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Inhibition of Gingival Fibroblast Growth by *Bacteroides gingivalis*

HANNU LARJAVA,[1]* VELI-JUKKA UITTO,[2] ERKKI EEROLA,[3] AND MARKUS HAAPASALO[4]

Departments of Periodontology[1] and Medical Microbiology[3] University of Turku, Turku, and Institutes of Periodontology[2] and Cariology,[4] University of Helsinki, Helsinki, Finland

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Human gingival fibroblasts were exposed in culture to cell extracts of different black-pigmented *Bacteroides* species, and their growth was monitored by determining thymidine uptake and counting cells. Of the *Bacteroides* species tested (*B. gingivalis*, *B. asaccharolyticus*, and *B. intermedius*), *B. gingivalis* gave the extract with the strongest inhibitory effect on fibroblast thymidine uptake. Linear inhibition reaching 80% of the control level was obtained with a dose of 100 μg of *B. gingivalis* extract protein per ml. The effect of *B. asaccharolyticus* resembled that of *B. gingivalis*, but even at the highest dose tested *B. intermedius* had only a slight inhibitory effect. When fibroblasts were counted after 2- and 4-day exposures to *B. gingivalis* extracts, a clear depression in the number of fibroblasts was found. The effects of extracts obtained from early and late growth phases of *B. gingivalis* cultures were similar. A fraction of *B. gingivalis* consisting essentially of lipopolysaccharides (LPSs) was obtained by degrading the extract proteins with proteinase K. Silver staining of polyacrylamide gels revealed a LPS pattern with a molecular mass ranging from 37 to 60 kilodaltons. This LPS-rich fraction caused inhibition of thymidine uptake by gingival fibroblasts similar to that caused by the native extract alone. Thus, the inhibition of gingival fibroblast growth by *B. gingivalis* appeared to be LPS mediated. This inhibitory effect of *B. gingivalis* on oral fibroblast growth may be a virulence factor of this bacterium.

Black-pigmented *Bacteroides* species is a group of microorganisms associated with adult and juvenile periodontitis. *B. intermedius* is associated with common, ulcerative, and pregnancy gingivitis. *B. gingivalis* is suggested to be a potent periodontopathogen. It is frequently found in lesions of periodontitis, although it is not always a high proportion of the microflora (for a review, see reference 24).

Bacteroides species may also be able to invade the gingival connective tissue, which may be an important pathogenetic mechanism in periodontitis (1, 2). Furthermore, *B. gingivalis* is also associated with active periodontitis in animal models (15). *B. intermedius* and *B. gingivalis* also seem to be frequently present in odontogenic abscesses (31).

Antibodies in serum against *B. gingivalis* are elevated in adult periodontitis (24). Antibody titers are sometimes even higher in the crevicular fluid, suggesting local antibody production against this bacterium (27). *Bacteroides* species avoid host defense systems in several ways. They resist complement-mediated killing and avoid phagocytosis by neutrophils, and they possess a large reservoir of proteolytic enzymes that are capable of degrading collagen, fibrin, immunoglobulins, and complement components (3, 24).

B. gingivalis lipopolysaccharides (LPSs) show low biologic activity (24), although their bone-resorbing capacity has been described previously (16, 17). This may be due to their unusual structure, for they lack heptose, 2-ketodeoxyoctonate, and hydroxydecanoic acid (17). *B. gingivalis* and *B. asaccharolyticus* supernatants have been shown to contain factors that are toxic to cultured kidney cells (30). Butyrate, which is produced by *Bacteroides* species, may be one such factor (29). Furthermore, culture filtrates of these *Bacteroides* species also seem to inhibit chondrocyte matrix production, although they do not affect cell proliferation (12, 28). However, no cell-associated effects of *Bacteroides* species on their target gingival cells have been reported.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains. *B. gingivalis* ATCC 33277, *B. intermedius* ATCC 25261, *B. asaccharolyticus* ATCC 27067, and a *B. gingivalis* strain we isolated from a root canal by one of the authors were cultivated for the study. Identification was based on usual bacteriologic tests and on the DNA base composition and production of collagenase and trypsinlike enzymes. All strains were stored freeze-dried, except for our strain, which was stored in glycerol-milk at −70°C. For this study the strains were grown on MCG agar containing 5% horse blood, 0.5% (wt/vol) yeast extract, 0.5 mg of menadione per liter, 500 mg of cysteine per liter, and 0.2% (wt/vol) glucose (8). The plates were cultivated in an anaerobic atmosphere at 37°C. Cultures were harvested at 3, 6, and 8 days of age, and the bacterial mass was suspended in distilled water. Unless otherwise stated the 6-day-old cultures were used in the experiments. The samples were homogenized on ice with a sonicator (B3; Branson Sonic Power Co., Danbury, Conn.) and centrifuged at 11,000 × *g*. Disruption of the bacteria was confirmed by microscopy. The cell-free supernatants were stored at −20°C and used for the experiments. The protein content of these cell extracts was determined (14). Dilutions of 1 to 100 μg of protein per ml were made with fibroblast culture media.

The LPS-rich fraction of *B. gingivalis* was prepared as follows. A fraction of 500 μg of protein extract was first heated for 10 min at 100°C to denature the extract enzymes. The extract proteins were then destroyed with proteinase K. Incubation (50 μg of proteinase K [Sigma Chemical Co., St. Louis, Mo.] per ml in a mixture of 0.01 M Tris hydrochloride–5 mM EDTA–0.5% sodium dodecyl sulfate (SDS) [pH 6.8] at 37°C for 60 min) was followed by heating (100°C, 10 min) and dialysis to inactivate the proteinase K and to remove the small peptides produced during the treatment. This procedure almost totally degraded the proteins but left the LPSs intact (9). Extracts of 6- and 8-day old cultures of *B. gingivalis* were treated as described above and analyzed

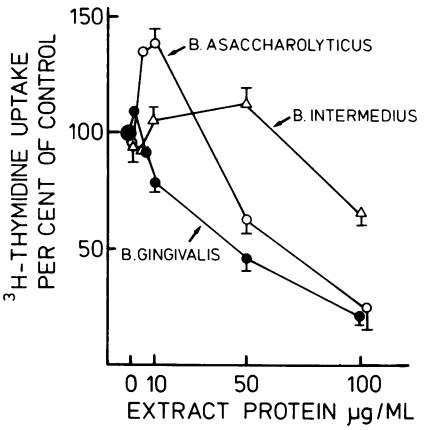

FIG. 1. Effects of black-pigmented *Bacteroides* species on gingival fibroblast thymidine uptake. The results represent means ± standard errors of the mean (SEM) of three different sets of experiments, each comprising three to five separate measurements, except for those without bars, which express single determinations. A value of 100% is given for the mean of the controls in each set.

by SDS-polyacrylamide gel electrophoresis (19) followed by silver staining, which visualized both protein and LPS (9).

Gingival fibroblast culture. Normal human gingival fibroblasts were cultured from a gingival biopsy (dens 17 distal surface) from a healthy 27-year-old patient with excellent oral hygiene. The cells were cultured as described previously (13) in Dulbecco modified Eagle medium (Flow Laboratories, Inc., McLean, Va.) containing supplements and 10% fetal calf serum (KC Biologicals, Lenexa, Kans.). For some experiments the serum was heated at 56°C for 30 min to denature the complement system. Cells from passages 3 to 11 were used and shown to be free of mycoplasma contamination (4).

Incorporation of [^{3}H]thymidine. Gingival fibroblasts were trypsinized, transferred to the wells of microtiter test plates (10,000 cells per well; Becton Dickinson Labware, Oxnard, Calif.), and grown in 200 μl of medium containing 10% fetal calf serum. The medium was changed 1 day after plating. The next day the medium was removed, and the cell layer was washed once with phosphate-buffered saline (100 μl). The washed fibroblasts were incubated for 24 h in complete growth medium containing different dilutions of *Bacteroides* sonicate (0 to 100 μg of protein per ml). The cultures were pulse-labeled with [^{3}H]thymidine (1 μCi per well; specific activity, 25 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 2 h at the end of the incubation period. The cells were then detached with trypsin and harvested with an automatic cell harvester. The radioactivity incorporated into the cells was determined as described previously (20).

Cell number. Cells were distributed among culture flasks at a low density (5,000 cells per cm^2, 21-cm^2 flasks; Nunc, Roskilde, Denmark). The medium was changed the next day, and the number of attached cells was counted with a chamber. After 2 days the cultures were at the beginning of the logarithmic growth phase. The *B. gingivalis* extract (50 μg of protein per ml) in complete growth medium was then added to the cells. Cell numbers were counted after exposure to the extract for 1, 2, and 4 days. The medium was not changed during this period (single exposure).

Incorporation of [^{3}H]proline. Gingival fibroblasts were distributed among microtiter test plates and cultured as described above. During the 24-h culture period with the *B. gingivalis* extract, the cells were labeled with [^{3}H]proline (30 μCi/ml; specific activity, 5.0 Ci/mmol; Amersham) for 22 h. The cells were then washed and harvested as described above for thymidine-labeled cells. The incorporated radioactivity was determined.

RESULTS

The gingival fibroblast reaction to extracts from the different *Bacteroides* species differed markedly. Inhibition of thymidine uptake by gingival fibroblasts was strongest with *B. gingivalis* and weakest with *B. intermedius* (Fig. 1). The effect of nonoral *B. asaccharolyticus* resembled that of *B. gingivalis*, except that a slight stimulatory effect was observed at low doses of the extract (Fig. 1). *B. intermedius* sonicate was inhibitory only at the highest dose used in the study (100 μg of protein per ml), causing a decrease to 65% of control values (Fig. 1). A depression of up to 80% of thymidine uptake by gingival fibroblasts was caused by the highest doses of the *B. gingivalis* and *B. asaccharolyticus* extracts (Fig. 1). The effect of the *B. gingivalis* sonicate was dose dependent. The inhibition was statistically significant at doses of 10 μg/ml or higher ($P < 0.001$ by Student's t test).

The effects of extracts of the type culture strain of *B. gingivalis* and of our strain were very similar (Fig. 2). Heat inactivation of the complement in fetal calf serum decreased the inhibitory effect of both *B. gingivalis* strains (Fig. 2).

The extracts from 3-, 6-, and 8-day-old *B. gingivalis* cultures inhibited the uptake of thymidine by gingival fibro-

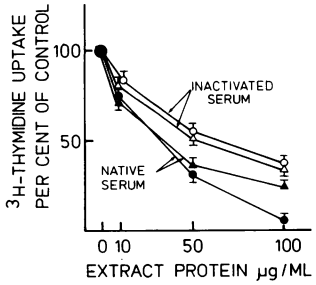

FIG. 2. Effects of extracts from two *B. gingivalis* strains on thymidine uptake by gingival fibroblasts in the presence of untreated serum (shaded symbols) and complement-depleted serum (open symbols). Circles represent the type culture strain of *B. gingivalis*, and triangles represent our isolated strain. A value of 100% is given for the controls, and the results indicate means ± SEM of five different cultures.

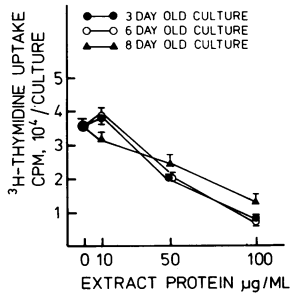

FIG. 3. Effects of extracts of *B. gingivalis*, cultured for various time periods, on the DNA synthesis of gingival fibroblasts. Extracts from 3-, 6-, and 8-day-old cultures of *B. gingivalis* were prepared similarly and added to the fibroblast culture media in dilutions with different protein concentrations. The values are means ± SEM of five replicate samples.

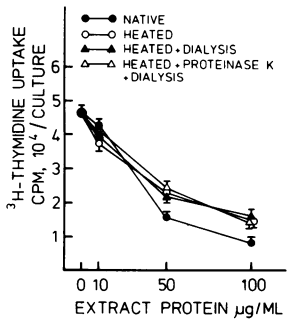

FIG. 4. Effects of heat treatment, dialysis, and proteinase K treatment of *B. gingivalis* extract on its action on gingival fibroblast DNA synthesis. Means ± SEM of five replicate samples are given.

blasts in a similar manner (Fig. 3). The heated *B. gingivalis* extract inhibited thymidine uptake by gingival fibroblasts almost as strongly as the native extract (Fig. 4). Dialysis of the *B. gingivalis* extract after heating did not cause any changes in the inhibitory action on fibroblasts (Fig. 4). This action was retained even after proteinase K treatment following dialysis of the extract (Fig. 4). The untreated sonicates from 6- and 8-day-old cultures of *B. gingivalis* contained few major proteins, as indicated by the silver-stained gels of the samples. The predominant fraction was a 54-kilodalton (kDa) polypeptide band. Proteinase K treatment effectively destroyed the proteins of the *B. gingivalis* extract. However, bands characteristic for LPSs and with molecular masses ranging from 37 to 60 kDa were obtained (Fig. 5).

Protein synthesis by gingival fibroblasts was significantly decreased when the cells were exposed to *B. gingivalis* at doses higher than 10 μg of protein per ml (Fig. 6). Proline uptake, however, was decreased much less than thymidine uptake. With a dose of 50 μg of protein per ml, the decrease was about 20%; and with 100 μg of protein per ml, the decrease was about 40%.

Gingival fibroblasts exposed to *B. gingivalis* extract (50 μg of protein per ml) at the beginning of the logarithmic growth phase showed a significant decrease after 2 and 4 days of growth in culture (Table 1). The effect appeared to be nontoxic, however, because the number of cells increased

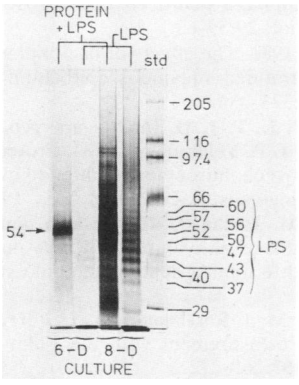

FIG. 5. SDS-polyacrylamide gel electrophoresis of *B. gingivalis* cell extracts. Native extracts (protein-LPS), and proteinase K-treated extracts (LPS) from 6- and 8-day-old cultures were run in 10% polyacrylamide gels and silver stained. The main cell extract protein band is indicated (54 kDa), as is the LPS pool (37 to 60 kDa) produced by treatment with proteinase K. Commercial molecular weight standards (std) are indicated. D, Day.

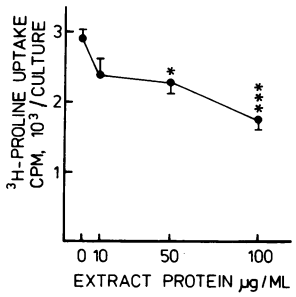

FIG. 6. Effect of *B. gingivalis* extract on the proline uptake by gingival fibroblasts. Means ± SEM of five replicate samples are shown. Symbols: *, $P < 0.05$; ***, $P < 0.001$ (by Student's *t* test).

slightly in comparison with the seeding density. Moreover, no changes in cell morphology could be detected by phase-contrast microscopy (data not shown).

DISCUSSION

Gingival fibroblasts are cells that maintain connective tissue in the periodontium. Their function in the pathogenesis of periodontal disease is poorly understood, however. Fibroblasts in gingival connective tissue may interact with inflammatory cells and their products, but they also interact directly with bacterial products. In addition, there is increasing evidence for direct interaction between fibroblasts and bacteria. Many studies have demonstrated bacteria in the gingival connective tissue (1, 2, 21–23). Members of the genus *Bacteroides* have also been found in connective tissue (1).

Fibroblast growth inhibition has been demonstrated in many oral gram-positive and -negative bacterial strains. Oral *Streptococcus* species (5, 6), *Actinomyces* species (5), *Capnocytophaga sputigena* (26), and *Actinobacillus actinomycetemcomitans* (25) extracts seem to inhibit fibroblast growth in vitro. Growth inhibition of cultured epithelial cells, but not of connective tissue cells, by *B. gingivalis* sonic extracts has been reported previously (11). During culture, however, *B. gingivalis* and *B. asaccharolyticus* seem to release components that are toxic to cultured fibroblasts (30). One such component has been proposed to be butyrate (29). Cell-associated factors from *B. gingivalis* affecting fibroblasts, however, have not been described. In this study we showed that gingival fibroblast growth is inhibited by *B. gingivalis* cell extracts. *B. asaccharolyticus* (a nonoral microorganism) inhibited gingival fibroblast growth to a similar degree, but *B. intermedius* had a much weaker effect. This fits well with the pathogenic features of

TABLE 1. Inhibition of gingival fibroblast growth by *B. gingivalis*[a]

Organism	Days in culture	No. of cells in[b]:	
		Expt 1	Expt 2
Control	2	1.055 ± 0.091	0.803 ± 0.015
B. gingivalis	2	0.638 ± 0.073[c]	0.533 ± 0.038[c]
Control	4	1.518 ± 0.090	1.143 ± 0.061
B. gingivalis	4	0.738 ± 0.068[c]	0.840 ± 0.085[c]

[a] *B. gingivalis* cell extract (50 μg of protein per ml) was added to fibroblast cultures at the early logarithmic growth phase, and cell numbers were counted after 2 and 4 days of culture. Results of two separate sets of experiments are given (mean ± standard deviation of three to four replicate samples).

[b] Cell number (10^6) per flask.

[c] $P < 0.001$, for difference between treated and control cultures (by Student's *t* test).

these bacteria, for *B. gingivalis* is more virulent than *B. intermedius* (24). Clinically, *B. intermedius* is associated with gingivitis, whereas *B. gingivalis* shows closer associations with nonreversible periodontitis. In in vitro studies culture filtrates of *B. gingivalis* and *B. asacchrolyticus*, but not of *B. intermedius*, have been shown to be toxic to cultured Vero cells (30). In this study the effect of *B. gingivalis* extract was apparently nontoxic; in spite of treatment with the extract, gingival fibroblasts still proliferated at reduced growth rates. The inhibition of thymidine uptake by gingival fibroblasts was probably induced by the LPS in the extract because proteinase K-treated preparations from *B. gingivalis* retained their inhibitory effect. *B. gingivalis* LPS has demonstrated low endotoxic activity in biological assays (24). This may be the reason for its low toxicity to fibroblasts. The LPS-rich fraction of *B. gingivalis*, however, has a strong bone-resorbing capacity (10, 16, 17).

Our preparation of *B. gingivalis* LPS was heterogeneous, with molecular masses of 37 to 60 kDa. The exact molecular masses may be different, however, because in this study the LPS fractions were compared with the weights of known globular proteins, which may differ from the LPS in SDS-polyacrylamide gel electrophoresis runs. Naito et al. (18) obtained a very similar pattern with a *B. gingivalis* LPS preparation, although they used a phenol-water extraction method. It should be taken into account that different extraction methods can produce LPS preparations with different biological activities (33). Our preparation method, including an enzymatic protein digestion step, resulted in an LPS-rich extract with apparently intact biological activity. Millar et al. (16) recently separated high- and low-molecular-mass LPSs from *B. gingivalis*. These two fractions had similar biological effects in bone resorption assays, however, and may represent the same original pool that was divided into high- and low-molecular-mass LPS fractions.

The outer membrane of *B. gingivalis* seems to contain only a few major polypeptide bands on silver staining. The predominant protein was a 54-kDa band. This is probably the same band that Williams and Holt (32) found in several *B. gingivalis* strains (57 kDa according to their measurements). They demonstrated that this protein is very close to the bacterial cell surface. The proteins of the extract, however, played only a minor role in gingival fibroblast inhibition. Their removal by proteinase K did not markedly affect the biological activity of the extract. The native proteins, such as enzymes, may have some additive effect on growth inhibition. This action could also be mediated by complement, because destruction of the complement system of fetal calf serum by heating slightly reduced the inhibition.

Proline uptake was decreased in fibroblasts that were treated with *B. gingivalis* extract. *B. gingivalis* LPS affected noncollagen and collagen synthesis in fetal rat bones (16). Protein synthesis was much less strongly inhibited than thymidine incorporation, although the longer labeling period may partly explain the difference. Growth may be inhibited more easily than protein synthesis, however. The nature of the interactions of LPS with mammalian cells is unclear. LPS is known to cause hyperadherence of leukocytes (33). If a similar phenomenon occurs in fibroblasts, it may partly explain the growth inhibition. Further studies are needed to characterize the fibroblast receptors for *B. gingivalis* LPS, the binding of LPS to these structures, and their effect on cell metabolism.

The inhibition of gingival fibroblast growth must be added to the list of the destructive arsenal of *B. gingivalis*. This inhibition seems to be mediated by LPS. The bursts in the progression of periodontal disease may involve the invasion of *B. gingivalis* in the connective tissue and the exertion of its harmful effects on the tissue fibroblasts.

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