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# Expression of CD13/aminopeptidase N on human gingival fibroblasts and up-regulation upon stimulation with interleukin-4 and interleukin-13

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*Background and objectives:* Aminopeptidase N (APN)/CD13 is a multifunctional ectoenzyme that is involved in anti-inflammatory reactions, control of immune reactions and differentiation of many cellular systems. Here, we hypothesized that CD13/APN would be expressed on human gingival fibroblasts (hGF) and would contribute to the regulation of immune responses in periodontal tissue.

*Methods and results:* CD13/APN was expressed on hGF at the mRNA and protein levels as determined by reverse transcriptase–polymerase chain reaction (RT–PCR) and flow cytometry, respectively. Enzymatic activities accompanying the expression were assessed by colorimetrical analysis using the synthetic substrate Leu-*p*-nitroanilide. We examined the possible regulation of CD13/APN expression on hGF in response to T cell-derived cytokines. T helper (Th) 2 cell type cytokines such as interleukin-4 and interleukin-13, but not interleukin-2 or interleukin-15, preferentially increased the expression of proteins as well as the enzymatic activities of CD13/APN in a dose-dependent manner. Receptors for these cytokines, the interleukin-4 receptor $\alpha$ chain, interleukin-13 receptor $\alpha$1 chain, and interleukin-2R common $\gamma$ chain, were expressed on hGF assessed by RT–PCR or flow cytometry. hGF exhibited inhibitory effects for formyl-methionyl-leucyl-phenylalanine (FMLP)-induced polymorphonuclear leukocyte-activation that was evaluated by Mac-1 expression, and this inhibitory effect was partially recovered by pre-treatment with the APN-specific inhibitor bestatin.

*Conclusions:* These findings suggested that CD13/APN expressed by hGF could contribute to the anti-inflammatory response in periodontal tissue, and may be involved in disease processes mediated by Th2 cells.

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It has been reported that cell surface peptidases mediate or block signals by processing external peptides, and are also involved in signal transduction cascades. These peptidases play essential roles not only in the regulation of immune function, but also in the control physiological processes such as growth, differentiation, cell activation, and cell to cell interactions (1). CD13 was originally used as a marker for the myeloid differentiation antigen (2, 3),

which is identical to the zinc metalloprotease, aminopeptidase N (APN) (4). CD13/APN is widely distributed in a variety of non-hemotopoietic cell types such as intestinal epithelial cells, synovial fibroblasts and the synaptic membranes of the central nervous system neurons (1). CD13/APN hydrolyzes a variety of substrates, including substance P, formyl-methionyl-leucyl-phenylalanine (FMLP), bradykinin and enkepharin (5, 6), and substrates comparable to those of CD10/neutral endopeptidase (NEP) have been suggested (7). CD13 is also capable of degrading interleukin-8 and inactivating its chemotactic activity, resulting in a limitation of the neutrophil inflammation response (8). Peptides bound to major histocompatibility complex class II molecules can be digested by CD13 with dramatic consequences for T cell antigen recognition (9). Furthermore, APN is a chemoattractant for T lymphocytes (10), and is also essential for capillary tube formation (11).

Gingival fibroblasts have been previously considered as important connective tissue cells that construct a supporting framework crucial for tissue integrity and repair of periodontal tissue. Recently, fibroblasts were found to be important sentinel cells in the immune system of the skin and mucosa (12). Fibroblasts actively define the structure of tissue microenvironments and regulate infiltrated hematopoietic cell functions by production of cytokines/chemokines and the extracellular matrix (12, 13). However, very little is known regarding how cell surface peptidases on human gingival fibroblasts (hGF) operate physiologically or pathologically in periodontal tissue.

It is well known that immune responses in periodontal tissue are regulated by various cytokine networks. In particular, the balance between T helper (Th) 1 cytokines, such as interleukin (IL)-2, interferon-γ, or interleukin-12, and Th2 cytokines, such as interleukin-4, interleukin-10, or interleukin-13, may be closely associated with periodontal disease severity (14–19). Therefore, we examined the possible expression of CD13/APN on hGF and how T cell-derived cytokines may regulate the expression of CD13/APN on hGF.

## Material and methods

### Reagents

Glutarylalanylalanyl-(4-methoxynaphthylamine) (Glut-Ala-Ala-Phe-MNA), L-leucine-*p*-nitroanilide (Leu-*p*-NA), *p*-NA phosphoramidon, phenylmethylsulfonyl fluoride, pepstatin, bestatin, aprotinin, 1,10-phenanthroline, bovine serum albumin, Cell Dissociation Solution® and FMLP were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Aminopeptidase M (porcine kidney) was from Calbiochem-Novabiochem (La Jolla, CA, USA). RPMI 1640 medium was from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Anti-CD13 monoclonal antibody (mAb) (WM15, mouse IgG1), anti-CD10 mAb (HI10a, mouse IgG1), anti-CD132 (interleukin-2Rγc) mAb conjugated with phycoerythrin were purchased from BD Biosciences PharMingen (San Diego, CA). Isotype control Ab (mouse IgG1), anti-CD124 (interleukin-4Rα) mAb conjugated with phycoerythrin and anti-CD11b (Mac-1) mAb (Bear-1) conjugated with fluorescein isothiocyanate were purchased from Immunotech, a Beckman Coulter Company (Marseille, France). Anti-interleukin-13 receptor α 1 chain mAb (GM-1C8, mouse IgG1) was from Abcam Ltd. (Cambridge, UK). Recombinant (r) human interleukin-2, interleukin-4, interleukin-13 and interleukin-15 were purchased from Pepro Tech EC Ltd. (London, UK).

### hGF

hGF cells were prepared from the explants of clinically non-inflamed gingiva from 8–25-years-old patients with informed consent, as reported previously (20). Explants were cut into pieces and cultured in 100-mm diameter tissue culture dishes (Falcon; Becton Dickinson Labware, Lincoln Park, NJ, USA) in α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum with a medium change every 3 days for 10–15 days until confluent cell monolayers were formed. The cells were detached with 0.25% tripsin-1 mM ethylenediamine tetraacetic acid (EDTA), washed with phosphate-buffered saline and subcultured in plastic flasks (Corning Coster, Acton, MA, USA). After three to four subcultures by trypsinization, homogeneous, slim, spindle-shaped cells grown in characteristic swirls were obtained. The cells were used as confluent monolayers at subculture levels 5 through 15.

### Isolation of human polymorphonuclear leukocyte (PMN)

Human PMN from heparinized (10 U/ml) peripheral venous blood were isolated by density gradient centrifugation on Mono-Poly resolving medium® (ICN Biomedical Co., Costa Mesa, CA, USA) at 300 *g* for 30 min at room temperature (21). The fraction containing the PMN was harvested and washed twice with phosphate-buffered saline at 4°C. The viability of these cells was greater than 98%, as judged by trypan blue dye-exclusion. The purity of the PMN was above 95% morphologically.

### Flow cytometry analysis

hGF cells in 24-well multiplates were collected using Cell Dissociation Solution®(non-enzymatic), so as to avoid possible proteolysis destruction of cell surface proteins, processed by passing through a nylon mesh filter (94 μm of mesh size), washed with washing buffer (phosphate-buffered saline containing 1% bovine serum albumin) three times, and used for staining. A total of $10^5$ hGF were directly or indirectly stained with each mAb or isotype-matched control IgG at 4°C for 20 min. For indirect staining, fluorescein isothiocyanate-conjugated goat anti-mouse IgG (BioSource International, Camerillo, CA, USA) was added at 4°C for 20 min. Staining was analyzed on FACScan® (Becton Dickinson, Mountain View, CA, USA). Measurements were collected for 5000 events, which were stored in list mode and then analyzed with Lysis II software (Becton Dickinson). The arithmetic mean was

used in the computation of the mean fluorescence intensity.

### Enzyme assay

Confluent hGF in 96-well flat-bottomed plates were used for enzyme assays in triplicate. NEP 24.11 activity was measured fluorometrically in a coupled assay using Glut-Ala-Ala-Phe-MNA as a substrate. The reaction mixture contained 0.1 mM substrate, 0.1 M 2-(*N*-morpholino)ethanesulfonate buffer at pH 6.5, and 0.12 M NaCl in a final volume of 0.1 ml. After incubation at 37°C for the indicated time, the harvested supernatant was incubated with aminopeptidase M (final concentration 0.5 U/ml) for a further 10 min at 37°C. The final fluorescent product, MNA, was measured in a VersaFluor spectrophotofluorometer (Bio-Rad Laboratories, Hercules, CA, USA) with excitation at 340 nm and emission at 425 nm. Data obtained in fluorescence units were converted to pmol/confluent hGF (approximately $2 \times 10^4$ cells) using a standard fluorescence curve for the product, MNA. APN activity was assayed using 8.36 mM Leu-*p*-NA as a substrate in reaction mixture (100 mM HEPES buffer, pH 7.6, containing 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose and 10 mg/ml bovine serum albumin) in a final volume of 0.1 ml for the indicated time at 37°C. Enzymatic activity was determined by measuring the amount of *p*-NA formed in the supernatant at 405 nm. Cell-free and substrate-free blanks were run in parallel. Results are expressed as nmoles of *p*-NA/confluent hGF (approximately $2 \times 10^4$ cells) using a standard *p*-NA curve. To examine the effect of potential inhibitors, confluent hGF was pre-incubated with various inhibitors for 15 min at 37°C before addition of the substrate to the reaction mixture.

### Reverse transcriptase–polymerase chain reaction (RT–PCR) assay

Total cellular RNA was extracted from hGF cultured in a six-well multiplate by Isogen® (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription of the RNA samples to cDNA was done using a TaKaRa RNA PCR™ Kit (AMV) Version 2.1 (TAKARA BIO, Shiga, Japan). To transcribe the total RNA into cDNA, 1 μg of RNA, 250 U/ml reverse transcriptase XL isolated from avian myeloblastosis virus, 5 mM $MgCl_2$, 1 mM dNTP mixture, 1000 U/ml RNase inhibitor, and 2.5 μM Random 9 mer were mixed in a PCR buffer (total volume of 20 μl). The reaction mixture was incubated for 10 min at 30°C, 30 min at 42°C, followed by 5 min at 95°C. The primers used for PCR are given in Table 1. The PCR mixture contained 5 μl of the cDNA mixture, 2 μl of 10 × PCR buffer, 0.2 mM deoxynucleoside triphosphate, 50 pmol of each primer, and 0.1 μl of Ex Taq DNA polymerase (Takara, Tokyo, Japan) in a total volume of 20 μl. Amplification was performed in an iCycler thermal cycler (Bio-Rad) with the cycle program shown in Table 1. Amplified samples were visualized on 2.0% agarose gels stained with ethidium bromide and photographed under ultraviolet light.

### Statistical analysis

All experiments in this study were performed at least three times to test the reproducibility of the results, and representative findings are shown. In some experiments, experimental values are given as means ± standard error (SE). The statistical significance of differences between two means was evaluated by one-way ANOVA and the Wilcoxon signed-ranks test (in Fig. 3), and *p*-values less than 0.05 were considered significant.

## Results

### Expression of CD13/APN on hGF

We first examined the possible expression of CD13/APN on hGF. In all hGF samples from eight donors, almost 100% of the cells from the confluent culture expressed CD13 by flow cytometry (Fig. 1A). The confluent monolayers of hGF from eight donors were subjected to APN assay with Leu-*p*-NA as a substrate, and all eight hGF samples expressed Leu-*p*-NA degradating activity, which

*Table 1.* Primer pairs used for polymerase chain reaction amplifications

| Gene (fragment) | Primer sequences | Denaturation/annealing/extension °C (s) | Cycle | Reference |
|---|---|---|---|---|
| IL-2Rb (437 bp) | 5′-CCGTGGCTCGGCCACCTC-3′<br>5′-TAGGGGTCGTAAGTAAAGTACACC-3′ | 94 (60)/62 (60)/72 (60) | 40 | 22 |
| IL-4Rα (335 bp) | 5′-GACCTGGAGCAACCCGTATC-3′<br>5′-CATAGCACAACAGGCAGACG-3′ | 94 (60)/53 (60)/72 (60) | 35 | 23 |
| IL-2R-γ (481 bp) | 5′-CCAGGACCCACGGGAACCCA-3′<br>5′-GGTGGGAATTCGGGGCATCG-3′ | 94 (60)/63 (60)/72 (60) | 40 | 23 |
| IL-13Rα1 (358 bp) | 5′-AGGATGACAAACTCTGGAG-3′<br>5′-CTCAAGGTCACAGTGAAGG-3′ | 94 (60)/58 (60)/72 (60) | 35 | 23 |
| IL-13R-α2 (426 bp) | 5′-ATACCTTTGGGACCTATTCC-3′<br>5′-TGAACATTTGGCCATGACTG-3′ | 94 (60)/58 (60)/72 (60) | 35 | 23 |
| β-actin (336 bp) | 5′-ATTGGCAATGAGCGGTTCCGC-3′<br>5′-CTCCTGCTTGCTGATCCACATC-3′ | 94 (60)/55 (60)/72 (60) | 30 | |

IL, interleukin.

ranged from 305.5 to 992.3 nmol/$10^6$ cells/h (average 573.4 nmol/$10^6$ cells/h, indicated with bar in figure) (Fig. 1B).

### CD13-associated aminopeptidase activity on hGF as ectoenzymes

We examined whether degradation of substrates by the monolayer cells resulted from enzymatic activity of APN on the cell surface. It is possible that incorporation of a substrate by hGF followed by intracellular degradation and release of products could occur or that intracellular enzyme was secreted. To eliminate this possibility, we carried out the following experiments. The confluent hGF monolayer was incubated at 37°C for 1 h with the substrate, and then the supernatant was harvested from the monolayer cells. The harvested supernatants were incubated for an additional 60 min without addition of any reagents, and monolayer cells were incubated in the new reaction mixture without substrate for an additional 60 min. As shown in Fig. 1(C), during the additional 60 min, the control group (without separation of cells from substrate) exhibited a linear and time-dependent enzymatic response. On the other hand, neither additional degradation of the substrate by the supernatant nor *p*-NA-release from the monolayer cells was observed during the same time period of separated culture. These findings rule out the possibility of secretion of intercellular enzymes and degradation of the substrates inside the cells followed by release of substrate in the monolayer cells. Furthermore, the possibility that enzymatic activity was released by lysed cells was ruled out because less than 2% of cells died during the experiment (Fig. 1D).

*Table 2.* Effects of various protease inhibitors on aminopeptidase N (APN) activity on human gingival fibroblasts (hGF)[a]

| Inhibitor | Concentration | % inhibition[b] APN activity |
|---|---|---|
| Phosphoramidon | 1 μM | < 1 |
| 1,10-Phenanthroline | 1 mM | 92.5 |
| EDTA | 5 mM | 35.5 |
| Phenylmethylsulfonyl fluoride | 1 mM | 4.5 |
| Bestatin | 1 mM | 83.7 |
| Pepstatin | 1 μM | < 1 |
| Aprotinin | 10 μg/ml | < 1 |

[a]Confluent hGF in 96-well plates were washed with phosphate-buffered saline three times, and examined by APN assays in the presence or absence of the indicated inhibitors.
[b]% inhibition was calculated as: [(enzymatic activity in the absence of inhibitor on hGF) − (enzymatic activity in the presence of inhibitor on hGF)]/(enzymatic activity in the absence of inhibitor on hGF) × 100. Findings are representative of three independent experiments. EDTA, ethylenediamine tetraacetic acid.

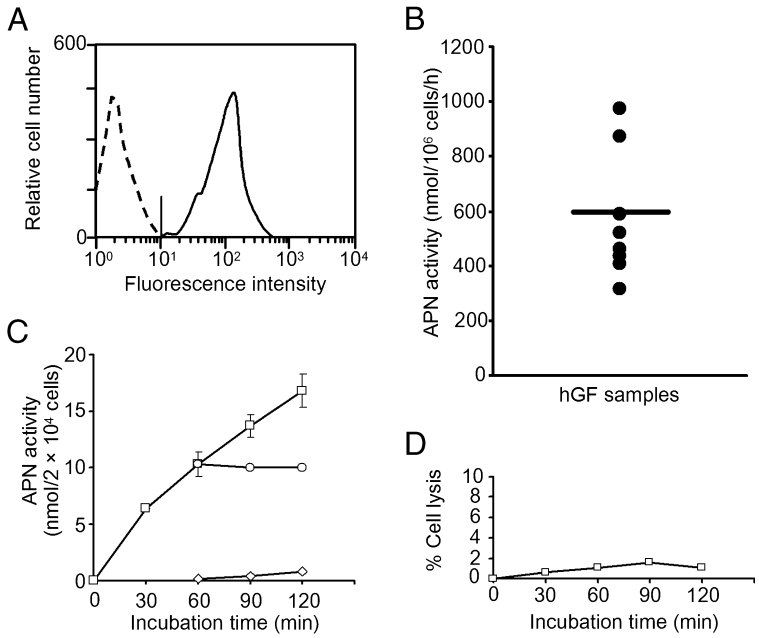


*Fig. 1.* Expression of CD13/aminopeptidase N (APN) on human gingival fibroblasts (hGF). (A) hGF was collected from confluent monolayers using Cell Dissociation Solution®. Expression of CD13 on the cell surface was assessed by flow cytometry. Isotype-matched antibody was used as the negative control (broken line). Findings are representative of eight independent experiments with eight different donors. (B) APN activity on confluent hGF from eight different donors was measured using L-leucine-*p*-nitroanilide (Leu-*p*-NA). The bar indicates the average APN activity. (C) Confluent monolayer cells were incubated with Leu-*p*-NA for APN assay as described in Materials and Methods. After a 60-min incubation, the supernatant and monolayer cells were separated. The supernatant and corresponding monolayer cells were resuspended in reaction mixture without the substrate and were incubated for an additional 60 min. (□),nmol *p*-NA formed by intact fibroblasts; (○),nmol *p*-NA formed by supernatant-resulting from 60 min of pre-incubation of cells with the substrate; (◇), nmol *p*-NA released by cells pre-incubated for 60 min with the substrate. (D) The percentage of lysed cells was counted by the trypan blue dye exclusion test at each time point. Representative findings of three independent experiments are shown as the mean ± SE of triplicate assays.

### Characterization of CD13-associated aminopeptidase activity

Bestatin is specifically used to inhibit APN activity (1). We examined whether Leu-*p*-NA degradating activities on hGF were sensitive to various protease inhibitors. As shown in Table 2,

the activities were strongly inhibited by bestatin (83.7% inhibition). Metalloprotease inhibitors 1,10-phenanthroline and EDTA inhibited APN activity by 92.5% and 35.5%, respectively, and other inhibitors, phenylmethylsulfonyl fluoride, aprotinin, and pepstatin, had no effects on the activity. These results clearly identified the enzyme expressed on hGF as APN.

### Regulation of CD13/APN expression on hGF in response to T cell-derived cytokines

As interleukin-4 is known for fibrogenic cytokines (24), we examined the possible regulation of CD13/APN and another ecto-peptidase, CD10/NEP, by interleukin-4 stimulation. Figure 2A shows that interleukin-4 markedly enhanced APN activity on hGF after 2 days stimulation. This response showed a time-dependent increase and reached a maximum at around day 2–4 (data not shown). However, it exhibited only a slight effect on NEP activity. Next, we examined how CD13 expression was regulated on hGF in response to T cell-derived cytokines, including interleukin-4. hGF were stimulated by various doses of interleukin-2, interleukin-4, interleukin-13 or interleukin-15 for 2 days and then stained with anti-CD13 mAb for flow cytometry. Figure 2(B) shows that CD13 expression was significantly increased upon stimulation by interleukin-4 or interleukin-13 in a dose-dependent manner compared to unstimulated controls. However, stimulation with interleukin-2 or interleukin-15 exhibited no significant changes at any of the concentrations tested. A similar response was obtained with APN enzymatic activity when hGF was stimulated with these cytokines for 2 days (Fig. 2C).

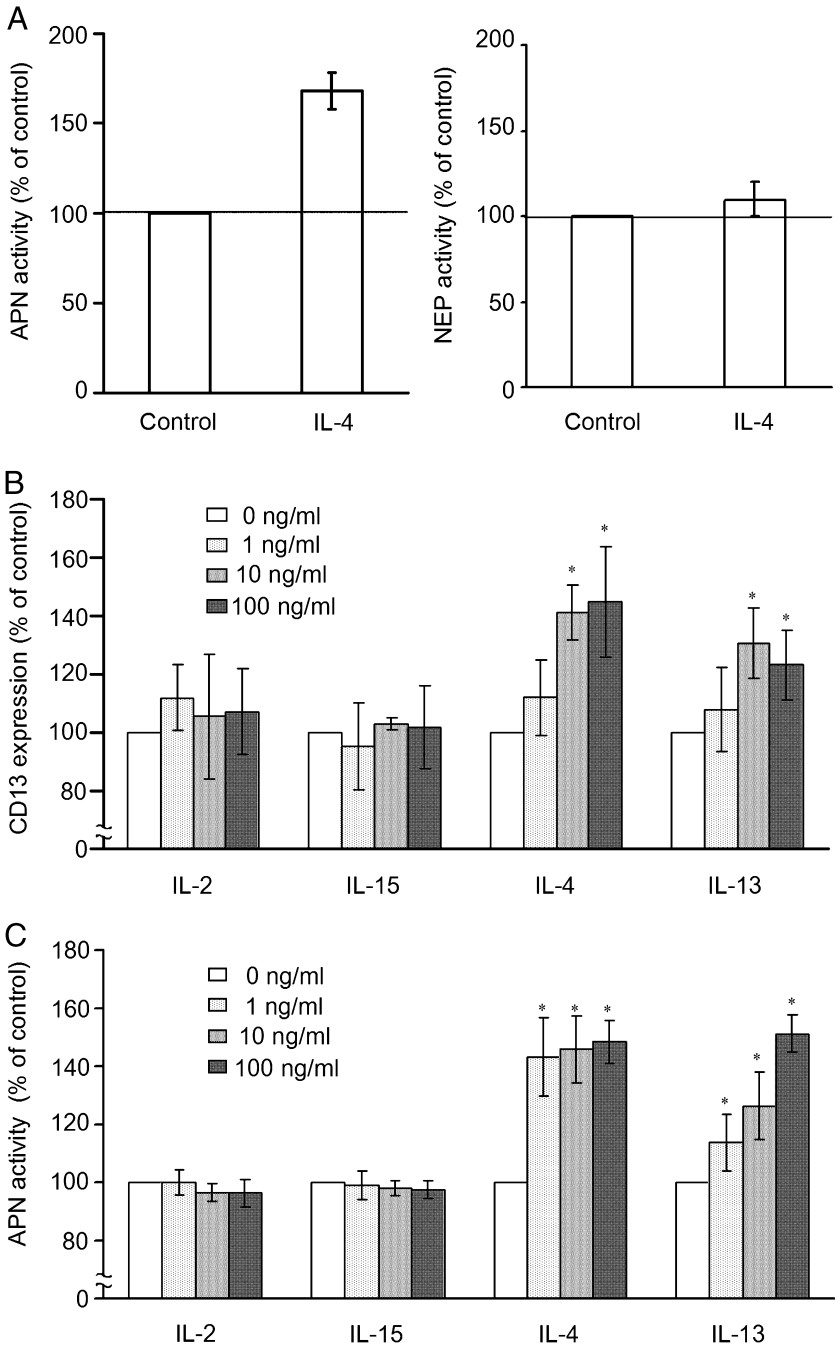


*Fig. 2.* Induction of CD13/aminopeptidase N (APN) on the cell surface of human gingival fibroblasts (hGF) in response to various cytokines. Confluent hGF was stimulated with 100 ng/ml interleukin (IL)-4 (A) or the indicated concentration of IL-2, IL-15, IL-4 or IL-13 for 2 days in α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum (B and C). After washing with phosphate-buffered saline three times, confluent monolayer cells were examined by APN assay (A and C) and neutral endopeptidase (NEP) assay (A). (B) After harvesting with Cell Dissociation Solution®, the cells were stained with anti-CD13 mAb and analyzed by FACS. The findings are shown as the mean ± SE of three donors and statistical significance is shown (*$p < 0.05$ vs. control).

### Expression of interleukin-4Rα chain, interleukin-13Rα1 chain, interleukin-13Rα2 chain, common-γ chain of interleukin-2R (interleukin-2Rγc) and interleukin-2/interleukin-15Rβ chain on hGF

Next, we examined which types of interleukin-4/interleukin-13R would be expressed on hGF by RT-PCR and flow cytometry. As shown in Fig. 3(A), mRNA for the interleukin-4Rα chain, interleukin-13Rα1 chain, interleukin-2Rγc chain, but not the interleukin-13Rα2 chain, was expressed,

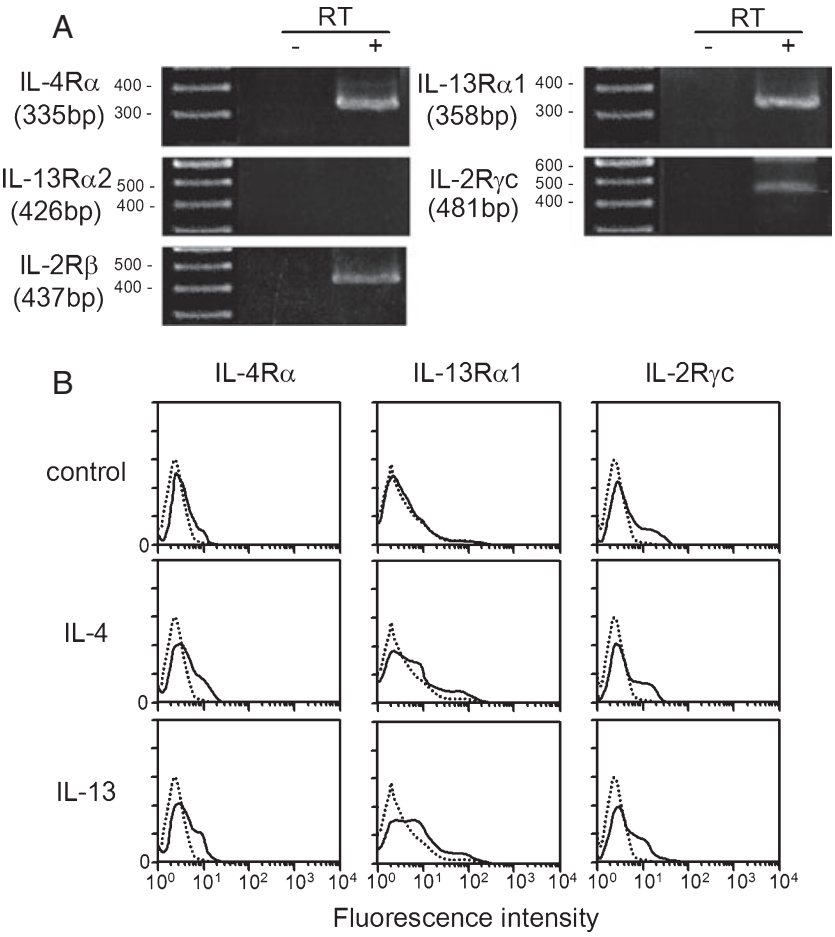


*Fig. 3.* Expression of cytokine receptors on human gingival fibroblasts (hGF). (A) Total RNA was extracted from confluent monolayer cells. Expression of interleukin (IL)-4Rα, IL-13Rα1, IL-13Rα2, IL-2Rγc, and IL-2/IL-15Rβ mRNA were assessed by reverse transcriptase–polymerase chain reaction (RT-PCR) (+). RT was omitted from the RT–PCR reaction for the negative control (–). (B) Confluent hGF was stimulated with 10 ng/ml of IL-4 and IL-13 for 2 days in α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum. After being harvested, cells were stained with anti-IL-4Rα, IL-13Rα1, and IL-2Rγc mAb and analyzed by FACS. Findings from (A) and (B) are representative of three independent experiments.

indicating that hGF expressed the interleukin-4R/interleukin-13R which likely utilized three components. Furthermore, mRNA for the interleukin-2/interleukin-15Rβ chain was also expressed on hGF, although neither interleukin-2 nor interleukin-15 induced CD13/APN expression. Flow cytometric analysis revealed that unstimulated confluent hGF expressed little interleukin-4Rα or interleukin-13Rα1 chains, which were, however, enhanced upon stimulation by interleukin-4 and interleukin-13. Expression of the interleukin-13Rα1 chain was hardly detected on unstimulated confluent hGF, and was induced slightly. Expression of the interleukin-2Rγc chain was observed on both unstimulated and stimulated hGF at almost the same level (Fig. 3B).

### Contribution of CD13/APN on hGF to inhibition of FMLP-induced PMN-activation

As FMLP is one of the substrates for APN and a stimulator for PMN, we examined whether CD13/APN on hGF could affect the FMLP-induced PMN-activation. FMLP was co-incubated with various cell numbers of fixed hGF for 1 h, then used for stimulation of PMN. After 30-min stimulation, Mac-1 expression of PMN was analyzed by flow cytometry. In order to exclude the possible interaction between PMN and soluble factor(s) that might be released from hGF, we used paraformaldehyde-fixed hGF, in which APN activity was unchanged by the fixation procedure (data not shown). As shown in Fig. 4(A), 100 nM but not 10 nM of FMLP co-incubated with hGF lost Mac-1-inducing activity in a cell-concentration-dependent manner compared with intact FMLP, and a significant inhibitory effect was observed at $5 \times 10^4$ hGF, which corresponded to a ratio of 1 : 20 (hGF : PMN). This inhibitory effect on Mac-1 expression was partially (34.7%) restored by pretreatment of hGF with the APN inhibitor bestatin, indicating that this inhibitory effect was partially dependent on CD13/APN (Fig. 4B, left). This inhibitory effect, however, was not enhanced by using interleukin-4-stimulated hGF instead of unstimulated hGF (Fig. 4B, right).

## Discussion

Fibroblasts are considered a rich source of ectoenzymes (1, 25, 26) including CD10/NEP (25–27), CD13/APN (25, 26, 28), CD73/5′-nucleotidase (29, 30), and CD26/dipeptidyl-peptidase IV (20, 25, 26). In the present study, we first demonstrated that hGF constitutively expressed CD13/APN at the protein and enzymatic activity levels (Fig. 1). CD13/APN is known to hydrolyze various substrates, such as FMLP (6) and interleukin-8 (8), both of which are chemotactic and activating factors for PMN. Substance P has been reported to be another substrate (26), and leads to vasodilatation, increased microvascular permeability and plasma extravasation, resulting in neurogenic inflammation (31), and has been detected in gingival connective tissue and gingival crevicular fluid in periodontitis (32, 33). These observations have suggested that CD13/APN has anti-inflammatory properties. In this study, we showed that CD13/APN contributed to the inhibition of FMLP-induced PMN activation (Fig. 4). Accordingly, CD13/APN on hGF could contribute to the anti-inflamma-

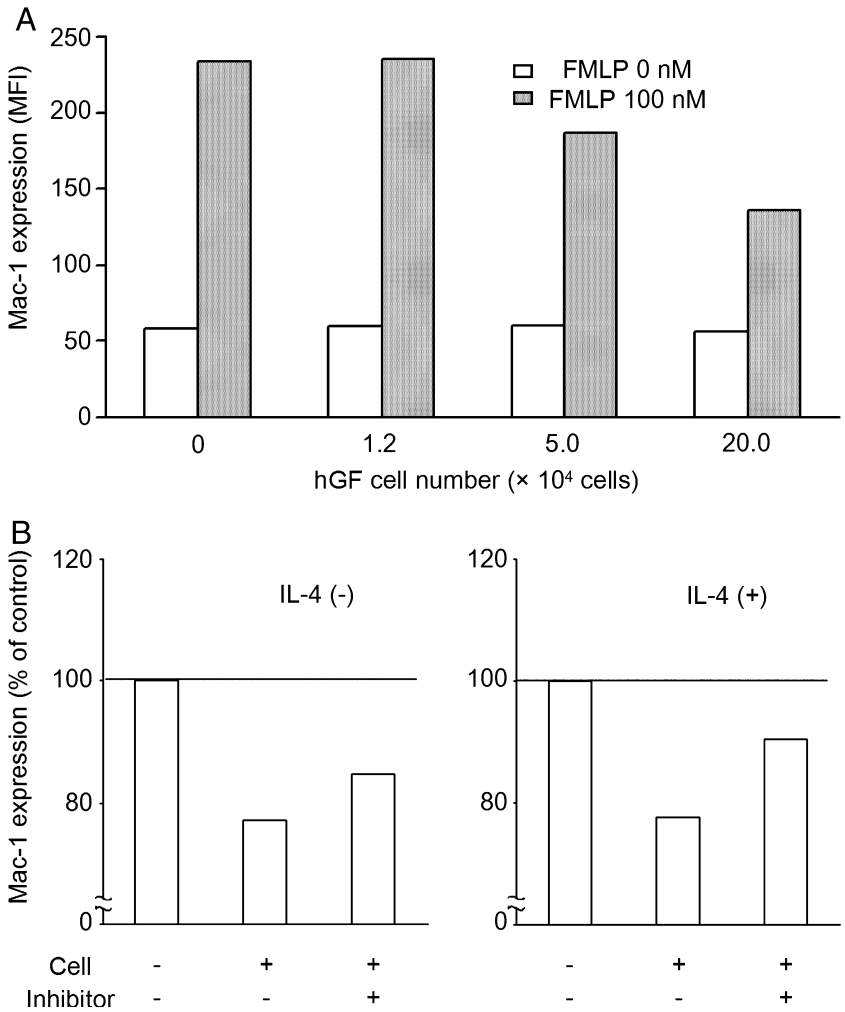


*Fig. 4.* Inhibitory effect of human gingival fibroblasts (hGF) for formyl-methionyl-leucyl-phenylalanine (FMLP)-induced Mac-1 expression on polymorphonuclear leukocyte (PMN). (A) The hGF were fixed with 1% paraformaldehyde in phosphate-buffered saline for 5 min at room temperature and washed three times. The indicated concentration of FMLP was co-incubated with the indicated numbers of fixed hGF cells for 30 min at 37°C in a total volume of 1 ml of RPMI1640, and then the supernatants were harvested by centrifugation. (B) The hGF was stimulated with or without 10 ng/ml interleukin-4 (IL-4) for 2 days before fixation. Then 100 nM of FMLP (total volume of 1 ml) was co-incubated for 30 min at 37°C with $5 \times 10^4$ fixed hGF, which had been pre-treated with or without 1 mM bestatin for 15 min at room temperature. (A and B) PMN ($1 \times 10^6$) from peripheral blood was incubated with 1 ml of the supernatant for 30 min at 37°C, followed by staining with anti-Mac-1 mAb and analyzed by FACS. Findings are representative of three independent experiments.

tory response in the inflamed region of the periodontal tissue. Although interleukin-4 enhanced the expression of CD13/APN (Fig. 2), interleukin-4-stimulated hGF did not enhance the inhibitory activity (Fig. 4B). This observation might be explained by the fact that other peptidases could limit this response, as FMLP is hydrolyzed by APN in combination with other peptidases such as CD10/NEP (6) which was rarely enhanced by interleukin-4 (Fig. 2A).

CD13/APN has been reported to be up-regulated by interleukin-4 in various types of cells, such as renal tubular epithelial cells (34), alveolar macrophages and endothelial cells (35), and recently dermal fibroblasts (36), and by interleukin-13 in renal tubular epithelial cells (34). Here, we first demonstrated that CD13/APN activity on hGF was up-regulated upon stimulation of interleukin-4 and interleukin-13. interleukin-4 and interleukin-13 are Th2-type cytokines, and both cytokines share their receptor and signaling pathways, giving them similar biological properties. interleukin-4 has anti-inflammatory properties such as inhibition of production of superoxide (37), prostaglandin $E_2$, interleukin-1β, tumor necrosis factor-α (38, 39), interleukin-8 (40) by monocytes, and interleukin-4 and interleukin-13 have suppressive effects on COX1 and COX2 gene expression in fibroblasts (41). Therefore, the up-regulation of CD13/APN expression by interleukin-4 and interleukin-13 can be a part of the anti-inflammatory mechanisms of interleukin-4/interleukin-13.

The molecular structure of the interleukin-4/interleukin-13 receptor appears to be diverse, with components including interleukin-4Rα, interleukin-13Rα1, interleukin-13Rα2, and interleukin-2Rγc (23, 42, 43). It has been suggested that the interleukin-4R, in lymphoid cells, is a heterodimer composed of the interleukin-4Rα and the interleukin-2Rγc chains. By contrast, in non-lymphoid cells, another interleukin-4R has been characterized to be composed of interleukin-4Rα chain and either the interleukin-13Rα1 or interleukin-13Rα2 chains (43). The existence of a heterotrimeric receptor (interleukin-4Rα/interleukin-13Rα/interleukin-2Rγc) in fibroblasts has also been reported (24). We detected mRNAs of interleukin-4Rα, interleukin-2Rγc, and interleukin-13Rα1 in hGF (Fig. 3), suggesting the possibility that the interleukin-4/interleukin-13R complex on hGF likely consists of three components. However, considering that hGF were suggested not to be a homogeneous population and to exist as subsets of cells (13, 44), it cannot be clarified if these three subunits were expressed on identical cells. Regarding the protein levels of interleukin-4Rα and interleukin-13Rα1, these were hardly detected in flow cytometry in unstimulated hGF. However, stimulation of hGF with interleukin-4 or interleukin-13 induced these proteins on the cell surface, suggesting that hGF could have an amplification

mechanism for interleukin-4/interleukin-13 stimulation.

The relative contribution of Th1 and Th2 cells in periodontitis has been widely studied (14–16). Current theories on T cell involvement state that Th1 cells are tightly localized at sites undergoing an active disease process, whereas the Th2 cells are widely distributed throughout the tissue and represent a more quiescent stage of the disease (17). In the present study, CD13/APN was up-regulated by Th2-cytokine, interleukin-4 and interleukin-13, but not Th1 cytokine, interleukin-2, or interleukin-15, which share the same receptor transducer component (45). We previously reported that hGF also expressed interleukin-2/interleukin-15R, and the binding of these cytokines resulted in the induction of intercellular adhesion molecule-1 expression on hGF (46), indicating enhancement of cell-mediated immunity. Taken together, our findings demonstrate that hGF actively responds to both Th1 and Th2 cytokines and function as specific effector cells, respectively. This may be consistent with the observation that the disease phenotype could be characterized by either Th1 or Th2 cytokines.

The present findings may provide an additional viewpoint to help clarify the mechanism of onset and development of inflammation involved in hGF.

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