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 N6-cyclopentyladenosine (selective A1 AR agonist), AB-N-MECA (selective A1 AR agonist), or CGS21680 (selective A2A AR agonist). KF17837 (selective A2AB 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 iodonotuberidin (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

Adenosine, a nucleoside long known as a “retaliatory” metabolite within the heart, has several physiologically significant effects on renal function.1 The biologic effects of adenosine are mediated by adenosine receptors (ARs), which exist in multiple subtypes (A1, A2A, A2B, and A3).1,2 However, participation of A1 and A2 ARs appears to be more important in renal biology, particularly with regard to reducing the risks and consequences of glomerular remodeling events associated with hypertension and diabetes.1 For example, activation of A1 receptors by adenosine attenuates the sympathetic nervous system by inhibiting the release of norepinephrine and attenuates the renin-angiotensin system by inhibiting renin release from juxtaglomerular cells.1 Both of these pathways are involved in the vascular and glomerular remodeling processes associated with hypertension, diabetes, and renal dysfunction. Through activation of A2 receptors, adenosine causes renal vasodilation, inhibits platelet aggregation, diminishes neutrophil adhesion to vascular endothelial cells, attenuates neutrophil-induced endothelial cell damage, and stimulates nitric oxide release from endothelial cells.3,4 These A2 receptor–mediated effects may increase renal medullary blood flow, resulting in decreased NaCl reabsorption, and may attenuate inflammation. Finally, adenosine induces apoptosis in glomerular mesangial cells (GMCs), an effect that may play an important role in regulating the glomerular remodeling process.5

Adenosine is a potent regulator of growth in both vascular and nonvascular cells.6–8 We have previously shown that adenosine inhibits growth of renal arteriolar9 and aortic smooth muscle 6 cells, which play a key role in vascular remodeling leading to vaso-occlusive disorders. Moreover, using receptor-specific agonists and antagonists as well as antisense oligonucleotides, we demonstrated that the antimitogenic effects of adenosine are mediated by activation of A2B receptors.6 The role of A2B receptors in mediating the antigrowth effects of adenosine is further supported by our recent
finding that this AR inhibits mitogen-induced growth of cardiac fibroblasts. 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. Therefore, it is important to determine the effects and mechanism of ARs on the growth of GMCs.

Because VSMCs are phenotypically similar to GMCs and because A_{3b} receptors are expressed in the kidney, we hypothesized that A_{3b} receptors mediate antiangiogenic actions in GMCs. Accordingly, the overall aim of the present study was to determine whether A_{3b} 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 A_{3b} receptors in inhibiting GMC growth, by using the sequence of the recently cloned A_{3b} receptor in rats, we designed antisense oligonucleotides to block A_{3b} receptor synthesis in rat GMCs.

### Methods

#### Materials

Adenosine, 2-chloroadenosine (Cl-Ad), *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA), and PDGF-BB were purchased from Sigma Chemical Co. N^6-cyclopentyladenosine (CPA), CGS21680, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), iodotubercidin (IDO), 1,3-dipropyl-8-p-sulphophenylxanthine (DPSPX), *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA), iodotubercidin (IDO).

To determine 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 A_{3b} receptors in inhibiting GMC growth, by using the sequence of the recently cloned A_{3b} receptor in rats, we designed antisense oligonucleotides to block A_{3b} receptor synthesis in rat GMCs.

#### Agents Used in the Study

**Pharmacological Agent**

<table>
<thead>
<tr>
<th>Pharmacological Agent</th>
<th>Pharmacological Characteristics</th>
</tr>
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<tbody>
<tr>
<td>2-Chloroadenosine (Cl-Ad)</td>
<td>Nonselective but stable adenosine analogue</td>
</tr>
<tr>
<td>5’-N-methylcarboxamidoadenosine (MECA)</td>
<td>Selective A_{3b} receptor agonist</td>
</tr>
<tr>
<td>4-Aminobenzyl-5’-N-methylcarboxamidoadenosine (AB-MECA)</td>
<td>Nonselective adenosine receptor agonist</td>
</tr>
<tr>
<td>5’-N-ethylcarboxamidoadenosine (NECA)</td>
<td>Selective A_{1} receptor agonist</td>
</tr>
<tr>
<td>N^6-Cyclopentyladenosine (CPA)</td>
<td>Nonselective adenosine receptor agonist</td>
</tr>
<tr>
<td>2-p-(2-Carboxyethyl)phenethylamino-5’-N-ethylcarboxamidoadenosine (CGS21680)</td>
<td>Selective A_{3b} receptor agonist</td>
</tr>
<tr>
<td>KF17837 (KF)</td>
<td>Selective A_{2} receptor antagonist</td>
</tr>
<tr>
<td>1,3-Dipropyl-8-p-sulphophenylxanthine (DPSPX)</td>
<td>Nonselective adenosine receptor antagonist</td>
</tr>
<tr>
<td>8-Cyclopentyl-1,3-dipropylxanthine (DPCPX)</td>
<td>Selective A_{1} receptor antagonist</td>
</tr>
<tr>
<td><em>Erythro</em>-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>

#### Growth Studies

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

#### MAPK Activity

MAPK activity in the cytosolic extracts of GCMs was quantified by our previously described radioactive method with myelin basic protein as the substrate and [32P]ATP. GCMs were pretreated with oligonucleotides for 72 hours and pretreated with other test agents for 24 hours before MAPK activity was measured.

#### Expression Studies With Western Blots

The expression of A_{3b} ARs in lysates from GCMs was analyzed by Western blotting and was probed with antibodies against A_{3b} receptors (rabbit anti-adenosine A_{3b} receptor polyclonal antibodies; Chemicon International Inc.). The expression of phosphorylated MAPK (p42MAPK/p44MAPK) in lysates from GCMs 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. In GCMs grown in parallel and treated similarly, the monolayers were inspected microscopically for intactness, and 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’s t-test.
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 severalfold 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 \( \times 10^{-6} \) 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). KF17837 and DPSPX, but not DPCPX, reduced the inhibitory effects of EHNA plus IDO on PDGF-BB–induced cell growth (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).
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 μmol/L (Figure 4D). At 0.5 μmol/L, antisense oligonucleotide did not cause cell toxicity, as assessed by trypan blue exclusion; however, at concentrations ≥1 μmol/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 pmol·min⁻¹·mg protein⁻¹. The MAPK inhibitor PD98059 (10 μmol/L) attenuated the stimulatory effects of PDGF-BB to 1.3±0.04 pmol·min⁻¹·mg protein⁻¹. PDGF-BB–mediated stimulation of MAPK activity was reduced in GMCs pretreated for 24 hours with 1 μmol/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).

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).

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).

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 and IDO) inhibited PDGF-BB–induced GMC DNA synthesis, collagen synthesis, cell proliferation, and MAPK activity. MECA, an adenosine agonist with high affinity for A2 receptors, expressed potency similar to that of Cl-Ad; however, neither sense nor scrambled oligonucleotide, to A2B receptors. Taken to-
Both MECA and NECA are AR agonists that activate multiple AR subtypes, including A2 receptors, and KF17837 and DPSPX are AR antagonists that block A2 and A3 receptors. The fact that MECA and NECA mimic the effects of Cl-Ad on GMC growth and the fact that KF17837 and DPSPX attenuate the inhibitory effects of Cl-Ad on GMC growth strongly support the conclusion that A2 receptors mediate the inhibitory effects on GMC growth. However, the mechanism by which A2 receptors mediate inhibition of GMC growth is not yet well understood. It has been suggested that A2 receptors may inhibit the growth of human and rat GMCs by reducing activation of the MAPK pathway.

The MAPK pathway is activated by multiple growth factors and is involved in pathologic conditions associated with glomerular remodeling and abnormal growth of GMCs. MECA and Cl-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. The inhibitory effects of MECA and Cl-Ad on PDGF-BB–induced MAPK activity are abolished in GMCs pretreated with antisense oligonucleotide to A2b receptors, which provides strong evidence that the inhibitory effects of endogenous GMC-derived adenosine are A2b receptor mediated. The aforementioned findings provide the first evidence that endogenous adenosine also inhibits GMC growth via A2b receptors.

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nyst, 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 $A_{2B}$ 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 $A_{2B}$ receptors, was abolished in GMCs treated with antisense, but not sense or scrambled, oligonucleotide. These findings confirm the conclusion that antisense oligonucleotide to the $A_{2B}$ receptor downregulates $A_{2B}$ 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 $A_{2B}$ 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, $A_{2B}$ receptors, and the glomerular...
remodeling process in hypertension, diabetes, and renal disease.

Recent studies by Shen et al. 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 A₁ 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 A₂B receptor (manuscript in preparation).

**Perspectives**

Our results provide evidence that adenosine inhibits GMC growth and that the inhibitory effects of adenosine are mediated via A₂B ARs. Because abnormal growth of GMCs contributes importantly to the glomerular remodeling process associated with glomerulosclerosis, hypertension, and diabe-

tes, our results suggest that abnormal or/and decreased expression of A₂B 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 A₂B receptors, as well as molecular targeting of the A₂B 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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