Modulation of Growth and Function of Human Gingival Fibroblasts by Fibroblast-Activating Factor Derived from *Porphyromonas gingivalis* W50

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Received 19 June 1992/Accepted 20 November 1992

The effect of a 24-kDa fibroblast-activating factor (FAF) isolated from outer membrane vesicles of *Porphyromonas gingivalis* W50 on the modulation of [³H]thymidine uptake and cell proliferation was examined in selected fibroblast and transformed cell lines. FAF enhanced the proliferation of human gingival fibroblasts, human skin fibroblasts, and human umbilical vein endothelial cells in subconfluent and confluent cells, suggesting that FAF might be functioning as a competence factor. The transformed cell lines, U-937 and Hep-2, were not responsive. FAF and several human-derived growth factors showed a synergistic effect on proliferation. [³H]proline and [³H]leucine were rapidly incorporated into fibroblasts in the presence of FAF; however, there was no selective induction of collagen synthesis. FAF was not active in the induction of interleukin-1 and interleukin-6. It is hypothesized that FAF from *P. gingivalis* functions as a growth factor for human fibroblasts but is without activity for transformed cells.

Porphyromonas gingivalis elaborates a variety of virulence factors in the in vitro environment. These include proteases, outer membrane proteins, lipopolysaccharides (LPS), and fimbriae (21). These putative virulence determinants have been shown to have an effect on host-parasite interaction in selected cell culture and animal models. Their roles in the destructive events of in vivo inflammation and bone and tissue destruction remain unclear.

We have reported the isolation of a novel outer membrane-associated protein from the outer membrane vesicles of *P. gingivalis* W50, W83, and ATCC 33277 (23). This protein, designated fibroblast-activating factor (FAF), had a significant proliferative stimulating effect on normal human gingival fibroblast (HGF) incorporation of [³H]thymidine. FAF is a heat-sensitive polypeptide with relative molecular masses of 24 kDa when heated at 100°C for 5 min and of 44 kDa when unheated.

Fibroblasts have been recognized as cells which control the metabolism of connective tissue by producing collagen and related compounds (2, 9, 10). In addition to this biosynthetic capacity, they also function as immunomodulating cells by producing such cytokines as interleukin-1 (IL-1) (18, 20), beta interferon (16), IL-6 (11), granulocyte macrophage colony-stimulating factor (35), and IL-8 (19). Therefore, fibroblasts more than likely function in the host in several capacities, including connective-tissue synthesis and repair in local inflammatory reactions (i.e., periodontal disease).

In the present study, we examined the modulatory effects of FAF on the growth and function of HGFs and several other host cells; we postulate that FAF plays a strong modulating role in periodontal tissue by regulating the metabolism and growth of HGFs.

MATERIALS AND METHODS

Preparation of FAF. FAF was isolated and purified from *P. gingivalis* W50 outer membrane vesicles according to a procedure previously described (23).

Human cells. The FAF function for various host cells was also examined (Table 1). All cells were cultivated in 100-mm culture dishes or 75-cm² culture flasks (Corning, New York, N.Y.) in their own suitable prescribed medium (Table 1) (15, 22, 23).

Measurement of effect of FAF on growth of host cells. FAF activity was measured as the amount of [³H]thymidine incorporated (23) into both confluent and subconfluent HGFs. HGFs were seeded at a concentration of 10⁴ cells per well in 96-well microtiter plates (Corning) in Dulbecco modified Eagle's minimum essential medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Hazleton, Lenexa, Kans.). The cells were incubated in a CO₂-humidified incubator at 37°C for 24 h to produce subconfluent cultures or for 72 h for confluent cultures. At 24 h, the medium was replaced with fresh DMEM plus FAF at various concentrations. Cultivation was continued for an additional 24 h; at 18 h, [³H]thymidine (specific activity, 74 GBq/mmol; ICN Radiochemicals, Irvine, Calif.) was added to each well at a concentration of 0.5 µCi (18.5 kBq). At the end of the incubation period, the HGFs were detached from the culture plate with 0.25% trypsin and 0.02% EDTA (JRH Biosciences, Lenexa, Kans.). The detached cells were harvested on a glass fiber filter (Cambridge Technology, Watertown, Mass.) with a cell harvester (model PHD; Cambridge Technology), and radioactivity was measured with a liquid scintillation counter (model LS1701; Beckman, Fullerton, Calif.).

Effect of FAF on total HGF protein synthesis. The effect of FAF on HGF protein synthesis was determined by the amounts of L-[2,3-³H]proline (specific activity, 1.04 TBq/mmol; Amersham, Arlington Heights, Ill.) and L-[4,5-³H]leucine (Amersham; specific activity, 4.92 TBq/mmol) that were incorporated into HGFs. HGFs were grown to confluency in DMEM plus 10% FBS (for 4 days), and the medium was

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TABLE 1. Human cell lines used in FAF experiments

Cell line	Origin	Growth medium ^a	Source ^b
JCE	Normal HGF	DMEM + 10% FBS	UTHSCSA
JNS	Normal HGF	DMEM + 10% FBS	UTHSCSA
HGF-1	Normal HGF	DMEM + 10% FBS	UTHSCSA
HSF	Normal human skin fibroblast	DMEM + 10% FBS	ATCC
HUVE	Normal human umbilical vein endothelial cell	M199 + ECGS + heparin + 20% FBS	UTHSCSA
HPDL-4	Human periodontal ligament cell	DMEM + 10% FBS	UTHSCSA
HEp-2	Human larynx epidermoid carcinoma cell	RPMI 1640 + 10% FBS	ATCC
U-937	Human leukemic cell	RPMI 1640 + 10% FBS	ATCC

^a ECGS, endothelial-cell growth supplement.^b UTHSCSA, University of Texas Health Science Center at San Antonio; ATCC, American Type Culture Collection.

replaced with DMEM supplemented with 50 µg of ascorbic acid per ml and FAF. For [³H]leucine incorporation, the medium was replaced with leucine-free Eagle's minimal essential medium (MEM) prepared from the MEM select Amine kit (GIBCO Laboratory, Grand Island, N.Y.) according to the manufacturer's instructions. After 48 h of culture, the medium was replaced with fresh leucine-free Eagle's MEM containing the same concentration of FAF plus 10 µCi of [³H]proline or [³H]leucine per ml with 50 µg of β-aminopropionitrile monofumarate (Sigma) per ml. The cells were incubated for an additional 24 h, and the plates were frozen (−70°C) and thawed (25°C). The thawed-lysed cells were removed, the wells were washed twice with 20 µl of 0.1 M NaOH, and the protein was precipitated by the addition of 200 µl of 50% (wt/vol) trichloroacetic acid (TCA)–5% tannic acid and incubation at 4°C overnight. The precipitate was recovered by centrifugation, washed twice with 1 ml of 10% TCA–1% tannic acid, and finally dissolved in 50 µl of 0.1 M NaOH. The amount of radioactivity in each sample was assessed by liquid scintillation counting (model LS1701; Beckman).

Effect of FAF on HGF collagen synthesis. As a measure of the effect of FAF on HGF-collagen synthesis, the amount of L-[2,3-³H]proline (Amersham) that was incorporated into collagenase-digestible protein (CDP) was determined according to the methods described by Peterkofsky and Diegelmann (25). HGFs (JCE and JNS, passage 5; 2 × 10⁵ cells) were seeded into 6-well culture dishes (Corning) and cultured in DMEM with 10% (vol/vol) FBS and 50 µg of ascorbic acid per ml until confluent. The cells were washed with DMEM (without FBS) and then cultured in DMEM with FAF (0, 50, or 500 µg/ml) for 72 h. At 48 h, the medium was replaced with fresh DMEM containing 5 µCi of [³H]proline, 50 µg of β-aminopropionitrile monofumarate (Sigma) per ml, and the appropriate concentration of FAF. At 72 h, both extracellular and intracellular fractions were collected. The intracellular fraction was obtained from cells treated with 1 N NaOH and subsequently neutralized with HCl. The extracellular fraction (total, 2 ml per well) consisted of the culture medium plus the phosphate-buffered saline used for cell washing. The protein in each fraction was precipitated with TCA and tannic acid, washed several times with the same reagent, and finally precipitated with ethanol-ether (1:1). The resulting precipitates were dissolved with 0.1 N NaOH and neutralized with 0.1 N HCl. A 400-µl volume of each sample (20 ml) was used for the detection of CDP. A 50-µl volume of a collagenase suspension consisting of 20 U of bacterial collagenase (type VII; Sigma), 1.2 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 25 mM *N*-ethylmaleimide (Sigma), and 5 mM CaCl₂ was added to each sample, and the mixtures were incubated for 2 h at

37°C. The undigested protein was precipitated with TCA and tannic acid, and the supernatant was collected by centrifugation. This procedure was repeated twice, and the amounts of radioactivity in both supernatants were measured by measuring the amounts of [³H]proline incorporated into CDP. The amount of nonincorporated [³H]proline in the medium supernatant was determined as described above; however, the supernatant was not treated with collagenase. The CDP was corrected for these nonspecific counts. The amount of radioactivity was determined on the basis of total cellular protein detected by the bicinchoninic acid assay (Bio-Rad Laboratories, Richmond, Calif.).

Measurement of FAF-induced cytokine activity. (i) **Preparation of FAF-stimulated fibroblast-conditioned media.** Both subconfluent and confluent HGFs (JCE [passage 6] cells in DMEM plus 10% FBS) were used. Cells were incubated with FAF in DMEM (FBS free) for 24 h (see description of the FAF assay above). The supernatants were collected and frozen at −20°C.

(ii) **Measurement of TAF activity.** To eliminate induction of IL-1, thymocyte-activating factor (TAF) activity was measured as described by Takada et al. (32). A 100-µl volume of thymocyte suspension (1.5 × 10⁷ cells per ml in RPMI 1640 containing 10% FBS) from C3H/HeJ mice (2 to 4 weeks old) was distributed to each well of a 96-well culture dish. A 50-µl volume of phytohemagglutinin (4 µg/ml; Wellcome Diagnostics, Temple Hill, United Kingdom) and 50 µl of conditioned medium diluted in RPMI 1640 were added, and the cultures were incubated for 72 h in a CO₂-humidified incubator at 37°C. Then, 0.5 µCi of [³H]thymidine (ICN Radiochemical) was added to each well during the final 16 h of cultivation. The thymocytes were then harvested with a cell harvester on a glass fiber filter, and the incorporated radioactivity was measured by scintillation counting (Beckman). TAF activity was expressed as a stimulation index (SI) as follows: SI = counts per minute in test culture/counts per minute in control (phytohemagglutinin).

(iii) **Measurement of IL-6 activity.** IL-6 activity in HGF-conditioned medium (see above) was determined by measuring the in vitro growth of the IL-6-dependent cell line B9 (14). A 50-µl volume of B9 cells (4 × 10⁴/ml) in Iscov's MEM supplemented with 5% (vol/vol) FBS and 50 µl of serially diluted conditioned medium were incubated in 96-well culture plates (Corning) for 48 h and pulse-labeled with 0.5 µCi per well of [³H]thymidine during the last 6 h of culture. The B9 cells were harvested as described for the thymocytes above. The amount of incorporated [³H]thymidine was determined by liquid scintillation counting. The amount of IL-6 was calculated on the basis of a dose-dependent curve obtained for standard human recombinant IL-6 (R&D Systems, Minneapolis, Minn.).

FIG. 1. Effect of FAF on incorporation of [3 H]thymidine into subconfluent and confluent HGFs. Subconfluent and confluent HGFs were stimulated with purified FAF at several concentrations for 24 h in DMEM without FBS. The results were expressed as amounts of incorporated [3 H]thymidine (counts per minute) per 10^4 HGFs on the basis of the terminal cell numbers. **, significantly different from value of FAF at 0 ng/ml ($P < 0.01$).

Comparison of proliferative effects between FAF with and FAF without selected growth factors. The effects of recombinant human platelet-derived growth factor (rPDGF) (Sigma), epidermal growth factor (rEGF; Sigma), and recombinant human basic fibroblast growth factor (rbFGF; Calbiochem, San Diego, Calif.) on FAF of HGF stimulation were examined in the proliferative-HGF assay (see above). Both confluent and nonconfluent HGFs were used. Growth factors were depleted from the confluent HGFs by culturing the cells during the last 4 h in FBS-depleted DMEM. The effects of FAF and growth factors on [3 H]thymidine incorporation were then determined by employing DMEM minus FBS as described above.

RESULTS

Effect of FAF on [3 H]thymidine incorporation into HGFs. Figure 1 shows the typical response of purified FAF to [3 H]thymidine incorporation into subconfluent and confluent HGFs. In confluent HGFs, there was a significant decrease in basal incorporation of [3 H]thymidine compared with that for the subconfluent cells. However, under both growth conditions, FAF had a dramatically dose-dependent proliferative effect compared with the respective controls (no FAF).

Effect of FAF on total protein synthesis and CDP synthesis by HGFs. The effect of FAF on HGF protein synthesis was determined by measuring the incorporation of [3 H]proline and [3 H]leucine into the total protein of confluent HGFs (Fig. 2). FAF at both 50 and 500 ng/ml had a significant effect on the incorporation of [3 H]proline and [3 H]leucine compared with that of the control. At these FAF concentrations, there was an almost twofold increase in the amount of [3 H]proline and [3 H]leucine incorporated compared with that of control HGFs in the absence of FAF. The ratio of [3 H]proline to [3 H]leucine as a function of FAF concentration remained the same. However, the effect of FAF on the [3 H]proline incorporation into both intracellular and extracellular CDP remained approximately the same (Table 2), indicating that FAF had no effect on HGF collagen synthesis in this experimental system.

Induction of IL-1 and IL-6 in HGF cultures by FAF

FIG. 2. Effect of purified FAF on the incorporation of [3 H]proline and [3 H]leucine into protein. Confluent HGFs were stimulated with or without FAF (50 or 500 ng of protein per ml) for 72 h. At 48 h, the HGFs were pulsed with [3 H]proline or leucine and radioactivity was measured in TCA-precipitable protein. Values are counts per minute of radioactivity in each culture per 10^5 HGFs $\pm$ standard errors. **, significantly different from control (no addition) ($P < 0.01$).

stimulation. The effect of FAF on the modulation of IL-1 and IL-6 is indicated in Table 3. Conditioned HGF medium (1:8 dilution) containing either 0.1 or 1.0 μ g of FAF protein from subconfluent or confluent HGFs per ml produced very little TAF (Table 3). At 10 μ g of FAF protein per ml, FAF was stimulatory for TAF production in confluent HGFs. *P. gingivalis* LPS showed significant enhancement of TAF activity at doses between 1 and 100 μ g/ml from subconfluent HGFs and between 10 and 100 μ g/ml from confluent HGFs.

FAF induced very little (<1 ng/ml) IL-6 in HGFs compared with that induced by *P. gingivalis* LPS (3.9 to 49.4 ng/ml) (Table 3). The tendency to produce IL-6 was similar in both confluent and subconfluent HGFs. The absence of FAF IL-1 induction combined with negative LPS staining on sodium dodecyl sulfate- and deoxycholate-polyacrylamide gel electrophoresis gels eliminates LPS contamination of the FAF preparation.

Effect of FAF on selected human cells. The FAF specificities for selected human cell lines were determined (Table 1; Fig. 3). FAF was stimulatory to HGFs and skin fibroblasts and to normal human endothelial cells. However, FAF was

TABLE 2. Effect of *P. gingivalis* W50 FAF on CDP synthesis

Treatment (concn)	Intracellular CDP (cpm/mg of protein) ^a	Extracellular CDP (cpm/mg of protein) ^a
Control (minus FAF)	9,895 $\pm$ 2,878	6,163 $\pm$ 1,049
FAF (50 ng/ml)	6,936 $\pm$ 763	6,598 $\pm$ 2,844
FAF (500 ng/ml)	10,810 $\pm$ 3,628	12,388 $\pm$ 1,040 ^b

^a Total synthesized CDP is the amount of [3 H]proline incorporated into CDP fractions. Data are based on milligrams of protein detected in cellular fractions.

^b Significantly different from control value. ($P < 0.05$).

TABLE 3. Induction of IL-1 and IL-6 in HGF culture supernatant by *P. gingivalis* W50 FAF and LPS

Assay and FAF concn ($\mu\text{g/ml}$)	SI or cpm with subconfluent HGFs ^a		SI or cpm with confluent HGFs ^a	
	FAF	LPS	FAF	LPS
TAF activity ^b				
0	1.07 $\pm$ 0.08	1.07 $\pm$ 0.08	1.14 $\pm$ 0.07	1.14 $\pm$ 0.07
0.1	1.62 $\pm$ 0.12	ND	0.98 $\pm$ 0.06	ND
1	1.59 $\pm$ 0.14	2.57 $\pm$ 0.49 ^c	1.57 $\pm$ 0.07	1.71 $\pm$ 0.08
10	1.73 $\pm$ 0.27	3.12 $\pm$ 0.73 ^c	2.13 $\pm$ 0.10 ^c	2.10 $\pm$ 0.27 ^c
100	ND ^d	3.47 $\pm$ 0.37 ^e	ND	2.53 $\pm$ 0.35 ^e
IL-6 induction ^c				
0	ND	ND	ND	ND
0.001	301	4,700	365	16,200
0.01	285	3,900	422	49,400
0.1	195	5,500	1,120	24,600
1	250	9,200	310	27,300

^a Values are stimulation indices (SI; see text) $\pm$ standard errors (for TAF activity) or counts per minute (for IL-6 induction). Values for [³H]thymidine incorporation in control thymocyte cultures are 1,083.3 cpm $\pm$ 883.2 (phytohemagglutinin alone) and 463.0 cpm $\pm$ 25.3 (no addition).

^b 1:8 diluted culture supernatants were incubated with thymocytes in the comitogenic assay (TAF activity), standard IL-1 detection assay (Materials and Methods).

^c In vitro growth of IL-6-dependent B9 cell line. Values are calculated on the basis of a standard curve of IL-6 standard (see Materials and Methods). [³H]thymidine incorporation into B9 cells increased in a dose-dependent manner at IL-6 concentrations of from 2.5 to 100 pg/ml (data not shown). No direct effect of *P. gingivalis* W50 LPS and FAF at the doses used was detected.

^d ND, no data.

^e Significantly different from control value (no addition) ($P < 0.05$).

not active in inducing [³H]thymidine incorporation into human periodontal ligament cells (data not shown) or into the two transformed cell lines, HEp-2 and U-937.

Patterns of response of FAF on HGFs in the presence and absence of selected growth factors. The effect of FAF in the presence and absence of added growth factors on [³H]thymidine incorporation into both confluent and nonconfluent HGFs is seen in Fig. 4. All factors significantly increased [³H]thymidine incorporation into the nonconfluent HGFs, in a range from 500% (FAF) to 3,000% (rPDGF). The confluent HGFs showed a lower incorporation of [³H]thymidine with *P. gingivalis* FAF (200%), rPDGF (900%), and rbFGF (130%). rEGF did not enhance FAF activity to any significant degree.

Additive effect of FAF with selected human growth factors

FIG. 3. Effect of FAF on incorporation of [³H]thymidine into the selected human cells JCE, JNS, HGF-1, HSF, and HUVE. Human cells were cultured in their appropriate growth media (Table 1), stimulated with various FAF concentrations, and compared with results for control (minus FAF, with 0 and 10% FBS). All cells were stimulated for 24 h under subconfluent condition, and FAF activity was determined by the standard [³H]thymidine incorporation assay (see Fig. 1).

on HGF proliferation. The effect of FAF and the selected growth factors, rPDGF, rbFGF, and rEGF, on HGF incorporation of [³H]thymidine is seen in Fig. 5. rPDGF, rbFGF, and rEGF all resulted in [³H]thymidine incorporation at

FIG. 4. Effect of FAF and human-derived growth factors rPDGF, rbFGF, and rEGF on [³H]thymidine incorporation into confluent and nonconfluent HGFs. HGFs were stimulated with various doses of *P. gingivalis* FAF and growth factors for 24 h. At 18 h of incubation, [³H]thymidine was added and the amount of incorporation into HGFs was measured as described in the legend to Fig. 1.

FIG. 5. Synergistic effect of FAF and human growth factors rPDGF, rbFGF, and rEGF on incorporation of [³H]thymidine into subconfluent HGFs (see Table 1). HGFs were stimulated with human growth factors alone or with human growth factors plus FAF (750 ng/ml).

suboptimal (i.e., low-dose) FAF concentrations (data not shown). At high (750-ng/ml) concentrations, there was an enhancement of the effect of growth factors on HGF proliferation.

DISCUSSION

FAF was characterized by Mihara and Holt (23) as a 24-kDa outer membrane-associated polypeptide. It was biologically active, functioning to enhance proliferation of human fibroblasts. The growth-enhancing ability of FAF on HGFs (JCE, JNS, and HGF-1) as well as on human skin fibroblasts was significant (Fig. 3). Interestingly, normal umbilical vein endothelial cells, a major cell type found to regulate inflammatory and immunological responses in local infections, were also activated by FAF. Human periodontal ligament cells, fibroblastlike cells derived from human periodontal ligament, did not respond to FAF (data not shown). The transformed cell lines, HEp-2 and U-937, were also not activated by FAF. Several growth factors which have been reported to function on normal cells have been observed to show inhibitory effects on transformed cells (for example, tumor necrosis factor [34], EGF [5], and IL-2 [31]). This difference in activity between normal and transformed cells could be due to the fact that in neoplastic cells, growth factors may lead to a reverse signal transduction compared with that in normal cells (13). It is possible that FAF functions in a similar manner, and its activity is suggestive of a specific target cell effect.

A variety of growth factors (i.e., insulin [1], thrombin [7], EGF [12], PDGF [29], FGF [8], insulinlike growth factor [33], transforming growth factor β [6], tumor necrosis factor [34], and IL-1 [30]) have been demonstrated to function as modulators of cell growth. Most of these factors are derived from the host, and in vivo, they are thought to modulate both the function and the growth of the host cell by a complicated and interconnected network (17, 32). Several proteolytic enzymes have also been shown to possess insulinomimetic protease activity (4, 26–28), and Blumberg and Robbins (3) have reported that several of these proteases may affect

HGF cell surface proteins (fibronectin) and hence activate the fibroblasts.

In the present report, we have compared the activities of three of the more-common growth factors (PDGF, FGF, and EGF) on HGFs with or without FAF. PDGF and FGF, reported to be competence factors elsewhere (24), were without significant activity in the studies reported here. However, the fact that the subconfluent (nonquiescent) HGFs (devoid of growth factor) were stimulated by FAF at low doses suggests that FAF might be functioning in a manner similar to that of PDGF and FGF.

Although FAF demonstrates a total-protein-synthesis enhancement, it was not active in the synthesis of collagen by HGFs. Also, FAF did not induce IL-1 (except at 10 μ g/ml) and IL-6, suggesting that FAF functions to modify HGF growth rather than HGF function. What role FAF plays in vivo in *P. gingivalis*-elicited infections is unclear; however, FAF does appear to have adequate ability to interfere with connective-tissue homeostasis by affecting growth and protein synthesis.

ACKNOWLEDGMENTS

We thank D. Thomas, M. P. Moyer, and B. McAllister for providing the various mammalian cell lines. We also thank D. Guerrero for photographic work and K. Lucas for her continued administrative assistance.

This study was supported by NIH grants DE08569 and DE07267.

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