Adenosine Inhibits PDGF-Induced Growth of Human Glomerular Mesangial Cells Via A2B Receptors

Raghvendra K. Dubey, Delbert G. Gillespie, Zaichuan Mi, Edwin K. Jackson

Abstract—The objectives of the present study were to determine whether adenosine attenuates proliferation of glomerular mesangial cells (GMCs), which adenosine receptor (AR) mediates the antimitogenic actions of adenosine, and the cellular mechanisms by which adenosine inhibits growth of GMCs. Studies were conducted in both human and rat GMCs. Platelet-derived growth factor (PDGF)-BB (25 ng/mL) increased DNA synthesis ([3H]thymidine incorporation), cellular proliferation (cell number), collagen synthesis ([3H]proline incorporation), and mitogen-activated protein kinase (MAPK) activity, and these effects were attenuated by 2-chloroadenosine (nonselective AR agonist) and 5′-N-methylcarboxamidoadenosine (MECA; nonselective AR agonist), but not by N′-cyclopentyladenosine (selective A1 AR agonist), AB-N-MECA (selective A1 AR agonist), or CGS21680 (selective A2A AR agonist). KF17837 (selective A2/3 AR antagonist) and 1,3-dipropyl-8-p-sulfophenylxanthine (nonselective AR antagonist), but not 8-cyclopentyl-1,3-dipropylxanthine (selective A1 AR antagonist), blocked the growth-inhibitory effects of 2-chloroadenosine and 5′-N-MECA. Antisense, but not sense or scrambled, oligonucleotides to the A2B receptor increased both basal and PDGF-induced DNA synthesis, cellular proliferation, and collagen synthesis, and the growth-inhibitory effects of 2-chloroadenosine, 5′-N-MECA, and erythro-9-(2-hydroxy-3-nonyl)adenine (inhibitor of adenosine deaminase) plus iodotubercidin (inhibitor of adenosine kinase) were abolished by antisense, but not scrambled or sense, oligonucleotides to the A2B receptor. We conclude that adenosine causes inhibition of GMC growth by activating A2B receptors coupled to inhibition of MAPK activity. A2B receptors may play an important role in regulating glomerular remodeling associated with GMC proliferation. Pharmacological or molecular biologic activation of A2B receptors may prevent glomerular remodeling associated with glomerulosclerosis, renal disease, and abnormal growth associated with hypertension and diabetes. (Hypertension. 2005;46:628-634.)

Key Words: adenosine ■ mesangium ■ receptors, adenine ■ remodeling ■ glomerulosclerosis ■ renal disease ■ kidney
A2B Adenosine Receptors Inhibit Growth

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Agents Used in the Study

<table>
<thead>
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<th>Pharmacological Agent</th>
<th>Pharmacological Characteristics</th>
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<tr>
<td>2-Chloroadenosine (Cl-Ad)</td>
<td>Nonselective but stable adenosine analogue</td>
</tr>
<tr>
<td>5'-N-methylcarboxamidoadenosine (MECA)</td>
<td>Nonselective adenosine receptor agonist</td>
</tr>
<tr>
<td>4-Aminobenzyl-5'-N-methylcarboxamidoadenosine (AB-MECA)</td>
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<td>5'-N-ethylcarboxamidoadenosine (NECA)</td>
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<td>5'-Cyclopentyladenosine (CPA)</td>
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<td>2-p-(Carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS21680)</td>
<td>Selective A2 receptor agonist</td>
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<tr>
<td>KF17837 (KF)</td>
<td>Selective A3 receptor antagonist</td>
</tr>
<tr>
<td>1,3-Dipropyl-8-p-sulfophenylxanthine (DPSPX)</td>
<td>Nonselective adenosine receptor antagonist</td>
</tr>
<tr>
<td>8-Cyclopentyl-1,3-dipropylxanthine (DPCPX)</td>
<td>Selective A1 receptor antagonist</td>
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<tr>
<td>Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA)</td>
<td>Adenosine deaminase inhibitor</td>
</tr>
<tr>
<td>Iodotubercidin (IDO)</td>
<td>Adenosine kinase inhibitor</td>
</tr>
</tbody>
</table>

Materials

Adenosine, 2-chloroadenosine (Cl-Ad), erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), and PDGF-BB were purchased from Sigma Chemical Co. 8'-Cyclopentyladenosine (CPA), CGS21680, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), iodotubercidin (IDO), 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX), 5'-N-ethylcarboxamidoadenosine (NECA), 5'-N-methylcarboxamidoadenosine (MECA), and 4-aminobenzyl-5'-N-MECA (AB-MECA) were purchased from Research Biochemicals International. KF17837 was obtained from Kyowa Hakko Kogyo Co. Ltd (Sunto). [3H]thymidine (specific activity, 11.8 Ci/mmol) and L-[3H]proline (23 Ci/mmol) were purchased from NEN. All other reagents were of tissue culture or the best grade available.

Methods

GMC Cultures

Human GMCs were obtained from Clonetics Corp. Rat glomerular GMCs were grown as explants from glomeruli isolated from Sprague-Dawley rats and as described previously.12 GMCs in the fourth passage were used for all experiments.

Growth Studies

[3H]thymidine incorporation (index of DNA synthesis), [3H]proline incorporation (index of collagen synthesis), and cell proliferation were conducted as previously described.6 GMCs were exposed to various treatments for 24 hours (thymidine incorporation studies), 48 hours (proline incorporation studies), or 5 days (cell proliferation studies).

Expression Studies With Western Blots

The expression of A2B ARs in lysates from GMCs was analyzed by Western blotting and was probed with antibodies against A2B receptors (rabbit anti-adenosine A2B receptor polyclonal antibodies; Chemicon International Inc). The expression of phosphorylated MAPK (p42MAPK/p44MAPK) in lysates from GMCs was analyzed by Western blotting and was probed with antibodies against phosphorylated MAPK (extracellular signal-regulated kinase [ERK]1/ERK2; anti-MAPK ERK1/ERK2, phosphospecific, human; Calbiochem). Antibodies against nonphosphorylated ERK1/ERK2 were used to measure total ERK1/ERK2.

cAMP Synthesis

Extracellular (supernatant) and intracellular (cellular fraction) portions of cAMP were pooled, and total cAMP levels were analyzed by high-performance liquid chromatography according to our previously described method.7 In GMCs grown in parallel and treated similarly, the monolayers were inspected microscopically for intactness, the protein content was measured, and the data were normalized to protein content.

Statistics

All growth experiments were performed in triplicate or quadruplicate with 3 or 4 separate cultures. Data are presented as mean±SEM. Statistical analysis was performed with ANOVA, paired Student t

finding that this AR inhibits mitogen-induced growth of cardiac fibroblasts.7 The same relation between abnormal vascular smooth muscle cell (VSMC) proliferation and vascular disease may hold for abnormal growth of GMCs and glomerular remodeling associated with glomerulosclerosis, renal disease, hypertension, and diabetes.10 Therefore, it is important to determine the effects and mechanism of ARs on the growth of GMCs.

Because VSMCs are phenotypically similar to GMCs10 and because A2B receptors are expressed in the kidney,11 we hypothesized that A2B receptors mediate anti-mitogenic actions in GMCs. Accordingly, the overall aim of the present study was to determine whether A2B receptors affect 4 major growth processes (DNA synthesis, collagen synthesis, cell proliferation, and mitogen-activated protein kinase [MAPK] activity) in GMCs. To accomplish this goal, we studied the effects of adenosine and its receptor-specific analogues and antagonists (the Table) on the growth of human and rat GMCs. Moreover, to provide direct evidence of a role for A2B receptors in inhibiting GMC growth, by using the sequence of the recently cloned A2B receptor in rats, we designed antisense oligonucleotides to block A2B receptor synthesis in rat GMCs.

Antisense Oligonucleotides for A2B Receptors

The antisense oligonucleotide 5’-CTCGTGTTCCAGTGACCAA-3’ was used for all experiments with rat GMCs. In a previous study, we showed that this antisense oligonucleotide is effective in modulating the effects of adenosine on rat VSMC growth.6 Sense (5’- TTGTCACGGAACACGAG-3’) and scrambled (5’-GCAGCTCTTATCTGCATG-3’) oligonucleotides were used as controls.

Materials

Adenosine, 2-chloroadenosine (Cl-Ad), erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), and PDGF-BB were purchased from Sigma Chemical Co. 8'-Cyclopentyladenosine (CPA), CGS21680, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), iodotubercidin (IDO), 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX), 5'-N-ethylcarboxamidoadenosine (NECA), 5'-N-methylcarboxamidoadenosine (MECA), and 4-aminobenzyl-5'-N-MECA (AB-MECA) were purchased from Research Biochemicals International. KF17837 was obtained from Kyowa Hakko Kogyo Co. Ltd (Sunto). [3H]thymidine (specific activity, 11.8 Ci/mmol) and L-[3H]proline (23 Ci/mmol) were purchased from NEN. All other reagents were of tissue culture or the best grade available.

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test, or Fisher least significant difference test, as appropriate. A value of \( P < 0.05 \) was considered statistically significant.

**Results**

In human GMCs, PDGF-BB increased by several fold all indices of cellular growth, including DNA synthesis, collagen synthesis, and cell proliferation. In PDGF-BB–treated cells, Cl-Ad concentration-dependently attenuated all indices of cell growth (Figure 1). With the various pharmacological agents (AR subtype–selective and nonselective agonists and antagonists) listed in the Table, we further assessed the role of various AR subtypes (A1, A2A, A2B, and A3) in mediating the antimitogenic actions in GMCs. Only very high (10^{-6} \text{mol/L}) concentrations of CPA and CGS21680 decreased PDGF-BB–induced cell growth (Figure 1). MECA was more potent than NECA, and MECA and NECA were more potent than AB-MECA, CPA, and CGS21680 in reducing PDGF-BB–induced increases in cell growth (Figure 1). KF17837 and DPSPX, but not DPCPX, blocked the effects of Cl-Ad on all indices of cell growth (Figure 2). Also, the inhibitory effects of MECA were antagonized by KF17837 and DPSPX, but not by DPCPX (Figure 2). EHNA and IDO, administered separately, inhibited PDGF-BB–induced DNA synthesis, collagen synthesis, and cell proliferation, and these effects were enhanced in cells treated with EHNA plus IDO (Figure 3). Trypan blue exclusion tests demonstrated that none of the aforementioned treatments altered cell viability. Western blots of human GMC lysates showed the presence of A2B ARs (Figure 3).

Similar to human GMCs, the PDGF-BB–induced growth of rat GMCs was inhibited by Cl-Ad, MECA, and NECA, but not by CPA, CGS21680, or AB-MECA (Figure 4A). Compared with NECA, both MECA and Cl-Ad were more potent in inhibiting GMC growth. Moreover, the inhibitory actions of both MECA and NECA were significantly reversed by KF17837 and DPSPX, but not by DPCPX (Figure 4B). Rat
IDO (0.1 \text{mol/L}) trypan blue exclusion; however, at concentrations of 0.2 to 0.5 \text{mol/L}, antisense oligonucleotide did not cause cell toxicity, as assessed by cell lysates from 3 separate GMCs and the effects of human GMCs used for the growth study (lanes 1, 2, and 3 have been caused by downregulation of the A2B receptor, whereas CPA and CGS21680, which are selective A1 and A2A receptor agonists, respectively, were only mildly inhibitory to A2B receptors. Taken together with Cl-Ad, MECA, or EHNA plus IDO but not pretreated with CGS21680, AB-MECA, or CPA (Figure 6A). DPSPX, but not by DPCPX, attenuated the inhibitory effects of Cl-Ad and EHNA plus IDO (Figure 6A).

Similar modulatory effects of adenosine analogues were observed in rat GMCs (data not shown). Moreover, in rat GMCs, antisense, but not sense or scrambled, oligonucleotide abolished the inhibitory effects of Cl-Ad or EHNA plus IDO on PDGF-BB–induced MAPK activity (Figure 6B). Western blot analysis of lysates collected from human GMCs treated with Cl-Ad, MECA, or EHNA plus IDO showed a significant decrease in the expression of phosphorylated MAPK (ERK1/ERK2; Figure 6C).

These experiments provide evidence that exogenous as well as GMC-derived adenosine inhibits PDGF-BB–induced growth in human GMCs used for the growth study (lanes 1, 2, and 3 specify cell lysates from 3 separate GMCs) and the effects of EHNA (10 \text{ \mu mol/L}), IDO (0.1 \text{ \mu mol/L}), or EHNA (10 \text{ \mu mol/L}) plus IDO (0.1 \text{ \mu mol/L}) on PDGF-BB–induced [\text{H}]thymidine incorporation (DNA synthesis; top panel), cell number (middle panel), and [\text{H}]proline incorporation (collagen synthesis; bottom panel) in the presence or absence of KF17837 (KF; 10^{-8} \text{ mol/L}), DPSPX (10^{-8} \text{ mol/L}), or DPCPX (10^{-8} \text{ mol/L}) in human GMCs. Values represent mean±SEM from 4 separate experiments, each conducted in quadruplicate. *P<0.05, vs control; #P<0.05, vs EHNA or IDO alone; §P<0.05, vs EHNA plus IDO (significant reversal of the inhibitory effect). Abbreviations are as defined in text.

GMC growth was also inhibited by both EHNA and IDO, and this effect was antagonized by KF17837 and DPSPX, but not by DPCPX (Figure 4B).

In rat GMCs, antisense oligonucleotide increased basal and PDGF-BB–induced DNA synthesis by 65% and 69%, respectively; however, neither sense nor scrambled oligonucleotide altered basal or PDGF-BB–induced DNA synthesis (Figure 4C). The effect of antisense oligonucleotide on DNA synthesis was biphasic, with maximal stimulatory effects observed at 0.2 to 0.5 \text{ \mu mol/L} (Figure 4D). At 0.5 \text{ \mu mol/L}, antisense oligonucleotide did not cause cell toxicity, as assessed by trypan blue exclusion; however, at concentrations ≥1 \text{ \mu mol/L}, cell shrinkage and cell death were evident. The induction of growth by antisense oligonucleotide alone may have been caused by downregulation of the A2B receptor, which would attenuate the growth-inhibitory effects of endogenously produced adenosine. The lack of stimulatory effects at high concentrations may have been caused by cell toxicity. Antisense oligonucleotide, but not sense or scrambled oligonucleotide, attenuated the inhibitory effects of Cl-Ad, MECA, and EHNA plus IDO on PDGF-BB–induced cell proliferation, DNA synthesis, and collagen synthesis (Figure 5).

In human GMCs, PDGF-BB increased MAPK activity, from 0.625±0.07 to 10.87±0.9 \text{ pmol \cdot min^{-1} \cdot mg \ protein^{-1}}. The MAPK inhibitor PD98059 (10 \text{ \mu mol/L}) attenuated the stimulatory effects of PDGF-BB to 1.3±0.04 \text{ pmol \cdot min^{-1} \cdot mg \ protein^{-1}}. PDGF-BB–mediated stimulation of MAPK activity was reduced in GMCs pretreated for 24 hours with 1 \text{ \mu mol/L} Cl-Ad, MECA, NECA, or EHNA plus IDO but not pretreated with CGS21680, AB-MECA, or CPA (Figure 6A). DPSPX, but not by DPCPX, attenuated the inhibitory effects of Cl-Ad and EHNA plus IDO (Figure 6A).

Discussion

These experiments provide evidence that exogenous as well as GMC-derived adenosine inhibits PDGF-BB–induced growth and MAPK activity via the A2B receptor. In support of this conclusion, we found that treatment of both human and rat GMCs with a metabolically stable adenosine analogue (Cl-Ad) or with agents that increase endogenous adenosine (EHNA or IDO) inhibited PDGF-BB–induced MAPK activity (Figure 4B). In control rat GMCs, Cl-Ad increased cAMP levels by 5.5-fold (Figure 7). The stimulatory effects of Cl-Ad on cAMP synthesis activity were completely abolished in GMCs pretreated with antisense, but not sense or scrambled, oligonucleotide (Figure 7). Rat GMCs expressed A2B ARs. In GMCs treated with antisense, but not sense or scrambled, oligonucleotide, the expression of A2B ARs was abolished, as analyzed by Western blot analysis (Figure 7).
Metabolism of adenosine to inosine and AMP by adenosine deaminase with EHNA and adenosine kinase, respectively,\textsuperscript{14,15} importantly regulates cellular levels of adenosine, and blockade of adenosine deaminase with EHNA and adenosine kinase with IDO increase endogenous levels of adenosine.\textsuperscript{8} Treatment of GMCS with EHNA plus IDO inhibits PDGF-BB–induced GMC growth, and the inhibitory effects of EHNA plus IDO on GMC growth are attenuated by KF17837 and DPSPX, but not by DPCPX. These findings provide strong evidence that the inhibitory effects of EHNA plus IDO are mediated via generation of adenosine and that these effects are A\textsubscript{3b} AR mediated. The observation that the growth-inhibitory effects of EHNA plus IDO are abolished in GMCS pretreated with antisense oligonucleotide to A\textsubscript{3b} receptors provides strong evidence that the inhibitory effects of endogenous GMC-derived adenosine are A\textsubscript{3b} receptor mediated. The aforementioned findings provide the first evidence that endogenous adenosine also inhibits GMC growth via A\textsubscript{3b} receptors.

The MAPK pathway is activated by multiple growth factors\textsuperscript{10} and is involved in pathologic conditions associated with glomerular remodeling and abnormal growth of GMCS.\textsuperscript{11} MECA and CI-Ad, but not CPA, AB-MECA, or CGS21680, attenuate PDGF-BB–induced MAPK activity, and these effects are antagonized by KF17837 and DPSPX, but not by DPCPX. In additional, the inhibitory actions of MECA and CI-Ad on PDGF-BB–induced MAPK activity are abolished by antisense oligonucleotide to A\textsubscript{3b} receptors. Also, treatment of GMCS with EHNA plus IDO inhibits MAPK activity, and this inhibition is antagonized by DPSPX, but not by DPCPX. The inhibitory effects of EHNA plus IDO are also abolished by antisense to A\textsubscript{3b} receptors. Taken together, these findings suggest that A\textsubscript{3b} ARs inhibit GMC growth in part by reducing activation of the MAPK pathway.

In the present study, we measured MAPK activity in the presence of a protein kinase inhibitor, a calmodulin antago-
nist, and a calcium chelator. Thus, kinase activity from protein kinase A, calmodulin-dependent kinases, and calcium-dependent forms of protein kinase C were excluded. In addition, stimulation of kinase activity, as measured by our assay, was abolished by PD98059, a selective MAPK kinase (MEK) inhibitor. Because MEK selectively activates p42MAPK (ERK1) and p44MAPK (ERK2), the results with PD98059 suggest that the kinase activity measured with our assay was caused by p42MAPK and/or p44MAPK. This conclusion is supported by our observation that the expression of phosphorylated p42MAPK and p44MAPK was significantly inhibited in GMCs treated with Cl-Ad, MECA, or EHNA plus IDO.

In the present study, the expression of A2B receptors was abolished in GMCs treated with antisense oligonucleotide, but not in cells treated with sense or scrambled oligonucleotide. Also, the effect of Cl-Ad on cAMP production, a second messenger activated by A2B receptors, was abolished in GMCs treated with antisense, but not sense or scrambled, oligonucleotide. These findings confirm the conclusion that antisense oligonucleotide to the A2B receptor downregulates A2B receptor expression in GMCs.

Our finding that adenosine inhibits GMC growth, together with our previous observation that adenosine inhibits growth of renal arteriolar SMCs, suggests that adenosine may play an important role in regulating the remodeling process within the glomeruli as well as in preglomerular arterioles. Because abnormal growth of renal arteriolar SMCs and glomerulosclerosis are observed in hypertension, it is possible that suppressed adenosine levels or decreased expression of A2B receptors may contribute to hypertension-induced renal injury. This idea is supported by our previous finding that adenosine levels are decreased in renal arteriolar SMCs obtained from spontaneously hypertensive rats. However, detailed studies are required to elucidate the association between adenosine levels, A2B receptors, and the glomerular...
remodeling process in hypertension, diabetes, and renal disease.

Recent studies by Shen et al.\textsuperscript{16} have demonstrated that adenosine stimulates, rather than inhibits, porcine coronary artery SMC number, cellular DNA content, protein synthesis, and proliferating cell nuclear antigen staining. Also, in these cells, the mitogenic effects of adenosine were mediated by the A\textsubscript{1} AR. The findings of Shen et al illustrate that the overall effect of adenosine on growth is dependent on the specific cell type and species. This underscores the need to perform growth studies in human cells wherever possible. Because the present study was conducted with human GMCs, the results are applicable to human kidneys. We are currently investigating the effects of adenosine and adenosine analogues on the growth of human coronary artery SMCs and our results to date indicate that adenosine inhibits growth of human coronary artery SMCs via the A\textsubscript{2B} receptor (manuscript in preparation).

**Perspectives**

Our results provide evidence that adenosine inhibits GMC growth and that the inhibitory effects of adenosine are mediated via A\textsubscript{2B} ARs. Because abnormal growth of GMCs contributes importantly to the glomerular remodeling process associated with glomerulosclerosis, hypertension, and diabetes, our results suggest that abnormal and/or decreased expression of A\textsubscript{2B} receptors or decreased synthesis of adenosine may contribute to the abnormal growth of GMCs in various renal pathologies. Moreover, development of pharmacological agents that activate A\textsubscript{2B} receptors, as well as molecular targeting of the A\textsubscript{2B} receptor to increase its expression, may be of therapeutic importance in protecting against glomerular remodeling associated with glomerulosclerosis, renal disease, and abnormal GMC growth associated with hypertension and diabetes.

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**References**

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