig. 6a** Effects of Bacterial Extracts

**Fig. 6b** Effects of Bacterial Extracts

**Fig. 6c** Effects of Bacterial Extracts

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**Fig. 6** Effect of soluble bacterial extracts (P.g., B.f., T.d.) and *E.coli* LPS on collagen content of control HPF (Fig. 6a), and HPF incubated with BAD (Fig. 6b) and DA (Fig. 6c) for 24 h at day 24. * P < 0.05, when compared with the control, BAD and DA groups without extracts at day 24. Each value represents mean ± S.E. from triplicate experiments.
facilitated the enhancement of ALP-positive cells. The extracellular matrix may also have a direct influence in promoting the increase of collagen and cell numbers in HPF cultures (51). Dexamethasone had an inductive effect on the osteoblast phenotype of adult rat bone marrow cells in vitro resulting from the expression of bone sialoprotein and osteocalcin (52). The L-ascorbic acid 2-phosphate (asc-P: stable ascorbic acid derivative) markedly stimulated the synthesis of marker proteins for osteoblastic differentiation such as alkaline phosphatase and osteocalcin as shown in a murine osteoblastic cell line, MC3T3-E1, suggesting that Asc-P could promote osteoblastic differentiation (53). In our study, however, the concerted action of these two agents, dexamethasone and ascorbic acid on the growing HPF cells resulted in an increase in bone-associated phenotypic markers despite the fact that it probably failed to promote total differentiation.

For testing bacterial influences on established HPF cultures we used 10 µg/ml of soluble bacterial extracts from P. gingivalis, B. forsythus, T. denticola and E. coli LPS on control cultures and HPF stimulated with BAD or DA at the end of the 24th day of cultivation. It is interesting to note that this concentration of soluble extracts obtained from the periodontal pathogens had at least a decreasing effect on the number of viable cells of the controls and both the BAD-and DA-stimulated HPF cells. In our study we found that the tested bacteria, especially P. gingivalis, may have inhibitory effects on the proliferative capacity of BAD-and DA-stimulated HPF cells even after 24 h. To further support this finding, a previous study on gingival fibroblasts exposed to Bacteroides gingivalis extracts with concentrations ranging from 10 µg to 100 µg protein/ml showed a significant decrease of growth after 2 and 4 days in culture which was apparently non-toxic (54). It has been shown in an earlier work that sonicates from B. gingivalis applied to IL-1 stimulated thymocytes at different concentrations (0.5 µg/ml, 2.8 µg/ml, 14.0 µg/ml, 70.0 µg/ml) demonstrated growth inhibitory effects which were related to the relative protein content and endotoxin activity of the sonicate preparations (55). The endotoxin from B. gingivalis caused a dose-dependent suppression of cultured human gingival fibroblasts ranging from 29% at 25 µg/ml to 45% at 200 µg/ml which could be attributed to the culture conditions and the particular preparation of endotoxin used (56).

The relative inhibition of ALP activity upon bacterial application for control, BAD-and DA-stimulated cells suggests that degradation products were present in the soluble bacterial extracts. Perhaps the extracts from P. gingivalis and B. forsythus as Gram (−) bacteria which contain not only soluble proteins, but also other components such as butyric acid, propionic acid, collagenases and endotoxins may have a direct influence on the HPF (34). This also indicates that the action of DA was diminished by the independent effects of bacterial extracts on ALP-positive cells. A study that used LPS of P. gingivalis led to the suppression of ALP mRNA expression in primary rat fetal calvarial cells, with an inhibitory pattern similar to that of enzyme activity observed. Results indicated that P. gingivalis-LPS inhibits osteoblastic cell differentiation and suggested that LPS-induced bone resorption in periodontal disease may be mediated by effects on osteoblastic as well as osteoclastic cells (57). Recent studies also have shown that sonicated extracts from Prevotella intermedia and Actinobacillus. actinomycetemcomitans decreased the alkaline phosphatase activity in a dose-dependent manner (58). In our study, we found moderate effects of P. gingivalis and B. forsythus in the reduction of ALP activity in both BAD-and DA-stimulated cells.

An earlier study showed that collagen production in murine calvarial cells may have been inhibited by surface-associated material (SAM) from bacteria implicated in the pathogenesis of periodontal disease such as Porphyromonas gingivalis and Eikenella corrodens (33). An in vitro study was done to test the biological activities of SAM from P. gingivalis. It was found to stimulate bone resorption at a concentration of 1.0 µg/ml. The SAM at a concentration of 10 ng/ml inhibited DNA and collagen synthesis in osteoblasts and murine calvaria. It was concluded that solubilised surface components from P. gingivalis could play a role in the pathogenesis of chronic periodontitis if these activities operate in vivo (59). In a study conducted utilizing two LPS species from P. gingivalis 381, the findings showed a 30-40% reduction in collagen protein formation at 10 µg/ml in fetal rat bones. It also further revealed that the higher molecular weight LPS species significantly inhibited non-collagen protein formation (60). It is an established fact that collagen has a significant role in maintaining cell morphology and promoting cell adhesion in the bone cells (61) as a result of tightly organized networks of type 1 collagen fibrils present extracellularly, and this may have affected the capability of these bacteria to disintegrate the matrix component of the BAD-and DA-stimulated HPF cells. We have speculated that the extracts of P. gingivalis or B. forsythus may at least cause a partial impairment to the BAD-and DA-stimulated HPF cells in a non-cytotoxic dependent manner owing to the bacteria’s low anti-collagenolytic properties. The observed effects of different bacterial species such as P. gingivalis and B. forsythus suggest differences in the molecular mechanism of their
actions which may lead to both quantitative and qualitative modifications of the matrix components (61-62). Extracts of *T. denticola* did not seem to contain collagen degrading components.

The findings of this study lead to the conclusion that stimulating agents such as BAD and DA may play different roles depending upon the target cell types and the availability of matrix components. The role of the extracellular matrix should be taken into consideration even if it does not directly promote regeneration, because it takes an active part in regulating the events necessary for regeneration (63).

Our data suggest that specific periodontopathic bacteria may suppress tissue regeneration in vivo not only by activating host defense mechanisms but also directly via a suppression of growth and differentiation of HPF and a reduction or destruction of the extracellular collagen matrix. For the process of pocket formation the direct influence of viable bacteria does not seem to be necessary. Additionally, the long-distance effects of bacteria harboured in periodontal pockets or in root canals might be of clinical significance.

This study has also made it clear that even a low grade infectious dose of bacterial extracts created a less favorable environment for the stages of HPF differentiation as manifested in this in vitro culture system through the use of BAD and DA agents. In actual cases, if the key periodontal bacteria are not totally eradicated, there is a possibility that wound regeneration in periodontally involved teeth would be delayed. Therefore, initial sealing and proper isolation of the periodontal site to prevent further tissue damage seem to be important.

The next step in gaining deeper insights into the mechanism of interactions between microbial actions and host responses should involve monitoring the changes in the HPF proteome as well as in osteoblastic markers such as ALP activity and collagen content during differentiation in the presence of bacterial extracts. Additionally, the effects of so-called beneficial bacteria should be included in such studies.

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