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Extracellular Adenosine Induces IL-6 Production through Activation of A2B Receptor and Epidermal Growth Factor Receptor in Human Keratinocyte HaCaT Cells

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Epidermal cells produce cytokines as a part of the body's response to various external stimuli. Though extracellular ATP-induced activation of P2 receptors is involved in cytokine production in epidermal cells, it is not known whether activation of P1 receptors by extracellular adenosine leads to IL-6 production in epidermal cells. Here, we show that activation of adenosine A2B receptor induces IL-6 production via phosphorylation of epidermal growth factor receptor (EGFR) in human keratinocyte HaCaT cells. We found that treatment of HaCaT cells with 100 μM adenosine or with A2B receptor-specific agonist BAY60-6583 induced IL-6 production, and the production of IL-6 was suppressed by pretreatment with A2B receptor-specific antagonist PSB603. Adenosine-induced IL-6 production was also suppressed by A2B receptor knockdown. In addition, adenosine- and BAY60-6583-induced IL-6 production was suppressed by treatment with EGFR antagonist AG1478. Furthermore, adenosine and BAY60-6583 induced EGFR phosphorylation, and this phosphorylation was suppressed by A2B receptor knockdown. Thus, our data indicate that the A2B receptor-EGFR pathway has a role in IL-6 production. This in turn suggests that extracellular adenosine is involved in skin inflammation.

Key words interleukin 6, adenosine, A2B receptor, epidermal growth factor receptor

INTRODUCTION

Skin forms a barrier that protects the interior of the human body from a range of chemical (cosmetics, detergents, drugs) and physical (ultraviolet light, heat, cold, dryness) insults.[1)] Furthermore, in response to such external stimuli, epidermal cells secrete many cytokines and chemokines, which promote the proliferation and regeneration of epidermal cells and mobilize inflammatory cells, often inducing an inflammatory response.[2)] Among these cytokines, interleukin 6 (IL-6) is produced rapidly and transiently in response to tissue damage and contributes to signaling for activation of repair functions.[2)] It also plays a central role in various inflammatory responses, and in protecting the host from threats such as infection. However, dysregulated IL-6 production can induce chronic inflammation and lead to autoimmune diseases.[2)] Indeed, IL-6 has been studied extensively as a therapeutic target for skin diseases,[3-5)] and several pathways of IL-6 production, including the epidermal growth factor receptor (EGFR) pathway, have been identified.[6-10)] The activation of EGFR is generally involved in cell proliferation and growth, and is essential for maintaining skin homeostasis. However, the mechanism of IL-6 production in epidermal cells has not yet been established in detail.

In recent years, it has been shown that ATP is released extracellularly in response to various physical or chemical stimuli, and its metabolites act as signal transduction molecules via purinergic receptors.[1,11,12)] Though we have previously reported the involvement of the ATP-P2Y11 receptor pathway in IL-6 production in epidermal keratinocytes (HaCaT cells),[13-16)] it is not known whether the adenosine-P1 receptor pathway also contributes to IL-6 production. P1 receptors (A1, A2A, A2B and A3 receptors) are G protein-coupled receptors. These receptors have different affinity for adenosine and various functions.[12)] P1 receptors have both pro-inflammatory and anti-inflammatory actions.[17)] Among them, the A2B receptor is activated in the presence of pathologically high concentrations of adenosine.[18)] It has been reported that A2B mediates IL-6 production in several cell lines,[19-24)] though it is not clear whether this also occurs in epidermal cells.

Since we have recently established that the A2B receptor is involved in the activation of EGFR,[25)] in this study, we investigated the involvement of A2B receptor and EGFR activation in adenosine-induced IL-6 production in HaCaT cells. Our data show that adenosine induces A2B receptor activation and EGFR phosphorylation, leading to IL-6 production in HaCaT cells.

MATERIALS AND METHODS

Reagents and Antibodies Adenosine was purchased from Sigma-Aldrich. BAY60-6583 and PSB603 were purchased from Tocris Bioscience. AG1478 was purchased from

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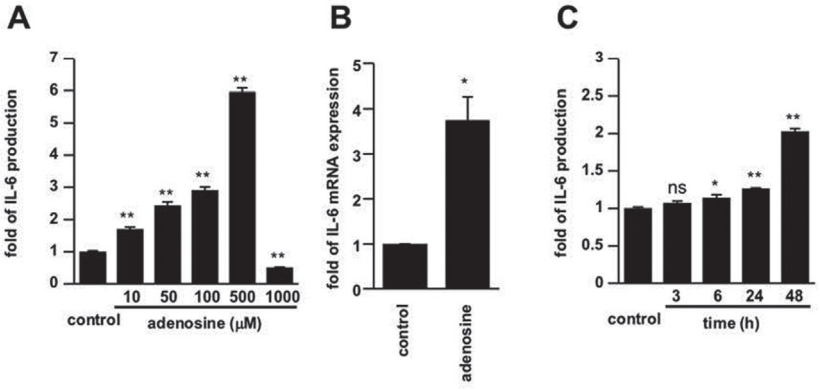

Fig. 1. Adenosine-Induced IL-6 Production in HaCaT Cells

(A) HaCaT cells were incubated with 10-1000 μM adenosine for 48 h, then the culture supernatant was harvested, and IL-6 production was measured by means of ELISA (n = 4). (B) HaCaT cells were incubated with adenosine (100 μM) for 48 h, and IL-6 mRNA was measured by real-time RT-PCR (n = 3, three independent experiments). (C) HaCaT cells were incubated with adenosine (100 μM) for the indicated period (3-48 h), then the culture supernatant was harvested, and IL-6 production was measured by ELISA (n = 4). (A-C) Each value represents the mean ± S.E. Significant differences from the control are indicated by ** ($p < 0.01$) or * ($p < 0.05$).

Biosource. Affinity-purified anti-human IL-6 monoclonal antibody (mAb) and biotin-conjugated anti-human IL-6 mAb were purchased from eBioscience. Rabbit anti-A2B receptor was purchased from Merck Millipore. Anti-EGFR, anti-phosphorylated EGFR (Tyr845) and anti-rabbit IgG horseradish peroxidase (HRP)-linked antibodies were purchased from Cell Signaling Technology. Anti GAPDH monoclonal antibody peroxidase conjugated was purchased from Wako Pure Chemical Industries.

Cell Culture Immortalized human-derived epidermal keratinocytes HaCaT cells were cultured as described previously.[16,26] HaCaT cells were kindly supplied by Drs. M. Ichihashi and M. Ueda (Kobe University School of Medicine, Kobe, Japan) with the permission of Dr. N. E. Fusening (German Cancer Research Center, Heidelberg, Germany).[27,28]

Cytokine Production (Enzyme-Linked Immunosorbent Assay (ELISA)) HaCaT cells (1.0×10^5 cells/mL) were seeded and incubated for 24 h. After incubation, the cells were stimulated with adenosine or BAY60-6583. IL-6 concentration in the culture supernatant was measured by means of ELISA, as described previously.[16,26]

Western Blotting Western blotting was performed as described previously.[16,25]

Real-Time RT-PCR Real-time RT-PCR was performed as described previously.[16,25]

siRNA Transfection Si-RNA transfection was performed as described previously.[25] SiRNA targeting A2B receptor and negative control siRNA (TriFECTa Kit® DsiRNA Duplex) were purchased from Integrated DNA Technology. HaCaT cells (1.6×10^5 cells in 24-well plates) were seeded in culture medium and transfected with A2B receptor duplex oligonucleotides (hs.Ri.ADORA2B.13.3) (100 nM) by using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. The cells were used for experiments (ELISA and western blotting) at 48 h after transfection.

Statistics Results were expressed as mean ± SE. The statistical significance (p values) of differences between two groups was calculated using Student's t test. Multiple groups were compared using ANOVA followed by pairwise comparisons with Dunnett's post hoc analysis. We statistically analyzed the experiments having two factors (e.g. adenosine and inhibitor) by two-way ANOVA followed by pairwise comparisons with Bonferroni's *post hoc* analysis. Calculations were done with the Instat version 3.0 statistical software package and the Prism version 5.0a (Graph Pad Software, USA). The criterion of significance was set at $p < 0.05$.

RESULTS

Adenosine-Induced IL-6 Production in HaCaT Cells When HaCaT cells were stimulated with adenosine (10-1000 μM), the production of IL-6 increased dose-dependently with a peak at 500 μM adenosine (Fig. 1A). However, production of IL-6 was reduced at 1000 μM adenosine. Subsequent experiments were conducted at 100 μM adenosine. Expression of IL-6 mRNA was also increased by treatment with 100 μM adenosine (Fig. 1B). When the time course of IL-6 production after stimulation with adenosine was examined, we found little change up to 24 h, but IL-6 production was significantly increased at 48 h after stimulation (Fig. 1 C).

Involvement of A2B Receptor in Adenosine-Induced IL-6 Production in HaCaT Cells Since adenosine induces IL-6 production (Fig. 1), we next examined which subtype of adenosine receptor is involved. We initially examined the mRNA expression of adenosine receptor subtypes (Fig. 2A). Among P1 receptors, A2B receptor was the most highly expressed. We also confirmed A2B receptor expression at the protein level in HaCaT cells (Fig. 2B). Based on these results, we examined the effect of an A2B receptor-specific antagonist (PSB603) on adenosine-induced IL-6 production. The increase of IL-6 production was significantly suppressed by pretreatment with PSB603 (Fig. 2C). Next, we examined the effect of an A2B receptor-specific agonist (BAY60-6583) on IL-6 production. BAY60-6583 significantly increased IL-6 production, and this increase was suppressed by pretreatment with PSB603 (Fig. 2D). We also confirmed that the solvent (DMSO) did not affect the production of IL-6. These results indicate that A2B receptor plays a key role in adenosine-induced IL-6 production.

Effect of A2B Receptor Knockdown on Adenosine-Induced IL-6 Production in HaCaT Cells To confirm the involvement of A2B receptor in adenosine-induced IL-6 pro-

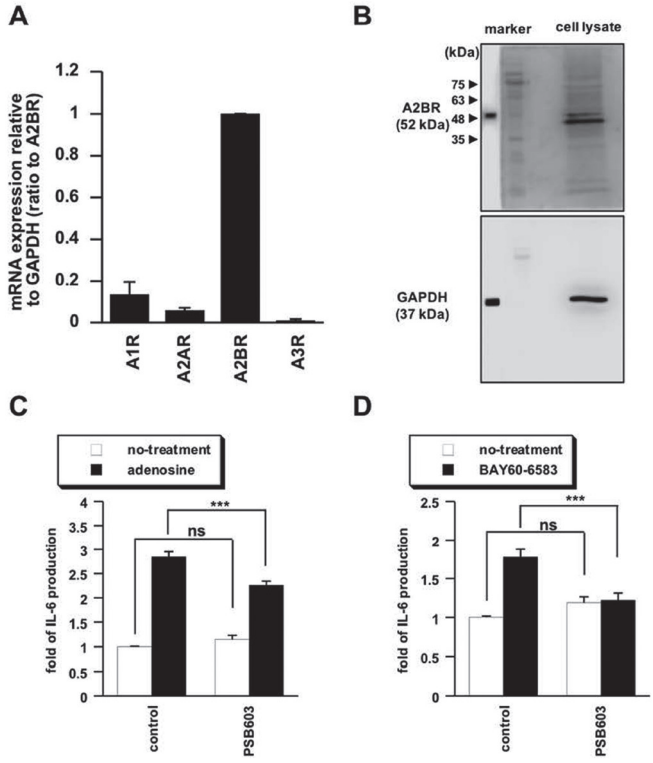

Fig. 2. Involvement of A2B Receptor in Adenosine-Induced IL-6 Production in HaCaT Cells

(A) Expression of P1 receptor mRNAs was measured by real-time RT-PCR (n = 3, three independent experiments). (B) Protein expression of A2B receptor was detected by western blotting. We put a luminescence marker (left small band) to indicate about 52 kDa for A2BR (upper panel) or about 37 kDa for GAPDH (lower panel). (C, D) HaCaT cells were incubated with adenosine (100 μM) or BAY60-6583 (1 μM) for 48 h. PSB603 (5 μM) was added 1 h before treatment of adenosine or BAY60-6583. The culture supernatant was harvested, and IL-6 production was measured by means of ELISA (n = 12, three independent experiments). Each value represents the mean ± S.E. Significant differences between indicated groups are indicated by *** ($p < 0.001$).

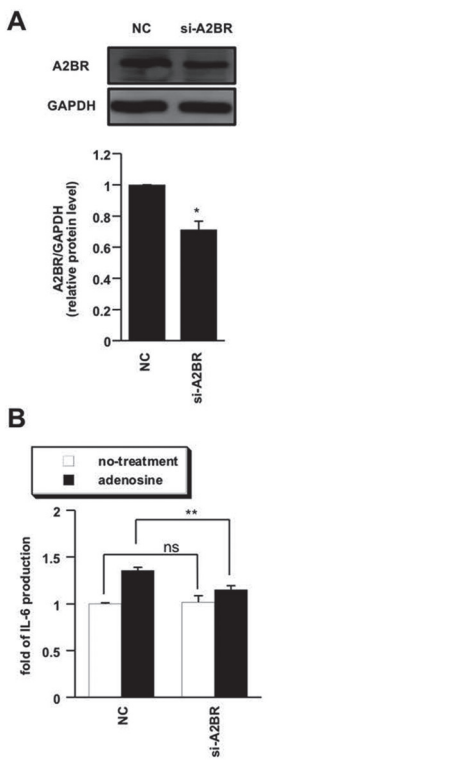

Fig. 3. Effect of A2B Receptor Knockdown on Adenosine-Induced IL-6 Production in HaCaT Cells

(A, B) HaCaT cells were transfected with negative control siRNA or siRNA targeting A2B receptor and incubated for 48 h. (A) Decrease of A2B receptor in HaCaT cells was detected by western blotting (n = 3, three independent experiments). (B) HaCaT cells were incubated with adenosine (100 μM) for 48 h. The culture supernatant was harvested, and IL-6 production was measured by means of ELISA (n = 12, three independent experiments). (A, B) Each value represents the mean ± S.E. (A) Significant difference from the negative control siRNA-transfected cells (NC) is indicated by * ($p < 0.05$). (B) Significant differences between indicated groups are indicated by ** ($p < 0.01$).

duction, HaCaT cells were transfected with siRNA targeting the A2B receptor (si-A2BR). As shown in Fig. 3A, A2B receptor expression in HaCaT cells transfected with si-A2BR was lower than that in negative control siRNA-transfected cells (NC), and adenosine-induced IL-6 production was significantly suppressed by the A2B receptor knockdown (Fig. 3B). These results support the idea that the A2B receptor is involved in adenosine-induced IL-6 production.

Role of EGFR in IL-6 Production Induced by Adenosine via A2B Receptor in HaCaT Cells Since EGFR is the major factor involved in IL-6 production,[3] we examined whether EGFR also has a role in adenosine-A2B receptor-mediated IL-6 production. AG1478 (an EGFR inhibitor) significantly suppressed IL-6 production induced by adenosine (Fig. 4A), and also suppressed IL-6 production induced by BAY60-6583 (Fig. 4B). Next, we examined whether adenosine and BAY60-6583 induce phosphorylation of EGFR. Both adenosine and BAY60-6583 caused increased phosphorylation of EGFR at 48 h after stimulation (Fig. 4C). Furthermore, knockdown of the A2B receptor significantly suppressed the adenosine-induced phosphorylation of EGFR (Fig. 4D). These results suggest that EGFR activation is involved in IL-6 production induced by adenosine via the A2B receptor.

DISCUSSION

It has not been reported whether or not adenosine induces IL-6 production in epidermal cells. The A2B receptor is known to be involved in proliferation[23,29] and is highly expressed in keratinocytes,[29,30] and we have previously found that extracellular ATP is involved in gamma-ray irradiation-induced IL-6 production through activation of P2Y11 receptor in keratinocytes.[16] Therefore, we hypothesized that adenosine and the A2B receptor might be involved in IL-6 production.

Indeed, IL-6 production was increased by adenosine in a dose-dependent manner (Fig. 1A), we performed subsequent experiments with 100 μM of adenosine. Though the concentration of extracellular adenosine in human epidermal keratinocytes is unclear, in general, extracellular adenosine usually exists at a low concentration but it rises up to about 50 times in a hypoxic state.[31] It is known that the epidermis becomes hypoxic in pressure ulcers, diabetic ulcers, and venous ulcers,[32] and this study is conducted assuming such diseases. In addition, adenosine-induced IL-6 production was suppressed by blockade of A2B receptor in HaCaT cells. Thus, we concluded that A2B receptor mediates the production of IL-6 induced by adenosine. As shown in Fig. 2C, D, PSB603 almost completely suppressed the increase in IL-6 production induced by BAY60-6583, but did not completely suppress

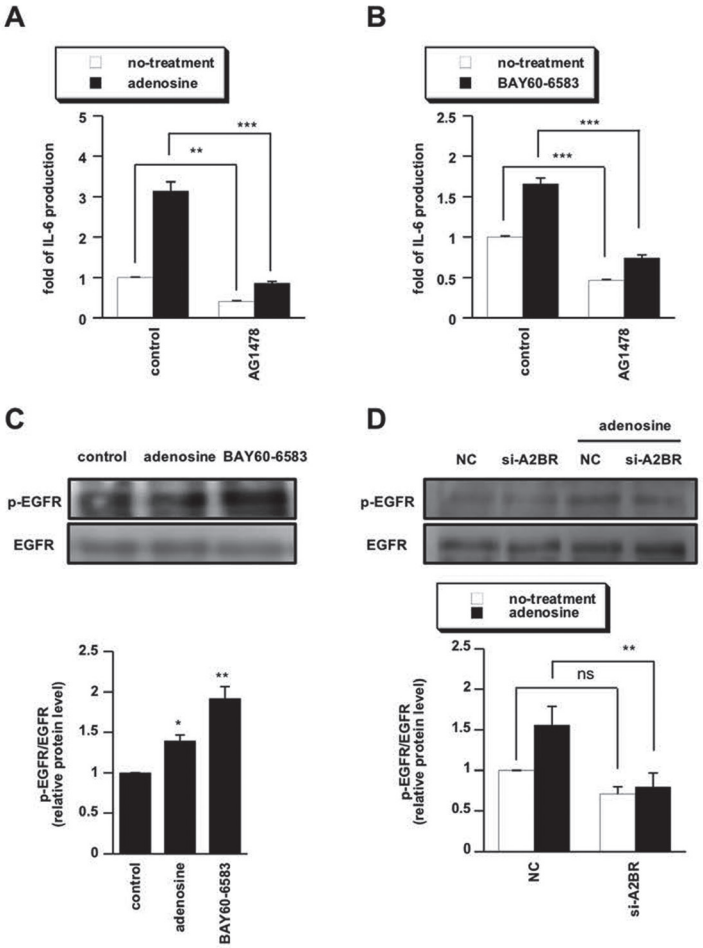

Fig. 4. Involvement of EGFR Phosphorylation in A2B Receptor-Mediated IL-6 Production in HaCaT Cells

(A, B) HaCaT cells were incubated with adenosine (100 μM) or BAY60-6583 (1 μM) for 48 h. AG1478 (10 μM) was added 1 h before treatment with adenosine or BAY60-6583. The culture supernatant was harvested, and IL-6 production was measured by means of ELISA (n = 12, three independent experiments). (D) HaCaT cells were transfected with negative control siRNA or siRNA targeting A2B receptor. (C, D) HaCaT cells were incubated with adenosine (100 μM) or BAY60-6583 (1 μM) for 48 h. Phosphorylation of EGFR was detected by western blotting (n = 4, four independent experiments). Each value represents the mean ± S.E. (A, B, D) Significant differences between indicated groups are indicated by ** ($p < 0.01$) and *** ($p < 0.001$). (C) Significant differences from the control are indicated by * ($p < 0.05$) or ** ($p < 0.01$).

that induced by adenosine. Consequently, there appear to be other mechanism(s) of adenosine-induced IL-6 production, besides the A2B receptor-mediated pathway. Though other P1 receptors might mediate adenosine-induced IL-6 production, we found that inhibitors of other P1 receptors did not significantly influence adenosine-induced IL-6 production (data not shown). This suggests that among P1 receptors, the A2B receptor is solely, or at least predominantly, involved in adenosine-induced IL-6 production. It is possible that adenosine is extracellularly phosphorylated to ATP, which would activate the P2Y11 receptor to induce IL-6 production, but the relationship between adenosine and ATP in keratinocytes is still unclear, and this issue requires further study.

We also established the involvement of EGFR in adenosine-induced IL-6 production downstream of A2B receptor activation. This is the first evidence indicating that A2B receptor and EGFR activation are involved in adenosine-induced IL-6 production in human keratinocytes. However, the pathways upstream and downstream of EGFR phosphorylation by adenosine remain unclear. It is known that EGFR (Tyr845) is often activated by Src,[33] and Src has been reported to be associated with G protein-coupled receptors (GPCRs),[34] including the A2B receptor. Since we previously found that the A2B receptor is involved in Src phosphorylation and EGFR phosphorylation/nuclear translocation,[25] we speculate that Src is involved in adenosine-induced IL-6 production upstream of EGFR.

EGFR phosphorylation mediates the production of pro-inflammatory factors via cyclooxygenase-2 and p38MAPK phosphorylation,[35,36] and it has also been reported that IL-6 production induced by A2B receptor activation is suppressed by a p38 MAPK inhibitor.[18,19] Therefore, adenosine-induced IL-6 production in HaCaT cells might be mediated by p38 MAPK activation following A2B receptor activation and EGFR phosphorylation.

Adenosine-induced IL-6 production takes 24-48 h (Fig. 1C), and it is thought that various factors might be involved in this time, such as balance of extracellular concentrations of adenosine and ATP leading to A2B receptor activation, Src activation, EGFR ligand production, EGFR activation/nuclear translocation, and p38 MAPK activation. These mechanisms might be complexly involved in adenosine-induced IL-6 production.

In conclusion, we have identified a new pathway of adenosine-induced IL-6 production through the A2B receptor and EGFR in HaCaT cells. Since A2B receptor-mediated IL-6 pro-

duction promotes cell proliferation and inflammation in mouse microglia,[23] these findings should be helpful to understand the role of the A2B receptor in inflammation in human keratinocytes. The A2B receptor might be a candidate for the treatment of skin inflammation.

Conflict of interest The authors declare no conflict of interest.

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