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Inflammatory Cytokines Regulate Function and Expression of Adenosine A2A Receptors in Human Monocytic THP-1 Cells

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Adenosine, acting at its receptors, particularly A2A receptors, is a potent endogenous anti-inflammatory agent that modulates the functions and differentiation of inflammatory and immune cells. Because the inflammatory milieu abounds in proinflammatory cytokines, we investigated the effects of Th1-inflammatory cytokines on function and expression of adenosine A2A receptors in the human monocytic cell line THP-1. We found that, consistent with previous reports, adenosine and 2-[[{2-(carboxyethyl)phenyl-ethylamino}]5'-N-ethylcarboxamidoadenosine (CGS-21680), a selective A2A receptor agonist, suppress IL-12 production but increase IL-10 production in LPS-activated THP-1 cells. These effects were blocked by the A2A receptor antagonist 4-[[7-aminomethylamino]-5-(2-furyl)[1,2,4-triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl]phenol (ZM-241385). More importantly, the suppressive effect of adenosine and CGS-21680 on IL-12 production was significantly enhanced in cells pretreated with either IL-1 (10 U/ml) or TNF-α (100 U/ml) but markedly attenuated in cells pretreated with IFN-γ (100 U/ml). Similarly, IL-1 and TNF-α treatment potentiated the stimulatory effect of adenosine and CGS-21680 on IL-10 production, whereas IFN-γ treatment almost completely abolished this effect. CGS-21680 stimulated an increase in intracellular cAMP in a time- and dose-dependent manner in IL-1- and TNF-α-treated cells but not in control or IFN-γ-treated cells. Both IL-1 and TNF-α increased A2A receptor mRNA and protein. In parallel with its effect on A2A receptor function, IFN-γ down-regulated A2A receptor message and protein. Because adenosine mediates many of the anti-inflammatory effects of drugs such as methotrexate, these observations suggest that local changes in the cytokine milieu may influence the therapeutic response to those drugs by altering the expression and function of adenosine receptors on inflammatory cells. The Journal of Immunology, 2001, 167: 4026–4032.

Adenosine, released by cells and tissues, is a potent endogenous regulator of inflammation. Adenosine diminishes the proinflammatory actions of inflammatory and immune cells via interaction with specific cell surface receptors, of which there are four known subtypes, A1, A2A, A2B, and A3 (see reviews in Refs. 1 and 2). Recent reports also indicate that adenosine, acting at its receptors, modulates the anti-inflammatory action of antirheumatic drugs such as methotrexate (3).

Monocytes and macrophages synthesize and release into their environment a variety of cytokines and other proteins that play a central role in the development of acute and chronic inflammation. There have been some lines of evidence suggesting a regulatory connection between adenosine and its receptors and inflammatory cytokines. In human and murine monocytes/macrophages, the activation of adenosine receptors, particularly A2A receptors, by adenosine or its analogues modulates the production of inflammatory cytokines including TNF-α, IL-10, and IL-12 (4–8). IL-12, a proinflammatory cytokine and a central inducer of Th1 responses and cell-mediated immunity, is suppressed by adenosine and its analogues, whereas secretion of IL-10, a protective cytokine that suppresses IL-12 and TNF-α release, is enhanced by adenosine and A2A receptor agonists both in vitro and in vivo (6–9).

In contrast, other lines of evidence have suggested that the expression and functions of adenosine receptors may be regulated by numerous endogenous factors involved in inflammation and cellular growth and differentiation such as glucocorticoids (10, 11), growth factors (12, 13), and other cytokines. For example, Xaus et al. (14) reported that IFN-γ up-regulates expression of A2B receptors and promotes macrophage activation. The effects of IFN-γ and other prominent inflammatory cytokines such as IL-1 and TNF-α on expression and function of A2A receptors, however, have not been documented.

We therefore investigated the effects of IL-1, TNF-α, and IFN-γ, the most prominent Th1-inflammatory cytokines in rheumatoid arthritis and other inflammatory diseases, on function and expression of adenosine A2A receptors. We found, to our surprise, that IL-1, TNF-α, and IFN-γ all modulate the effects of A2A receptor occupancy on secretion of IL-10 and IL-12 as well as cellular accumulation of cAMP in human monocytic THP-1 cells. In accord with their effects on A2A receptor function, IL-1, TNF-α, and IFN-γ also regulate expression of A2A receptors in THP-1 cells.

Materials and Methods

Reagents

Human rIL-1α, TNF-α, and IFN-γ were purchased from R&D Systems (Minneapolis, MN). The A2A receptor agonist 2-[[{2-(carboxyethyl)phenyl-ethylamino}]5'-N-ethylcarboxamidoadenosine (CGS-21680)1 was purchased from Sigma (St. Louis, MO). The A2A Receptor agonist antagonist 4-[[7-
amino-2-[2-furyl][1,2,4-triazolol[2-3-[1,3,5]triazin-5-ylamino]ethyl] phenol (ZM-241385) was purchased from Tocris Cookson (Ballwin, MO). LPS (from Escherichia coli, serotype K-235) and adenosine were obtained from Sigma. The murine mAb (7F6-G5-A2) against human A2A receptor, with a high affinity and specificity (15) was a gift from Dr. J. Linden (University of Virginia School of Medicine, Charlottesville, VA).

Cell culture and cytokine treatment

THP-1 cells, a human monocytic leukemia cell line (16), were maintained in culture with RPMI 1640 medium supplemented with 10% FBS, penicillin (50 U/ml) and streptomycin (50 μg/ml) at 37°C in a humidified atmosphere consisting of 5% CO2. At the onset of each experiment the cells were placed in fresh medium and then cultured in the presence of IL-1α (10 U/ml), TNF-α (100 U/ml), IFN-γ (100 U/ml), or medium alone for 3 h or overnight (18 h) followed by further analyses.

Assays for IL-10 and IL-12

THP-1 cells were pretreated overnight with IL-1, TNF-α, or IFN-γ and then placed in 24-well tissue culture plates at ~106 cell/ml with 1.5 ml medium/well. The cells were further cultured overnight in the presence or absence of LPS (5 μg/ml). The A2A receptor agonist CGS-21680 or adenosine with or without the A2A receptor antagonist ZM-241385 was added to cultures 20 min before the addition of LPS. Culture supernatants were collected and subjected to assays for human IL-12 p70 (the heterodimer form), IL-12 p40 subunit, and IL-10 using ELISA Quantikine kits purchased from R&D Systems.

cAMP assay

THP-1 cells were pretreated overnight with the cytokines. On the day of the cAMP assay, the cells were placed in fresh medium and incubated with CGS-21680 at different concentrations (0.01–100 μM) or 1 μM CGS-21680 for varying periods of time (0–25 min). Cells were then harvested, lysed, and assayed for cellular cAMP accumulation using the cAMP enzyme immunoassay system kit (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer’s instructions.

Isolation of mRNA and semiquantitative RT-PCR

mRNA was isolated from THP-1 cells using a MicroFastTrack kit (Invitrogen, Carlsbad, CA) and transcribed into cDNA (RT). Aliquots of RT, after serial dilutions, were subject to PCR using an RNA-PCR Core kit (PerkinElmer, Branchburg, NJ). The PCRs were performed using primers specific for adenosine A2A receptors and GAPDH, a housekeeping gene. The cycle number was adjusted to allow the PCR to proceed in a linear range. Briefly, PCR conditions for A2A receptors were 32–35 cycles of 45 s at 94°C, 45 s at 62°C, and 1 min at 72°C with 1× and 2× diluted RT as templates. PCR for GAPDH was run for 22–25 cycles with RT of 100× and 200× dilutions as templates. The primer sequences were 5′-ACCTG CAGAACGCTCAACCAAC-3′ (forward) and 5′-TCTGTTACGCTGTGTCG CAC-3′ (reverse) for A2A receptor and 5′-ACCATATCCTGCCGTCTT AC-3′ (forward) and 5′-CCTGCTTGCTGACCCAAAT-3′ (reverse) for GAPDH. All primers were designed to amplify cDNA that crossed an intron in the genomic DNA, and the amplicon was sequenced to confirm the identity of the cDNA amplified. Aliquots of PCR product were loaded onto ethidium bromide-stained agarose gel, visualized with an ultraviolet transilluminator, and digitally photographed. The amplicon was quantitated densitometrically using Kodak Digital Science software and all values were normalized to the GAPDH amplicon.

Membrane protein extraction and Western blot analysis

All Western blots were done on cell membrane preparations after overnight cytokine treatment. Crude membrane protein was isolated after sonication according to the membrane was blocked with 3% nonfat milk in Tris-buffered saline containing 0.3% Tween 20. The membranes were then incubated for 1–2 h with a mAb against adenosine A2A receptors (15). After three washes of 5 min each in 0.3% Tween 20-TBS, the blots were incubated with an alkaline phosphatase-conjugated secondary Ab for 1 h. After extensive washes, the blots were exposed to a fluorescent enhanced chemiluminescency (dithranolamine) substrate (Amersham Pharmacia Biotech, Sunnyvale, CA) and scanned using the Storm PhosphorImager system (Molecular Dynamics, Sunnyvale, CA). The band density was then directly quantitated using Image Quant software (Molecular Dynamics).

Data analysis

Data are presented as mean ± SEM. One-way and two-way ANOVAs were used to determine statistical significance between means or curves. Differences with a p value of <0.05 were considered significant. All statistical analysis was performed using the SigmaStat program (SPSS, Chicago, IL).

Results

Inflammatory cytokines modulate A2A receptor-mediated suppression of IL-12 production

Without LPS stimulation, production of IL-12 in monocytic THP-1 cells (either cytokine-treated or control) was undetectable (data not shown). In control LPS-activated THP-1 cells (without cytokine pretreatment), CGS-21680, a selective A2A agonist, suppressed production of IL-12 p70 and p40 in a dose-dependent manner (IC50 ~ 100 and 60 nM, respectively) as previously reported (7, 8). CGS-21680 itself, at concentrations up to 10 μM, inhibited LPS-induced IL-12 production by up to 40% (for IL-12 p70) and 60% (for IL-12 p40). In the cells pretreated with IFN-γ, LPS stimulated greater IL-12 p70 and p40 secretion than control, IL-11-treated, and TNF-α-treated cells, and the inhibitory effect of CGS-21680 on secretion of IL-12, especially IL-12 p40, was markedly attenuated (p < 0.05, IFN-γ vs control, for both IL-12 p70 and IL-12 p40). In contrast, pretreatment of the cells with either IL-1 or TNF-α significantly enhanced the effect of CGS-21680 on suppression of both active heterodimer IL-12 p70 (p < 0.05 and p < 0.01 vs control, respectively; IC50 ~ 50 nM) and IL-12 p40 subunit (p < 0.01 for both vs control, IC50 ~ 10 nM). Under all treatment conditions, the changes in levels of p40 subunit were more marked than those of p70 heterodimer (Fig. 1, A and B). The inhibitory effect of CGS-21680 on IL-12 production was reversed by the specific A2A receptor antagonist ZM-241385 (10 μM, Fig. 1C), further supporting the role of A2A receptors in modulation of IL-12 release.

To further understand the role of inflammatory cytokines in modulating adenosine receptor function, we examined the responsiveness of cytokine-treated THP-1 cells to adenosine. Like CGS-21680, adenosine itself inhibited LPS-induced IL-12 production in control, IL-1-treated, and TNF-α-treated cells but not in IFN-γ-treated cells, although it seemed that adenosine was less potent than CGS-21680 at the same concentration, probably due, in part, to prompt uptake and metabolism of adenosine by THP-1 cells (Fig. 2A). Moreover, the effect of adenosine was abrogated by ZM-241385 (Fig. 2B), providing further evidence that the A2A receptor is primarily involved in regulation of IL-12 production.

Inflammatory cytokines modulate A2A receptor-mediated stimulation of IL-10 production

Like IL-12, no detectable IL-10 was produced by THP-1 cells without LPS stimulation (not shown). The concentrations of IL-10 in supernatants of control LPS-activated THP-1 cells remained low but were increased in a dose-dependent manner by CGS-21680 (EC50 ~ 100 nM). The stimulatory effect of CGS-21680 on IL-10 production was significantly greater in THP-1 cells pretreated with IL-1 or TNF-α (p < 0.05 and p < 0.01 vs control, respectively; EC50 ~ 50 nM for both) and reached increases of 160 and 170%, respectively, in IL-10 secretion in response to 10 μM CGS-21680, compared with an increase of 141% in controls. In contrast, CGS-21680 did not increase IL-10 production in THP-1 cells that had been pretreated with IFN-γ (p = 0.01 vs control; Fig. 3A). ZM-241385 reversed the effect of CGS-21680 on IL-10 production by untreated THP-1 cells and THP-1 cells pretreated with IL-1 or TNF-α (Fig. 3B).
Like CGS-21680, adenosine enhanced LPS-induced secretion of IL-10 in untreated THP-1 cells, although to a lesser extent, and the effects of IL-1, TNF-α, and IFN-γ on IL-10 secretion were very similar to those observed with CGS-21680 (Fig. 4A). The enhancement in IL-10 levels by adenosine in control, IL-1-treated, and TNF-α-treated cells was completely abolished in the presence of ZM-241385 (Fig. 4B). These results indicate that, as with IL-12, the A2A receptor is the adenosine receptor primarily responsible for modulation of IL-10 production.

**Inflammatory cytokines modulate A2A receptor-mediated accumulation of cAMP**

Both A2A and A2B receptors signal via Gs protein-cAMP pathways. To further determine whether inflammatory cytokines modulate A2A receptor function, we studied the cAMP response of cytokine-activated THP-1 cells after agonist ligation. Surprisingly, the basal levels of cAMP in IL-1- and TNF-α-treated cells were higher than those in either control or IFN-γ-treated cells (p < 0.05 vs control for TNF-α; Fig. 5).

The addition of 1 μM CGS-21680, a concentration at which CGS-21680 is still highly selective for A2A receptors, to control cells did not significantly stimulate cAMP accumulation at any time point up to 25 min (Fig. 5A). Moreover, there was only a slight increase of cAMP production in control cells with a very high concentration (100 μM) of CGS-21680 (Fig. 5B). The CGS-21680-induced cAMP response of IFN-γ-treated cells was very similar to that of controls. Although the cAMP levels induced by high doses (>1 μM) of CGS-21680 in IFN-γ-treated cells were slightly lower than in control cells, the overall responses to CGS-21680 stimulation by IFN-γ-treated and control cells were not significantly different (p > 0.05, two-way ANOVA; Fig. 3). In contrast, CGS-21680 induced striking cAMP generation by IL-1- and TNF-α-treated cells, as much as 3- to 4-fold higher than basal levels, and this increment differed significantly from the response of control cells (two-way ANOVA, p < 0.001). The peak levels of cAMP generation in IL-1- and TNF-α-treated cells occurred as early as 2–5 min after CGS-21680 stimulation (Fig. 5A) and in a dose-dependent manner (EC50 ~40 and 30 nM, respectively; Fig. 5B). These observations together with those on IL-10 and IL-12 production are consistent with the hypothesis that IL-1, TNF-α, and IFN-γ regulate A2A receptor levels and/or function.

**Inflammatory cytokines regulate A2A receptor expression**

To gain greater insight into the mechanism by which the Th1 cytokines IL-1, TNF-α, or IFN-γ modulate A2A receptor sensitivity and function, we examined the effects of these cytokines on the expression of A2A receptors in THP-1 cells. As shown in Fig. 6, the message for A2A receptors was significantly altered on treatment of the cells with each of the cytokines studied. A 3-h incubation with IL-1 or TNF-α led to an increase in expression of A2A message to 149 ± 16 and 159 ± 22% of control, respectively (n = 9, p < 0.01 vs control for both). In contrast, a 3-h treatment with IFN-γ decreased the A2A message to 69 ± 5% of control (n = 9, p < 0.01). The effects of the cytokines on A2A message remained consistent after overnight incubation; the message levels for A2A receptors were 144 ± 12, 167 ± 16, and 65 ± 8% of control in IL-1-, TNF-α-, and IFN-γ-treated cells, respectively (n = 9, p < 0.01 vs control for IL-1 and IFN-γ, and p < 0.001 for TNF-α).

To determine the significance of the change in mRNA levels for the A2A receptors, we examined protein expression by Western blot analysis using a mAb against A2A receptors. The immunoblots of membrane preparations from THP-1 cells pretreated overnight with medium or cytokines revealed a single band of a 45-kDa protein, the size of A2A receptors (15), under all treatment conditions (Fig. 7A). Compared with controls, protein levels of A2A receptors were increased by IL-1 and TNF-α to 127 ± 9 and 136 ± 9% (n = 5, p < 0.05 vs control for IL-1, and p < 0.01 for TNF-α) and decreased by IFN-γ to 73 ± 4% of control (n = 5, p < 0.05; Fig. 7B).

**Discussion**

The effects of adenosine and its analogues on cytokine secretion have been studied using human and murine monocytes/macrophages and human and murine monocyte/macrophage cell lines...
with very similar results. Based on the published data, the A2A receptor is the most prominent regulator of cytokine secretion. We observed similar effects of the A2A receptor agonist CGS-21680 on LPS-induced production of IL-12 and IL-10 in resting (control) THP-1 cells as had previously been reported for human peripheral blood mononuclear cells and isolated monocytes by Link et al. (8). More importantly, in the current study we found that IL-1, TNF-α, and IFN-γ, the most prominent cytokines secreted by monocyte/macrophages and Th1 cells, modulated the capacity of A2A receptor occupancy to regulate secretion of IL-10 and IL-12 in LPS-activated THP-1 cells. Whereas IL-1 and TNF-α clearly potentiate the A2A receptor function, IFN-γ attenuates it. These data also extend the previous observation that the suppressive effect of adenosine on IL-12 production by murine macrophages was much less pronounced in cells activated with both LPS and IFN-γ than in those activated with LPS alone (7). It is well recognized that cytokines stimulate or inhibit the production of other cytokines in an autocrine and paracrine manner, and our findings suggest that adenosine receptors may play a role in mediating this mutual interaction among cytokines. The functional modulation of adenosine receptors is likely a key factor in the regulation of inflammatory conditions involving numerous cytokines.

It has been shown previously that adenosine suppresses IL-12 production in murine macrophages by A2A-dependent and A3-dependent mechanisms (7). However, our finding that adenosine and CGS-21680 regulate cytokine secretion by THP-1 cells, both untreated and cytokine treated, and that the effects of both agents were blocked by ZM-241385 is most consistent with regulation via the A2A receptor alone. Absence of a role for A3 receptor in regulating cytokine production by human monocytes is consistent with the previous findings of Link et al. (8).

The A2A receptor-mediated increase in intracellular cAMP accumulation provides further evidence for the regulatory effects of inflammatory cytokines on A2A receptor function and A2A-mediated signaling. Although some cell types respond to A2A receptor occupancy with a brisk cAMP response (18), we observed a minimal cAMP response of resting THP-1 cells to CGS-21680, consistent with previous findings in the same cell line by Munro et al.
The minimal accumulation of cAMP in resting and IFN-γ/H9253-treated THP-1 cells reported here may have resulted from a reduction in functional A2A receptor expression, desensitization of A2A receptors on THP-1 cells, or diminished signal transduction machinery. The in vitro up-regulation of A2A receptor function by IL-1 and TNF-α treatment parallels the observation that in vivo cAMP production is enhanced in ciliary epithelial bilayers from IL-1 or TNF-α-inflamed eyes (20). Our observation that there was an increased cAMP response after treatment with an A2A receptor agonist in IL-1- and TNF-α-treated THP-1 cells suggests that the elevated cAMP production in these models may have resulted, at least in part, from the activation of an increased number and/or sensitivity of A2A receptors. Because elevated intracellular levels of cAMP are generally associated with suppression of inflammatory responses, it is likely that the up-regulation of A2A receptors along with endogenous adenosine release at inflamed sites constitutes a feedback loop to diminish or terminate the inflammatory response.

In parallel with the observed functional changes, expression of A2A receptor message and protein was found to be regulated on cytokine treatment. The Th1 cytokines IL-1, TNF-α, and IFN-γ play a critical role in the pathogenesis of many severe inflammatory conditions such as rheumatoid arthritis (see review in Refs. 21 and 22). Although they are all regarded as proinflammatory cytokines, the differences in their actions on adenosine A2A receptor expression may reflect a complex mechanism of receptor regulation, especially at inflamed sites (e.g., inflamed synovium) at which numerous cytokines are secreted. It has been reported that IFN-γ up-regulates A2B receptor expression in murine bone marrow-derived macrophages (14). Our data indicate that IFN-γ has an opposing effect on A2A receptors. This difference in the regulatory action of IFN-γ on the two receptors may be significant in modulating adenosine-mediated functions in cells and tissues where subtypes of adenosine receptors have different patterns of expression and distribution.

It is still not clear how the inflammatory cytokines studied here regulate adenosine A2A receptor expression. Direct regulation of transcription or altered mRNA stability after cytokine stimulation may explain our observations. Computerized analysis of upstream
regions of the A2A gene obtained from chromosome sequence databases demonstrates numerous potential regulatory elements including several NF-κB-binding sites. The presence of NF-κB-binding sites likely explains, at least in part, the effect of IL-1 and TNF-α on increased receptor expression, although which of these binding sites is involved is not yet known. Nevertheless, the effect of inflammatory cytokines on expression of A2A receptors cannot entirely explain the observed changes in receptor function with respect to either cAMP generation or regulation of cytokine secretion. It has been reported that elevated cAMP inhibits NF-κB-mediated transcription of numerous genes (23). Thus, the increase in cAMP levels, although it seems transient, in IL-1- and TNF-α-treated cells observed here may have a feedback control on the increased expression of A2A receptors by those cytokines. We and others have demonstrated that adenosine, acting at one or more of its receptors, mediates the anti-inflammatory effects of drugs such as methotrexate and sulfasalazine, commonly used and effective disease-modifying antirheumatic agents, in in vitro and in vivo models of acute inflammation and chronic arthritis (26–31). Despite its proven efficacy in the treatment of rheumatoid arthritis, many patients do not respond or only partially respond to methotrexate therapy. Higher levels of or greater sensitivity to IFN-γ in methotrexate-resistant patients may be an explanation for the diminished therapeutic response.

Our study demonstrates that function and expression of adenosine A2A receptors are differentially regulated by the inflammatory cytokines IL-1, TNF-α, and IFN-γ. This finding suggests that the effects of adenosine and its analogues at inflamed sites may be
modified in the short and long term by inflammatory mediators. We speculate that the activation or inhibition of adenosine receptors by inflammatory cytokines may partly account for the variability in response to adenosine-mediated anti-inflammatory agents used in the treatment of inflammatory diseases such as rheumatoid arthritis.

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