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Expression of CD73/ecto-5′-nucleotidase on human gingival fibroblasts and contribution to the inhibition of interleukin-1α-induced granulocyte-macrophage colony stimulating factor production

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Background and objectives: CD73/5′-nucleotidase (5′-NT) is an ectoenzyme that participates in immune/inflammatory reactions. We examined the possible expression of CD73/5′-NT on human gingival fibroblasts (hGF), which are important to the immune/inflammatory system in periodontal tissue.

Methods and results: We demonstrated that CD73/5′-NT was expressed on hGF by flow cytometry. We found that pre-treatment of hGF with 5′-AMP induced marked inhibition of granulocyte-macrophage colony-stimulating factor (GM-CSF) production from hGF upon stimulation with interleukin-1α (IL-1α) by enzyme-linked immunosorbent assay (ELISA). A specific inhibitor of 5′-NT, adenosine 5′-[α,β-methylene] diphosphate blocked the inhibition of GM-CSF production, suggesting that adenosine converted from 5′-AMP acts on the inhibitory effects. The GM-CSF inhibition suggested that A3 receptor might be involved. The rank order of agonists was found to be (N^6-benzyl-5′-*N*-ethylcarboxamidoadenosine) A3 receptor agonist ≥ (2-chloroadenosine) non-selective agonist > (CGS-21680) $A2_A$ receptor agonist > adenosine ≥ (N^6-cyclohexyladenosine) A1 agonist. Further support for the main role of A3 receptor was the binding A3 antagonist [9-chloro-2-(2-furanyl)-5-([phenylacetyl]amino)[1,2,4]-triazolo[1,5-*c*]quinazdine] reversed the effect of adenosine, but no significant reverse was observed by A1 (1,3-dipropyl-8-cyclopentylxanthine), A2 [3,7-dimethyl-1-(2-propargyl)xanthine], $A2_A$ [8-(3-chlorostyryl)caffeine], and $A2_B$ (alloxazine) antagonists. The CD73/5′-NT expression was increased upon stimulation with gamma-interferon, but not other stimulants such as tumor necrosis factor-alpha, IL-4, lipopolysaccharide from *Porphyromonas gingivalis* and *Escherichia coli*, and fimbriae from *P. gingivalis*, and this increase was correlated with the enhanced GM-CSF inhibition by 5′-AMP but not adenosine.

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Conclusions: These findings suggested that CD73/5′-NT on hGF exerts an anti-inflammatory effects in periodontal disease by conversion from 5′-AMP to adenosine.

CD73/5′-nucleotidase (5′-NT) is a glycosyl phosphatidylinositol (GPI)-anchored purine salvage enzyme (EC 3.1.3.5), which catalyzes the dephosphorylation of purine and pyrimidine ribo- and deoxyribonucleoside monophosphates to the corresponding nucleosides (1). Although 5′-NT hydrolyzes a variety of nucleoside 5′-monophosphates, it has greatest affinity for 5′-AMP with K_m values in the micromolar range (1). In normal tissue, CD73 has been detected on subsets of T cells, B cells, monocytes, germinal center follicular dendritic cells, umbilical vein endothelial cells, intestinal epithelial cells and reticular fibroblasts (2–6). It was reported that CD73/5′-NT participates in adenine nucleotide metabolism on the surface on endothelial cells (2, 4, 7) and epithelial cells (3, 6, 8). 5′-AMP, which is known to be released from activated neutrophils (2, 3), is rapidly converted to adenosine via CD73/5′-NT on the cell surface, and adenosine released into the extracellular space directly activates adenosine receptors as a paracrine mediator. These pathways act on promotion of endothelial (2, 4) and epithelial (8) barrier functions, inhibition of platelet aggregations (7), and stimulation of epithelial electrogenic chloride secretion (3, 6). Moreover, the anti-inflammatory properties of methotrexate and sulfasalazine are mediated by adenosine release and require ect-5′-nucleotidase-mediated conversion of adenine nucleotides (9). Studies of ligation of adenosine receptors by adenosine have revealed that adenosine possesses potent anti-inflammatory properties such as inhibition of pro-inflammatory cytokine release (10–12), inhibition of neutrophil adherence to endothelial cells (13), inhibition of T cell adherence to fibroblasts (14), neutrophil degranulation (15) and enhancement of anti-inflammatory cytokine, interleukin (IL)-10 (16). CD73 also functions as a co-stimulatory molecule in human T cells (5) and adhesion molecules in lymphocyte-endothelial cells binding (5).

Fibroblasts were previously considered important connective tissue cells that construct a supporting framework crucial for tissue integrity and repair (17). Recently, fibroblasts have been suggested to be important sentinel cells in the immune system (18). Fibroblasts actively define the structure of tissue microenvironments and regulate infiltrated hematopoietic cell functions by production of cytokines/chemokines (17, 18) in which systemic granulocyte-macrophage colony stimulating factor (GM-CSF) is an important cytokine with an ability to augment many of the functions of granulocyte, monocyte/macrophage and dendritic cells to strengthen host defenses against a broad spectrum of invading organisms (19). Furthermore, fibroblasts are known to express various ectoenzymes (20, 21), such as CD10/neutral endopeptidase, CD13/animopeptidase N, and CD26/dipeptidylpeptidase IV, all of which play essential roles in the regulation of immune/inflammatory functions (22).

Those observations led us to investigate the possible expression of CD73/5′-NT on fibroblast and whether this molecule contributes to fibroblasts functions in the inflammatory and immune systems in periodontal tissue. In this study, we first found that GPI-anchored CD73/5′-NT was expressed on not only human gingival fibroblasts (hGF) but also dermal and lung fibroblasts, and CD73/5′-NT was involved in not only the inhibition of IL-1α-induced GM-CSF production from 5′-AMP pre-incubated hGF but also in the regulation of other cytokine/chemokine production. We further investigated the mechanism of the inhibition of GM-CSF production by 5′-AMP and the possible regulation of CD73/5′-NT expression on hGF in response to inflammatory cytokines and cell surface components from periodontal disease-associated bacteria.

Materials and methods

Reagent

Phorbol 12-myristate 13-acetate, Cell Dissociation Solution® (non-enzymatic) and *Bacillus cereus* phosphatidylinositol-specific phospholipase C (PI-PLC), 5′-AMP, adenosine, adenosine 5′-[α, β-methylene] diphosphate (APCP) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2-Chloroadenosine (6-amino-2-chloropurine riboside), N^6-benzyl-5′-*N*-ethylcarboxamidoadenosine (N^6-benzyl-NECA), 2-*p*-(2-carboxyethyl)phenethylamino-5′*N*-ethylcarboxamidoadenosine (CGS-21680), N^6-cyclohexyladenosine, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, A1 antagonist), 3,7-dimethyl-1-(2-propargyl)xanthine (DMPX, A2 antagonist), 8-(3-chlorostyryl)caffeine ($A2_A$ antagonist), alloxazine ($A2_B$ antagonist), and 9-chloro-2-(2-furanyl)-5-([phenylacetyl]amino)[1,2,4]-triazolo[1,5-c]quinazdine (MRS1220, A3 antagonist), and dipyridamole (adenosine transport inhibitor) were purchased from Research Biochemicals International (Natick, MA, USA). Anti-CD73 monoclonal antibody (mAb) (AD2, mouse IgG1) was purchased from Pharmingen (San Diego, CA, USA). Isotype control mAb (mouse IgG1) was purchased from Immunotech, a Beckman Coulter Company (Marseille, France). α-Minimum essential medium (MEM) and 0.25% trypsin-1 mM EDTA were from Gibco BRL (Rockville, MD). Recombinant human (rh) IL-4, rhIL-1α, rh gamma-interferon (IFN-γ) and rh tumor necrosis factor-alpha (TNF-α) were purchased from Pepro Tech EC Ltd (London, UK).

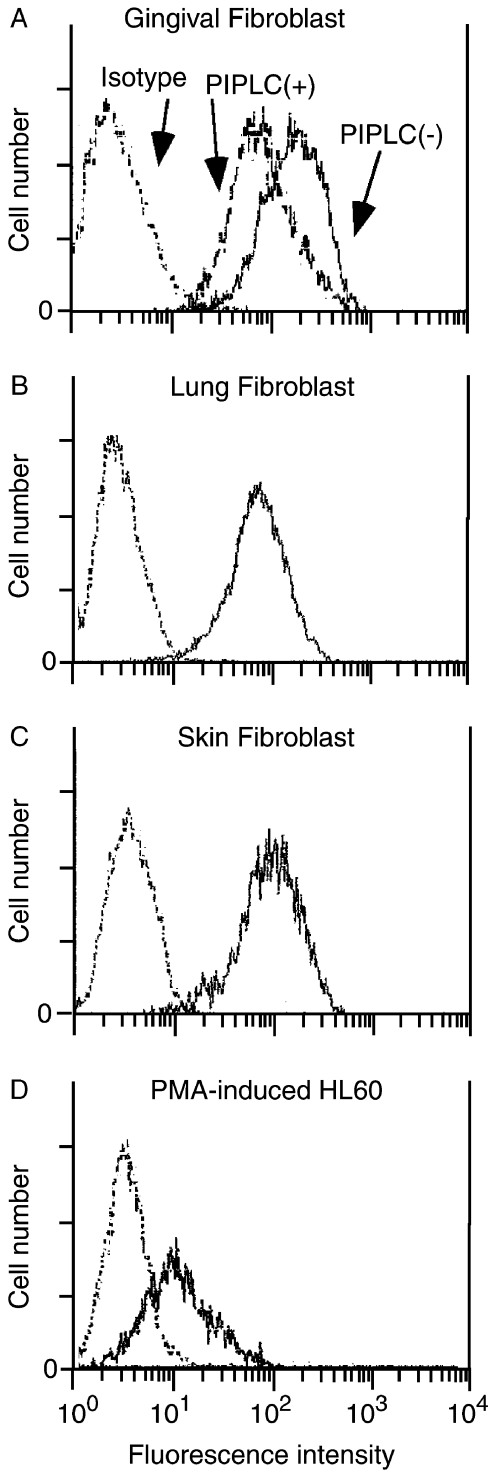

Fig. 1. Expression of CD73 on human normal fibroblasts of various tissues. Fibroblasts derived from gingival (A), lung (B) and skin (C) were collected from confluent monolayers using Cell Dissociation Solution® and Monocytic differentiated HL-60 (D) was collected after stimulation with phorbol 12-myristate 13-acetate for 3 d. hGF collected from confluent monolayers were incubated in α-MEM with or without of 5 U/ml of PI-PLC for 60 min at 37°C (A). CD73 on the cell surface were assessed by flow cytometry as described in Materials and Methods. Isotype-matched antibody was used as the negative control (broken line). Findings are representative of three independent experiments with three different donors.

Cells

hGF was prepared from the explants of normal gingiva from 18- to 40-year-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-MEM supplemented with 10% fetal bovine serum (Flow Laboratories, McLean, VA, USA) with a medium change every 3 d for 10–15 d until confluent cell monolayers were formed. The cells were detached with 0.25% trypsin–1 mM 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. Human skin fibroblasts and human lung fibroblasts were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, USA). These fibroblasts were maintained in α-MEM supplemented with 10% fetal bovine serum. HL-60 cells were maintained in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) with 10% fetal bovine serum, 2 mM glutamine and antibiotics. Monocytic differentiation of HL-60 cells was initiated by the addition of phorbol 12-myristate 13-acetate (final concentration; 10 ng/ml) to the culture medium and cultured for 3 d (23).

PI-PLC treatment

A total of 10^5 hGF was incubated for 60 min at 37°C in 200 μl of α-MEM with or without of 5 U/ml of PI-PLC in microcentrifuge tubes, and then washed two times with phosphate-buffered saline.

Preparations of bacterial components

Lipopolysaccharide was prepared from *Porphyromonas gingivalis* 381 by the hot phenol–water extraction method as described previously (24). Hot

phenol–water-extracted lipopolysaccharide from *Escherichia coli* (O127: B8) was purchased from Sigma Chemical Co. Fimbriae prepared from *P. gingivalis* 381 (25) were generously supplied by T. Ogawa (Asahi University Dental School, Gifu, Japan).

Stimulation of hGF

The hGF monolayer in 24-well multiplates was stimulated in α-MEM with 5% fetal bovine serum with IFN-γ (100 U/ml), TNF-α (100 ng/ml), IL-4 (100 U/ml), *P. gingivalis* lipopolysaccharide (1 μg/ml), *P. gingivalis* fimbriae (1 μg/ml), or *E. coli* lipopolysaccharide (1 μg/ml) for 3 d.

Fluorescence-activated cell sorting (FACS)

hGF in 24-well multiplates were collected using Cell Dissociation Solution®, washed with phosphate-buffered saline three times, and used for staining. A total of 10^5 hGF were stained with each mAb or isotype-matched control IgG at 4°C for 20 min. Following washing, fluorescein isothiocyanate-conjugated goat anti-mouse IgG (BioSource International, Camerillo, CA) was added at 4°C for 20 min. Staining was analyzed on a FACScan® (Becton Dickinson, Mountain View, CA). 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 (MFI).

Adenine nucleotide-treatment of hGF

Confluent monolayers of hGF (approximately 2×10^4 cells) in 96-well multiplates were washed with phosphate-buffered saline three times, pre-treated with the indicated concentration of 5′-AMP or adenosine for 10 min at 37°C, followed by addition of IL-1α (final concentration of 10 ng/ml) in 200 μl of α-MEM with 1% fetal bovine serum, which had been inactivated at 64°C for 2 h to destroy 5′-NT activity of fetal bovine serum (26). After stimulation for the indicated time, the supernatants were collected and kept at −20°C until use.

Detection of cytokines using enzyme-linked immunosorbent assay (ELISA)

The level of GM-CSF, IL-8, IL-6 and macrophage chemoattractant protein-1 (MCP-1) in the supernatants was determined using a human GM-CSF, IL-8 and IL-6 ELISA kit (Endogen, Woburn, MA, USA), and OptEIA™ human MCP-1 ELISA kits (PharMingen, San Diego, CA, USA), respectively. The assays were performed precisely as instructed by the ELISA manufacturer. The concentrations of GM-CSF, IL-8, IL-6 and MCP-1 in the supernatants were determined using the Softmax data analysis program (Molecular Devices Co., Menlo Park, CA, USA). Each sample was assayed in triplicate.

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 Wilcoxon signed-ranks test (in Fig. 6A), and p-values less than 0.05 were considered significant.

Results

Expression of CD73 on hGF

CD73 expression on fibroblasts derived from gingiva, skin and lung was investigated by flow cytometry. Since HL-60 differentiated into the macrophage lineage by phorbol 12-myristate 13-acetate expresses CD73 (21), we used this cell line as a positive control and compared the expression of these cells. In hGF tested in this study ($n = 6$), almost 100% of cells from confluent culture expressed CD73 antigen on the cell surface with brighter intensity (MFI 156.4 ± 45.7) compared with that of differentiated HL-60 (MFI 22.5 ± 3.5) (Figs 1A and D). Fibroblasts from skin and lung also expressed CD73 on the cell surface with a degree similar to that of hGF (Figs 1B and C). Since CD73/5′-NT was reported to be the GPI-anchored protein on the plasma membrane of many cell types (1), we examined the sensitivity of CD73 expressed on hGF to PI-PLC treatment. hGF was treated with 5 U/ml of PI-PLC for 1 h and analyzed by flow cytometry for the expression of CD73. Figure 1(A) shows that PI-PLC treatment induced a 46.6% decrease in the expression of CD73 compared with untreated hGF

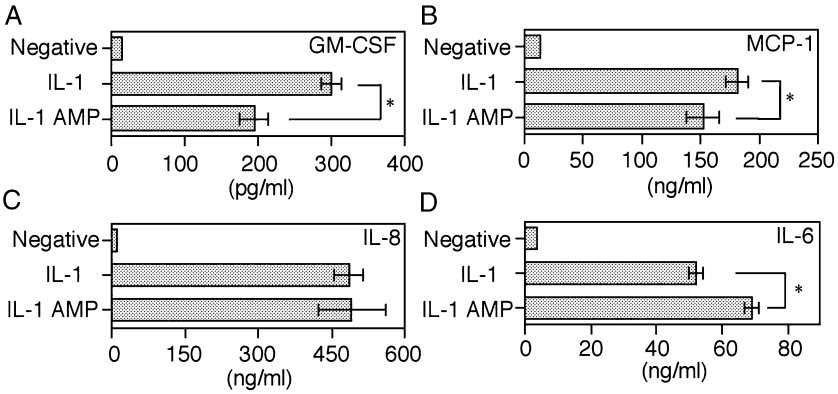

Fig. 2. 5′-AMP regulates IL-1α-induced cytokines/chemokines production from hGF. The hGF monolayer was pre-treated with 100 μM of 5′-AMP for 10 min at 37°C before stimulation with 10 ng/ml IL-1α for 12 h at 37°C. The amounts of GM-CSF (A), MCP-1 (B), IL-8 (C) and IL-6 (D) in the supernatants were analyzed by ELISA. Representative findings of four independent experiments with four different donors are shown as the mean ± SE of triplicate assays. Statistical significance is shown (*$p < 0.05$ vs. respective control).

[MFI 104.4 (PI-PLC), 195.5 (untreated)], suggesting that GPI anchors of CD73 is partially sensitive to PI-PLC hydrolysis.

5′-AMP influences IL-1α-induced cytokine/chemokine production from hGF

CD73/5′-NT converts extracellular 5′-AMP to adenosine, which acts in a variety of physiological signaling processes (2, 3, 6), and hGF releases various pro-inflammatory cytokines/chemokines such as GM-CSF upon stimulation with IL-1α (27). Therefore, we examined whether the addition of 5′-AMP to hGF culture could alter IL-1α-induced GM-CSF production. Confluent monolayer hGF was pre-treated with 100 μM of 5′-AMP, then stimulated with IL-1α for 12 h, and the amount of GM-CSF in the supernatants were determined. Figure 2(A) shows that the pre-treatment of hGF with 5′-AMP exhibited marked inhibition of IL-1α-induced GM-CSF production at approximately 35.1%. Furthermore, we tested the effect of 5′-AMP on other cytokines/chemokines production from IL-1α-stimulated hGF. As shown in Figs 2(B) and (C), the production of MCP-1 was also inhibited by 16.0%, but IL-8 production was not significantly ($p < 0.05$) changed. In contrast, a significantly enhanced production (31.5%) was observed in IL-6 (Fig. 2D). These results suggest that 5′-AMP not only inhibited GM-CSF production but also influenced MCP-1 and IL-6 production negatively and positively, respectively.

Inhibition of ecto 5′-NT attenuates the inhibition of IL-1α-induced GM-CSF production by 5′-AMP pre-treatment

Since it is assumed that adenosine converted from 5′-AMP by CD73/5′-NT on hGF could act as a local messenger, we examined whether the 5′-NT specific inhibitor, APCP, abrogates the inhibition of IL-1α-induced GM-CSF production by 5′-AMP pre-treatment. Figure 3(A) shows that addition of 1000 μM of APCP before 5′-AMP pre-treatment exhibited an approximate 90% recovery for the inhibition. However, addition of 1000 μM of APCP did not exhibit significant change in the adenosine-mediated inhibition (Fig. 3B). These results suggest that the inhibition of GM-CSF production by 5′-AMP pre-treatment is involved in CD73/5′-NT activity and adenosine converted from 5′-AMP by CD73/5′-NT can act as a local messenger. Next, we attempted to confirm whether adenosine induces the inhibitory effects similar to 5′-AMP. As shown in Figs 4(A) and (B), the concentration kinetics experiments revealed that significant ($p < 0.05$) inhibition of GM-CSF production was observed in 100 μM of

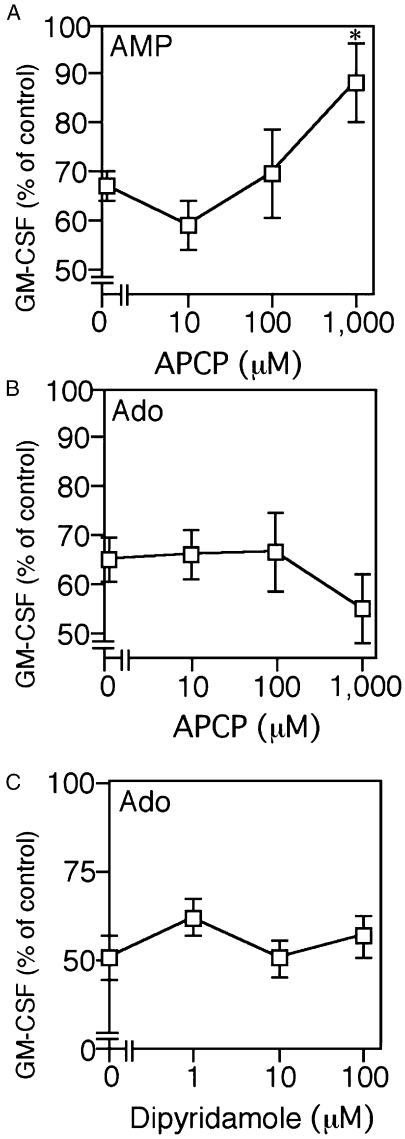

Fig. 3. Inhibition of ecto 5′-NT attenuates the inhibition of IL-1α-induced GM-CSF production by 5′-AMP pre-treatment. (A and B) The hGF monolayer was pre-treated with the indicated concentration of APCP for 15 min at 37°C followed by incubation with 100 μM of 5′-AMP or adenosine for 10 min at 37°C, and then stimulated with 10 ng/ml IL-1α for 12 h, (C) The hGF monolayer was pre-treated with the indicated concentration of dipyridamole for 10 min followed by incubation with 100 μM adenosine for 10 min at 37°C, and then stimulated with 10 ng/ml IL-1α for 12 h at 37°C. The amount of GM-CSF in the supernatants was analyzed by ELISA. Representative findings of three independent experiments with three different donors are shown as the mean ± SE of triplicate assays. Statistical significance is shown (*$p < 0.05$ vs. without inhibitor).

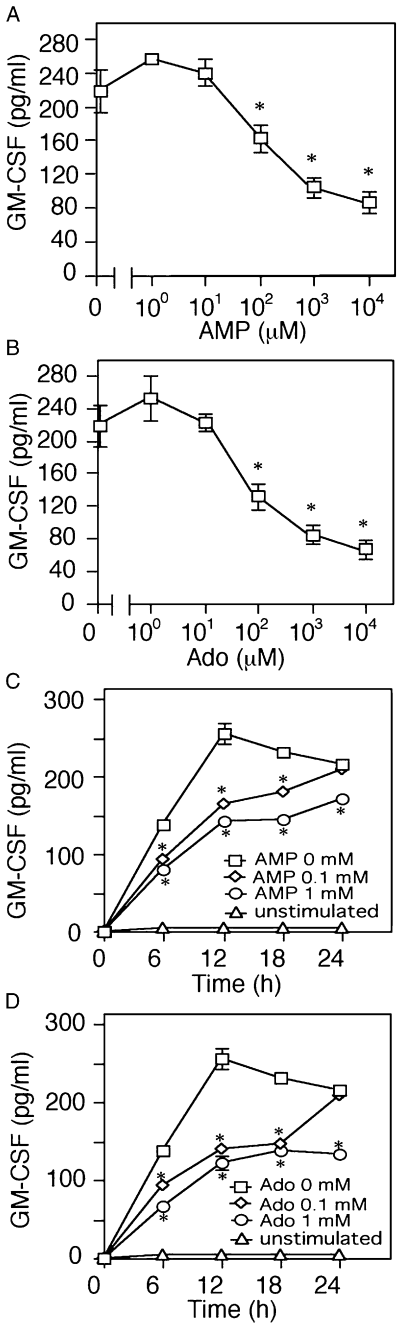

Fig. 4. Concentration- and time-dependent inhibition of GM-CSF production by 5′-AMP or adenosine pre-treatment. The hGF monolayer was pre-treated with the indicated concentration (A and B) or 100 μM (C and D) of 5′-AMP or adenosine for 10 min at 37°C before stimulation with 10 ng/ml IL-1α for 12 h (A and B) or the indicated time (C and D) at 37°C. The amount of GM-CSF in the supernatants was analyzed by ELISA. Representative findings of five independent experiments with five different donors are shown as the mean ± SE of triplicate assays. Statistical significance is shown (*$p < 0.05$ vs. respective control). Ado, adenosine.

both 5′-AMP and adenosine. Slight enhancement of the production was observed in 1 μM of both 5′-AMP and adenosine, but not at a significant ($p < 0.05$) level. Adenosine exerted a somewhat more potent inhibitory effect on GM-CSF production compared with equimolar concentrations of 5′-AMP, but significance ($p < 0.05$) was not reached (Figs 4A and B). From the response curves of 5′-AMP and adenosine from five independent experiments, IC_{50} (i.e. the concentration of adenosine or 5′-AMP producing 50% of maximal inhibition) was calculated to range between 100 and 200 μM. In time kinetics experiments shown in Figs 4(C) and (D), GM-CSF production induced by IL-1α reached maximum at 12 h, and a marked inhibition of GM-CSF production was observed at 6 and 12 h in both 5′-AMP and adenosine.

Adenosine and receptor-specific adenosine analogues inhibit IL-1α-induced GM-CSF production

It is most likely that adenosine converted from 5′-AMP by CD73/5′-NT acts on the specific adenosine receptors for the inhibitory effects, so we compared the inhibitory capacity of adenosine with that of analogues. N^6-cyclohexyladenosine and N^6-benzyl-NECA are selective A1 and A3 receptor agonists, respectively. CGS-21680 is a specific $A2_A$ receptor agonist (28) and 2-chloroadenosine is a non-selective agonist that binds to A1 and A3 receptor (12, 29) and also has affinity for A2 receptor at high micromolar concentrations. As shown in Fig. 5(A), 2-chloroadenosine and N^6-benzyl-NECA exert a much stronger inhibitory effect than adenosine on IL-1α-induced GM-CSF production. The inhibitory effect of 2-chloroadenosine on 100 μM was less than 100 μM of N^6-benzyl-NECA, though still stronger than adenosine. CGS-21680 showed a significant inhibitory effect only at a 1-μM concentration, but no additional increase in inhibitory effect was observed even with increasing concentrations and there was no significant difference with adenosine at a 100-μM concentration. N^6-cyclohexyladenosine was less effective than 2-chloroadenosine, N^6-benzyl-NECA and CGS-21680 for the inhibition and showed no significant difference than that of equimolar concentrations of adenosine. These findings suggested that the mechanism of GM-CSF inhibition might involve A3 receptor and also only partially $A2_A$ receptor activation. To confirm these observations,

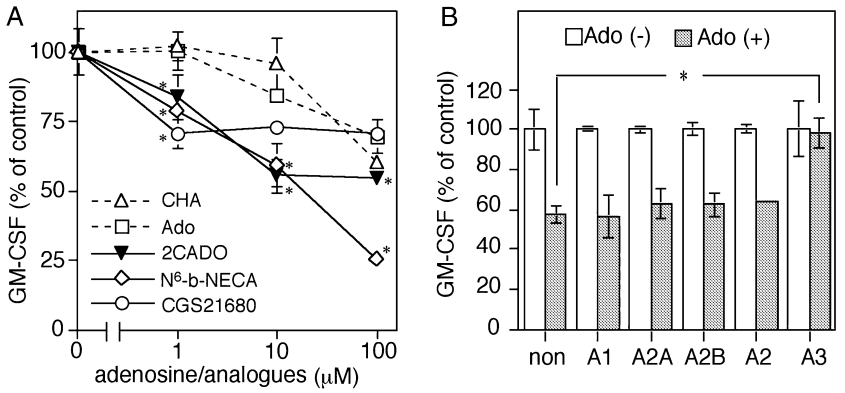

Fig. 5. Adenosine and receptor-specific adenosine analogues inhibit IL-1α-induced GM-CSF production. (A) The hGF monolayer was pre-treated with the indicated concentration of either adenosine (Ado), 2-chloroadenosine (2CADO), CGS-21680, N^6-cyclohexyladenosine (CHA) or N^6-benzyl-NECA (N^6-b-NECA) for 10 min at 37°C before stimulation with 10 ng/ml IL-1α for 12 h at 37°C. The amount of GM-CSF in the supernatants was analyzed by ELISA. GM-CSF level from control was 421.4 ± 33.7 pg/ml. (B) The hGF monolayer was pre-treated with/without 10 μM of either A1 antagonist DPCPX, A2 antagonist DMPX, $A2_A$ antagonist 8-(3-chlorostyryl)caffeine, $A2_B$ antagonist alloxazine and A3 antagonist MRS1220 for 10 min at 37°C followed by treatment with 100 μM of adenosine for 10 min, then stimulated with 10 ng/ml IL-1α for 12 h at 37°C. The amount of GM-CSF in the supernatants was analyzed by ELISA. Representative findings of four independent experiments with four different donors are shown as the mean ± SE of triplicate assays. Statistical significance is shown (*$p < 0.05$ vs. adenosine (A) and vs. no antagonist (B), respectively).

we examined the ability of selective receptor antagonists to block the effect of adenosine. As shown in Fig. 5(B), there was no significant reverse effect of GM-CSF inhibition by pre-treatment with A1 antagonist (DPCPX), $A2_A$ antagonist [8-(3-chlorostyryl)caffeine], $A2_B$ antagonist (alloxazine), and A2 antagonist (DMPX). However, A3 antagonist (MRS1220) reversed the action of adenosine almost completely ($p < 0.05$, compared with adenosine alone). These observations support the agonist findings, but it remains unclear whether adenosine converted on the cell surface may be taken up by hGF for the GM-CSF inhibition. To clarify this, hGF monolayer was pre-treated with an inhibitor of adenosine transport, dipyridamole (30), before the addition of adenosine. As shown in Fig. 3(C), dipyridamole showed no significant effect on inhibition of IL-1α-induced GM-CSF production by adenosine. These results suggested that the inhibition of GM-CSF production resulted from mainly A3 and only partially $A2_A$ receptor activation by adenosine but did not require cellular uptake of adenosine.

IFN-γ influences the expression of CD73/5′-NT on hGF and enhances inhibitory effect of GM-CSF inhibition by 5′-AMP pre-treatment

We examined whether CD73/5′-NT expression might be influenced upon stimulation with various stimulants. The hGF was stimulated with IFN-γ, IL-4, TNF-α, and bacterial components, *P. gingivalis* lipopolysaccharide, *P. gingivalis* fimbriae, and *E. coli* lipopolysaccharide for 3 d and expression of CD73/5′-NT was analyzed by flow cytometry. Figure 6(A) shows that IFN-γ increased the expression of CD73/5′-NT significantly ($p < 0.05$, 178.2 ± 40.9% induction of the expression over the unstimulated control). Other stimulants showed no significant effect on the expression compared with unstimulated hGF. Next, we examined whether the priming of hGF with IFN-γ enhances the GM-CSF inhibition by 5′-AMP pre-treatment. As shown in Fig. 6(B), a significant ($p < 0.05$) inhibition of GM-CSF production from IFN-γ-primed hGF was observed in 10 μM 5′-AMP pre-treatment at which concentration, no inhibitory effect was observed in unprimed hGF. Furthermore, IFN-γ priming enhanced the inhibitory effect observed in 100 μM 5′-AMP pre-treatment compared with control. However, IFN-γ priming had no significant effects for the inhibition at any concentrations of adenosine pre-incubation (Fig. 6C).

Discussion

In the present study, we showed that GPI-anchored CD73/5′-NT was expressed on hGF and contributed to the influence of IL-1α-induced cytokine/chemokine production. Fibroblasts are not a homogeneous population, but can differ in morphology, phenotype, such as CD14 (31) and CD26 (20), and function between and even within tissue types (32). With regard to CD73/5′-NT, we showed that all fibroblasts derived from skin, lung and gingiva express CD73/5′-NT by flow cytometry, suggesting that this regulatory mechanism exists on various tissues where fibroblasts are involved in inflammatory response.

GM-CSF is an important pro-inflammatory cytokine for host defenses, which activates not only granulocytes and macrophages to enhance many functions, but also dendritic cells to generate antigen-specific T cell response (19, 33). On the other hand, chronic cascade of inflammation triggered by GM-CSF can exist (19) with availability of increased numbers of inflammatory granulocytes and macrophages by GM-CSF-dependent enhanced survival and even local proliferation in macrophages. Therefore, the inhibition of GM-CSF production from hGF might be a negative-feedback mechanism for inflammatory process. MCP-1, a chemoattractant for monocytes but also for activated T cell, NK cells, and basophils (34), was also significantly inhibited, although percentage inhibition was lower than GM-CSF, indicating a partial negative-feedback mechanism. In contrast to GM-CSF and MCP-1, IL-6 production was significantly enhanced by pre-treatment with 5′-AMP, which is consistent with the previous finding that adenosine enhances IL-6 production from hGF stimulated by IL-1β (14).

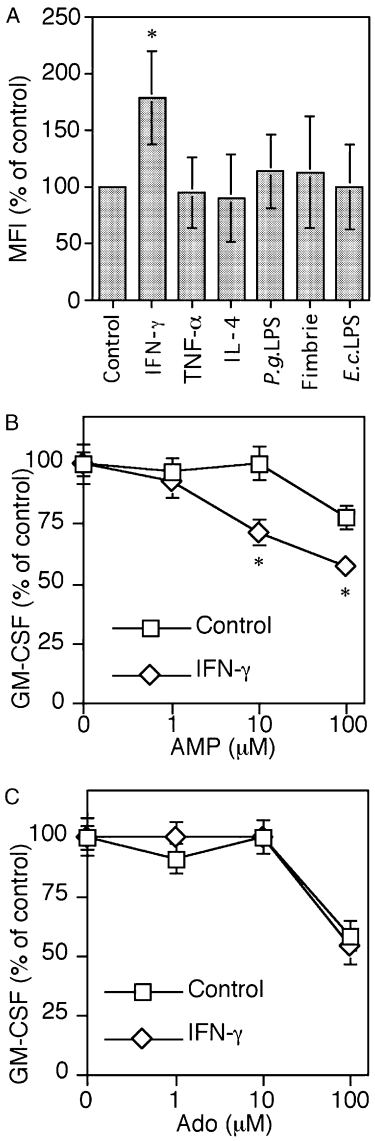

Fig. 6. IFN-γ up-regulates the expression of CD73/5′-NT on hGF. (A) The hGF monolayer in 24-well multiplates was stimulated in α-MEM with 5% fetal bovine serum with IFN-γ (100 U/ml), TNF-α (100 ng/ml), IL-4 (100 U/ml), *P. gingivalis* lipopolysaccharide (*P.g.* LPS, 1 μg/ml), *P. gingivalis* fimbriae (1 μg/ml), or *E. coli* lipopolysaccharide (*E.c.* LPS, 1 μg/ml) for 3 d. After being harvested, cells were stained with anti-CD73 mAb and analyzed by FACS. The results from (A) are shown as MFI (mean fluorescence intensity) ± SE of six donors and statistical significance is shown (*$p < 0.05$ vs. control). (B and C) The hGF monolayer was primed with 100 U/ml of IFN-γ for 3 d, and pre-incubated with the indicated concentration of 5′-AMP or adenosine for 10 min at 37°C followed by stimulation with 10 ng/ml IL-1α for 12 h at 37°C. The amount of GM-CSF in the supernatants was analyzed by ELISA. The GM-CSF levels from control and IFN-γ-primed fibroblasts were 331.0 ± 16.6 pg/ml and 393.5 ± 31.5 pg/ml, respectively. Representative findings of three independent experiments with three different donors are shown as the mean ± SE of triplicate assays. Statistical significance is shown (*$p < 0.05$ vs. respective control).

IL-6 is a pleiotropic cytokine that has both pro- and anti-inflammatory actions. In contrast to the acute phase response in various tissues via its broad pro-inflammatory actions (35), IL-6 was reported to have suppressive effects on fibroblasts (36–38) and macrophages (39), for example by an induction of IL-1 receptor antagonist, suppression of TNF-α, inhibition of proliferation of fibroblasts and induction of protease inhibitor. Consistent with these anti-inflammatory effects, IL-6 reduces inflammation in animal models such as zymosan-induced arthritis (40) and inflammatory lung disease (41). Furthermore, a mutation that partially inhibited IL-6 signal transduction induces development of spontaneous arthritis (36). From these points of views, it is suggested that CD73/5′-NT on hGF exerts an anti-inflammatory effect through its activity to convert 5′-AMP to adenosine.

A series of studies has demonstrated that adenosine exerts potent anti-inflammatory properties, mediated through the occupancy of A2 or A3 receptors in various cell types described in the Introduction (10–16), and also exerts the promotion of wound healing (28) through $A2_A$ receptor. On the other hand, pro-inflammatory properties were also reported that occupancy of A1 receptors enhances neutrophil adherence to endothelium and chemotaxis (13, 42). In the present study, we first demonstrated that the inhibition of GM-CSF production from hGF is mediated mainly via A3 receptor activation and only partially via $A2_A$ receptor activation. The former conclusion is based on the following observations: (i) N^6-benzyl-NECA, a selective A3 receptor agonist, strongly inhibited the GM-CSF production; (ii) 2-chloroadenosine, a non-selective agonist with the great affinity for A1 and A3 receptor (12, 29), was also equally as effective as N^6-benzyl-NECA; (iii) N^6-cyclohexyladenosine, an A1-specific agonist that also can bind to A3 receptor (12), showed a lower inhibition but was equally as effective as adenosine at high concentrations (100 μM), probably due to binding to A3 receptor; (iv) A3 receptor antagonist reversed the effect of adenosine. On the other hand, the antagonist of $A2_A$ receptor showed no significant effect to reverse the inhibition. This was probably because most of the inhibition was caused by A3 receptor activation and $A2_A$ receptor was only partially involved in the inhibition.

It is unclear whether the inhibition of GM-CSF production could occur *in vivo*. Previous studies demonstrated

that neutrophils release 5′-AMP upon activation (3) and supernatant from activated neutrophils contains micromolar concentrations of bioactive 5′-AMP and adenosine (2, 3). Other cell types also release adenosine nucleotides and adenosine, such as endothelial cells (9), eosinophils (43) and platelets (44). As for the local inflammatory site, the number of neutrophils may increase by even 100-fold (45, 46) and neutrophils are capable of adhering to fibroblasts (47), which may create microenvironments, allowing that adenine nucleotides and adenosine could be locally concentrated. Actually, CD73/5′-NT is a critical mediator of methotrexate- and sulfasalazine-induced anti-inflammatory activity *in vitro* and *in vivo* (9) and adenine nucleotides, released from cells, were suggested to be the source of extracellular adenosine (9). Considering these previous findings, it is likely that 5′-AMP or adenine nucleotide released from locally accumulated neutrophils or other types of cells could act on the inhibition of GM-CSF production from hGF at inflammatory sites *in vivo*.

We demonstrated that CD73 expression was increased upon stimulation with IFN-γ, and this increase was correlated with enhanced GM-CSF inhibition by 5′-AMP- but not adenosine pre-treatment. IFN-γ is known to play an important role as a pro-inflammatory molecule (48); however, it was reported to have dual functions (i.e. enhancing and inhibiting inflammatory responses) for fibroblasts via regulation of IL-8 gene expression (49, 50). In human periodontitis gingiva, it was reported that IFN-γ is widespread in the connective tissue and increases with the severity of inflammation (51). Therefore, IFN-γ might exert a counter-inflammatory effect on hGF by increase of CD73 expression. This enhanced inhibition of GM-CSF by IFN-γ is likely to result from the production of marked amounts of adenosine by increased CD73/5′-NT. However, from the standpoint of metabolism of adenosine either through phosphorylation by adenosine kinase or deamination by adenosine deaminase, the net generation of adenosine from 5′-AMP depends on the relative activities of 5′-NT, adenosine deaminase and adenosine kinase (23). Therefore, the exact mechanism of enhanced inhibition by IFN-γ is unspecified at present.

In conclusion, excessive inflammatory responses in which the overproduction of cytokines/chemokines interrupts the smooth transition from acute inflammation to acquired imune responses, should be controlled to maintain the physiological balance in periodontal tissue. The present finding that the contribution of CD73/5′-NT expressed by hGF to the inhibitory effect on IL-1α-induced GM-CSF production suggests a natural feedback mechanism in an inflammatory process in periodontal tissue. The present findings may provide an additional viewpoint to help clarify the mechanism of onset, development and reduction of inflammation involved in fibroblasts in periodontal tissue.

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