Extracellular Adenosine Induces Apoptosis of Human Arterial Smooth Muscle Cells via A2b-Purinoceptor

Marie-Line Peyot, Alain-Pierre Gadeau, Frédéric Dandre, Isabelle Belloc, Françoise Dupuch, Claude Desgranges

Abstract—Apoptosis of arterial smooth muscle cells (ASMCs) could play an important role in the pathogenesis of atherosclerosis and restenosis. Recent studies have demonstrated that extracellular adenosine induces apoptosis in various cell types. Our aim was to delineate the capacity of this nucleoside to induce ASMC apoptosis in arterial diseases. We demonstrate that adenosine dose-dependently triggers apoptosis of cultured human ASMCs. Apoptotic cell death was quantified by analysis of nuclear chromatin morphology and characterized by DNA laddering. The involvement of adenosine receptors was suggested, because neither an adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride, nor an inhibitor of cellular nucleoside transport, dipyridamole, was able to inhibit adenosine-induced ASMC apoptosis. In contrast, an A1/A2-adenosine receptor antagonist, xanthine amine congener, totally inhibited adenosine-induced apoptosis. Furthermore, among more selective inhibitors of P1 purinoceptor subtypes, only alloxazine, an antagonist of A1- and A2-adenosine receptors, completely inhibited adenosine-induced ASMC apoptosis, suggesting that adenosine triggers ASMC apoptosis via either 1 or both of these receptors. However, 8-cyclopentyl-1,3-dipropylxanthine, 8-(3-chlorostyryl) caffeine, and 3-ethyl-5-benzyl-2-methyl-4-(6)-dihydropyridine-3,5-dicarboxylate, which are A1-, A2a-, and A3-adenosine receptor antagonists, did not inhibit adenosine-induced apoptosis, suggesting an involvement of the A2b-receptor in this process. Moreover, the cAMP increase followed by cAMP-dependent protein kinase activation appears essential to mediate adenosine-induced ASMC apoptosis, thus confirming the previous hypothesis. These results indicate that adenosine-induced apoptosis of ASMCs is essentially mediated via A2b-adenosine receptor and involves a cAMP-dependent pathway. (Circ Res. 2000;86:76-85.)

Key Words: apoptosis ■ adenosine ■ arterial smooth muscle cell ■ A2b purinoceptor ■ cAMP

Migration of arterial smooth muscle cells (ASMCs) from the media to the intima and their proliferation in the intimal space is thought to be responsible for intimal ASMC hyperplasia and consequently for intimal atherosclerotic thickenings.1 ASMCs from normal adult artery are quiescent because of the balance between the growth promoters and growth inhibitors locally present.1,2 Disruption of this balance in favor of growth promoters, possibly occurring after endothelial dysfunction, could trigger ASMC proliferation.3 However, in advanced atherosclerotic lesions, the rate of ASMC growth is close to baseline levels.4 In addition, a decrease in cellular density has been described in atherosclerotic lesions, leading to acellular zones.5 This cell decrease can be attributed to cell oncisis of the various cell types constituting the intimal lesions, ie, ASMCs, macrophages, and T lymphocytes, in response to accumulation of toxic factors such as oxidized LDLs.6 Moreover, recent studies have demonstrated that this cell death could also be related to cell apoptosis, although cell oncisis remains possible.7-11 Cell apoptosis can be distinguished from cell oncisis by morphological and biochemical criteria.12 Indeed, cells undergoing death by oncisis are characterized by membrane disruption, swollen cell organelles, and lack of chromatin condensation. In contrast, apoptotic cells show a nuclear and cytoplasmic condensation followed by DNA degradation into multiples of 180-bp internucleosomal fragments.13 The major cell types undergoing apoptosis in human atherosclerotic lesions are ASMCs7,8,10 and macrophages.9 ASMC apoptosis occurs not only in atherosclerotic lesions but also in human restenotic intimal lesions.8 Furthermore, ASMC apoptosis has also been described in animal models of intimal thickenings.14 The role of apoptosis in intimal thickening is uncertain. Apoptosis of intimal ASMCs may be a normal process involved in the control of hyperplasia in evolving intimal thickenings. In contrast, the high rate of ASMC apoptosis detected in advanced atherosclerosis may contribute to the destabilization of the fibrous lesion and, consequently, promote plaque rupture and its clinical consequences.

The factors and mechanisms triggering apoptosis in atherosclerosis essentially remain unknown. However, recent
reports have shown that apoptosis of cultured ASMCs can be induced by physical agents; by growth factor deprivation; or by treatment with NO donors, oxidized LDL, reactive oxygen species, or various cytokines. Cyclic nucleotides probably participate in the ASMC apoptotic process via protein kinase activation. Because extracellular adenosine not only induces apoptosis of various cell types but also mediates an increase in NO production and cyclic nucleotides levels in vascular muscle, the effect of extracellular adenosine on ASMC apoptosis was evaluated. The various effects of extracellular adenosine were mediated by plasma membrane adenosine receptors of the P1 purinoceptor family. Currently, 4 adenosine receptors, termed A1, A2a, A2b, and A3, have been cloned and characterized by pharmacological studies. All of these P1 receptors belong to the family of 7 transmembrane G-protein–coupled receptors. However, A2a and A3 receptors activate adenylyl cyclase, whereas A1 and A2b receptors inhibit this enzyme. All 4 P1 receptors have been demonstrated in ASMCs.

In the present work, we show that extracellular adenosine induces apoptosis in cultured human ASMCs and demonstrate that this effect is essentially mediated via the A2b-adenosine receptor present on the ASMC surface membrane. Furthermore, we show that the cAMP-dependent pathway is involved in adenosine-induced apoptosis.

Materials and Methods

Materials

Propidium iodide (PI) and 9-(tetrahydro-2′-furyl)adenine (SQ 22586) were products of Calbiochem. Adenosine, dibutyl cAMP, forskolin, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), alloxazine, dipyridamole, erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA), Hoechst 33342 (H33342), and DNase-free RNase were purchased from Sigma. Xanthine amine congener (XAC), N′-cyclopentyladenosine (CPA), 2-(2′-[2-carboxyethylphenylethylamino])-5′-N-ethylcarboxamidoadenosine (CGS-21680), A8′(3′-iodobenzylo)-5′-N-methylcarboxamido adenosine (IB-MECA), 8-(3′-chlorostyryl) caffeine (CS), enprofylline, 3-ethyl-5-benzyl-2-methyl-4-phenethyl-6-phenyl-1,4-(±)-dihydropyridine-3,5 dicarboxylate (MRS 1191), and protein kinase inhibitor 5-24 (PKI 5-24) were obtained from Research Biochemicals, Inc. F-10 HAM medium, FCS, and trypsin-EDTA were purchased from GIBCO.

ASMC Isolation and Culture

Human ASMCs were isolated from tunica media of aorta as previously described. The cells were cultured in F-10 HAM medium supplemented with 10% FCS, penicillin (100 U/mL), streptomycin (100 μg/mL), and 10 mmol/L HEPES buffer. Cells were identified as ASMCs by their characteristic hills-and-valleys growth pattern and by immunofluorescence detection of α-actin using an anti-α-smooth muscle actin monoclonal antibody (Sigma). Cells were passaged by trypsinization when they reached confluence and were plated at a density of 10^4 cells/cm² in polystyrene 75-cm² flasks (Nunc, Inc). Culture media were changed every 2 days. Cells from passages 5 to 10 were used in this study.

Determination of Apoptosis

Experimental Protocols

ASMCs (1.6×10^5) in 250 μL of F-10 HAM medium supplemented with serum were seeded on coverslips (14-mm diameter) placed in 12-well culture plates (Falcon). After adhesion, 2 mL of F-10 HAM medium containing 10% FCS were added to each well. After 24 hours, the cells were rinsed with serum-free F-10 HAM medium (SFM) and then incubated in 2 mL of this medium with or without various agents to test for their susceptibility to induce apoptosis. Generally, ASMCs were incubated for 24 hours.

Quantitative Analysis of Apoptotic Nuclei by Fluorescent Microscopy

Cell apoptosis was detected after incubation of cells with the membrane-permeable fluorescent DNA-binding dye H33342. Indeed, nuclei from normal cells demonstrated a normal uniform chromatin pattern, clearly different from the characteristic condensed or fragmented chromatin pattern of apoptotic cells. The membrane-permeable DNA-binding dye PI was used to identify nonviable cells (cells in oncosis) with normal nuclei. At the end of each incubation period with the tested compounds, H33342 (10 μg/mL) and PI (1 μg/mL) were added to each well for an additional 30-minute incubation at 37°C. Coverslips were then placed upside down on a glass slide and immediately observed by fluorescent microscopy using a photomicroscope (Nikon Microphot FXA) equipped with an epifluorescence device. For each experiment, 300 to 400 nuclei from 5 random fields of each coverslip were examined at high magnification (×400). The percentage of apoptotic nuclei was calculated as follows: (number of apoptotic nuclei/total number of nuclei)×100. Each experiment was repeated 3 times. In these experiments, apoptosis was quantified on the basis of the number of adherent cells present after 24 hours of treatment. However, for high concentrations of adenosine (>500 μmol/L), some apoptotic cells were detached from the glass coverslips. Indeed, for 1 mmol/L adenosine, ~10% of total cells became nonadherent. Therefore, for these concentrations, the percentage of apoptotic nuclei was slightly underestimated.

Analysis of Internucleosomal DNA Fragmentation: DNA Laddering

ASMCs grown at confluence in 10% FCS in 75-cm² flasks were incubated for an additional 24 hours in SFM in the presence or absence of adenosine. DNA fragmentation was determined using an adaptation of a previously described technique. Briefly, after shaking the flasks, weakly adherent and nonadherent apoptotic cells were collected by centrifugation of the cell culture medium. Cells of the pellet were incubated at 37°C for 3 hours in a lysis buffer consisting of, in mmol/L, Tris-HCl (pH 8.0) 10, EDTA 5, and NaCl 100, as well as 0.5% (vol/vol) SDS and 10 μg/mL, proteinase K (Sigma) under agitation. This incubation was followed by dropwise addition of 5 mol/L NaCl to a final concentration of 1 mol/L and an incubation at 4°C for 1 hour. After centrifugation at 12 000 rpm for 30 minutes at 4°C, supernatants were recovered, and DNAs were extracted with an equal volume of 25:24:1 phenol/chloroform/isooamyl alcohol (vol/vol/vol) once. Cells were resuspended in cold lysis buffer containing 1% (wt/vol) Triton X-100; 0.25% e-amino-n-caprylic acid; and 25 mg/L α1-antitrypsin. The cells were then incubated for 45 minutes on ice and centrifuged at 12 000 rpm for 10 minutes at 4°C in a microcentrifuge. The supernatants were stocked at 4°C. DNA electrophoresis was carried out in 1.5% agarose gels containing 0.5 μg/mL ethidium bromide, and DNA fragments were visualized under UV light.

Western Blot Analysis

Protein extracts were obtained from cultures by rinsing the cells with ice-cold NaCl 9% (wt/vol) once. Cells were resuspended in cold lysis buffer containing, in mmol/L, Tris-HCl (pH 7.5) 50, NaCl 150, EDTA 3, and AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride; Interchim) 0.1; 1% (vol/vol) Triton X-100; 0.25% e-amino-n-caprylic acid; and 25 mg/L α1-antitrypsin. The cells were then incubated for 45 minutes on ice and centrifuged at 12 000 rpm for 10 minutes at 4°C in a microcentrifuge. The supernatants were stocked at 4°C. The protein concentrations were measured by the method of Bradford with a Bio-Rad protein assay kit according to the manufacturer’s instructions. BSA was used as a protein assay standard. Samples (80 μg protein) were denatured with SDS loading buffer at 95°C for 5 minutes and then separated under reducing conditions on a SDS/9% polyacrylamide gel with a 5% stacking gel in SDS/Tris/glycine running buffer. The protein was electrophoretically transferred to an Immobilon-P membrane (Millipore), which
Excess of the 16-amino acid peptide (ATNNSTEPWDGTT-)

was then blocked with 5% (wt/vol) nonfat milk in TTBS buffer

was demonstrated for an adenosine concentration of

which induced apoptosis by adenosine was confirmed

these necrotic cells represented only a low percentage

induction of ASMC apoptosis by adenosine was confirmed

was expressed as pmol cAMP/10^6 cells.

Statistical Analysis

Data are expressed as mean±SEM of 3 independent experiments. Paired data were evaluated by Student t test. A 1-way ANOVA was used for multiple comparisons. Significance was established when the probability value was <0.01.

Results

Induction of ASMC Apoptosis by Extracellular Adenosine

In controls, the majority of cells demonstrated a uniformly stained nucleus after staining with the membrane-permeable DNA-binding dye H33342 (Figure 1A). A 24-hour exposure of ASMCs to 1 mmol/L adenosine induced morphological changes typical of apoptosis in ~50% of the cells, such as membrane blebbing, cytoplasm condensation, and nucleus fragmentation with condensed chromatin, detected by staining with H33342 (Figure 1B). The nucleus of the remaining cells was uniformly stained with H33342. The nucleus of apoptotic cells was not stained by PI, a membrane-impermeable DNA-binding dye, demonstrating that these cells had not undergone a necrotic process. However, in ASMCs treated with high concentrations of adenosine (≥500 μmol/L), some cells had become permeable to PI. These necrotic cells represented only a low percentage (<2%) of total cells.

Induction of ASMC apoptosis by adenosine was confirmed by the presence of internucleosomal DNA fragmentation of adenosine-treated cells into multimers of 180-bp nucleosomal units (Figure 1C). In control cells, only a slight DNA fragmentation, probably due to growth factor deprivation, was detected.

Adenosine-induced ASMC apoptosis, measured by detection of chromatin condensation, was strongly dependent on the extracellular adenosine concentration (Figure 2A). A faint but significant increase in the percentage of apoptotic nuclei was demonstrated for an adenosine concentration of 1 μmol/L (9±1% versus 7±0.1% in control cultures, n=3; P<0.01). The number of apoptotic cells regularly increased up to 250 μmol/L adenosine and then dramatically increased for higher adenosine concentrations. An ED₅₀ of ~14 μmol/L can be deduced from the first part of the curve corresponding
to lower adenosine concentrations (1 to 250 μmol/L). Maximal apoptosis induction, reached for 1 mmol/L adenosine (49 ± 1% of apoptotic nuclei at 24 hours), was maintained for higher concentrations.

To assess the onset of adenosine-induced apoptosis, a time-course study was performed (Figure 2B). Addition of 1 mmol/L adenosine to human ASMCs resulted in the onset of apoptosis within 8 hours. Maximal apoptosis induction was reached within 28 hours. At this time, 60% to 65% of ASMCs were in apoptosis. A significant increase in the number of apoptotic cells was detected in ASMCs incubated with SFM (2.3 ± 0.6% at 4 hours versus 8.7 ± 1.2% at 8 hours [n=3]; P<0.01). However, this percentage remained low in comparison with that obtained with 1 mmol/L adenosine.

Adenosine induced apoptosis even in the presence of low percentages (0.1% and 0.5%) of serum in the culture medium, ie, in culture conditions adequate for a good survival of cultured SMCs, as demonstrated by the presence of cell divisions and the decrease in basal apoptosis (Table 1). Indeed, in the presence of 0.5% FCS, there was a significant decrease in the percentage of apoptotic nuclei of untreated ASMCs (4.3 ± 0.5% versus 7.1 ± 0.5% in SFM). In these conditions, increases in SMC apoptosis induced by 1 to 100 μmol/L adenosine were close to those induced in the absence of serum. The presence of platelet-derived growth factor (20 ng/mL), an inhibitor of growth factor depletion–induced apoptosis,16 in SFM decreased the basal level of SMC apoptosis. Adenosine (1 μmol/L)–induced apoptosis was also faintly decreased (Table 2). In contrast, platelet-derived growth factor did not inhibit the apoptosis induced by higher doses of adenosine (10 and 100 μmol/L).

### Cellular Mechanisms of Adenosine-Induced ASMC Apoptosis

Adenosine-induced ASMC apoptosis might occur in 1 of the following 3 different ways: (1) via a catabolic product of adenosine, (2) intracellularly after adenosine uptake by ASMCs, and (3) after adenosine binding to 1 or more of its P1-specific receptors.

### Table 1. Effect of FCS on Adenosine-Induced ASMC Apoptosis

<table>
<thead>
<tr>
<th>Adenosine, μmol/L</th>
<th>% FCS in Medium</th>
<th>% Apoptotic nuclei*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>7.1 ± 0.5</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>5.7 ± 0.5</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>10.3 ± 0.5</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>10.1 ± 0.5</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>8.3 ± 1.7</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>15.7 ± 0.9</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>14.0 ± 1.4</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>12.3 ± 1.7</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>21.2 ± 0.5</td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>19.1 ± 0.5</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>17.6 ± 0.6</td>
</tr>
</tbody>
</table>

*Cells were incubated with different quantities of FCS in culture medium for 24 hours and stained with H33342 and PI. Apoptotic nuclei exhibiting characteristic chromatin condensation were counted by fluorescent microscopy. Values are mean ± SEM of 3 experiments.

### Table 2. Effect of PDGF on Adenosine-Induced ASMC Apoptosis

<table>
<thead>
<tr>
<th>Adenosine, μmol/L</th>
<th>PDGF, ng/mL</th>
<th>% Apoptotic nuclei*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>7.5 ± 0.5</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>12.5 ± 2.5</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>7.5 ± 1.5</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>16.5 ± 2.5</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>15.0 ± 2.0</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>22.5 ± 1.5</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>22.5 ± 2.5</td>
</tr>
</tbody>
</table>

*Cells were incubated with PDGF (20 ng/mL) in SFM for 24 hours and stained with H33342 and PI. Apoptotic nuclei exhibiting characteristic chromatin condensation were counted by fluorescent microscopy. Values are mean ± SEM of 3 experiments.
As shown in Figure 3A, the adenosine deaminase inhibitor EHNA, which blocked the catabolism of adenosine to inosine, was unable to inhibit adenosine-induced ASMC apoptosis. Indeed, percentages of apoptotic nuclei after treatment of ASMCs in the presence of adenosine (100 μmol/L), and adenosine (100 μmol/L) plus EHNA (10 μmol/L), were 21±2% for adenosine and 21±1% for adenosine plus EHNA, a condition in which adenosine degradation was entirely inhibited. This result suggests that adenosine induces ASMC apoptosis by itself, but not via one of its catabolic products, as confirmed by the fact that the first adenosine catabolic product (inosine) only induced a faint apoptosis in comparison with adenosine (21±2% for 100 μmol/L adenosine versus a mean of 10±1% for 100 μmol/L inosine and 8±1% in control).

To determine whether adenosine-induced apoptosis requires adenosine entry into the cells, the action of dipyridamole, an inhibitor of facilitated intracellular transport of adenosine, was studied. As shown in Figure 3B, coadministration of 1 μmol/L dipyridamole with 100 μmol/L adenosine had no effect on adenosine-induced ASMC apoptosis compared with 100 μmol/L adenosine alone. Indeed, the percentages of apoptotic nuclei in ASMCs incubated for 24 hours with 100 μmol/L adenosine in the presence and absence of dipyridamole were 20±0.1% with 1 μmol/L dipyridamole and 19.7±0.6% without (n=3, P>0.01).

Because adenosine did not induce apoptosis of human ASMCs either via adenosine catabolic products or by an intracellular pathway, the role of P1-adenosine receptors was considered. To study this pathway, we used XAC, an A1/A2-adenosine receptor antagonist. Adenosine-induced apoptosis was dose-dependently inhibited by the simultaneous application of XAC (Figure 3C). At 10 μmol/L, XAC entirely inhibited apoptosis induced by 100 μmol/L adenosine in human ASMCs. These findings suggest that adenosine induces apoptosis via a P1-purinoceptor.

**Role of A2b Purinoceptor in Human ASMC Apoptosis**

To determine the adenosine receptor subtypes involved in ASMC apoptosis induction, we first studied the effects of various adenosine receptor agonists. The partially selective agonists used in our experiments were CPA, an A1-adenosine receptor agonist; CGS-21680, an A2a-adenosine receptor agonist; and IB-MECA, an A3-adenosine receptor agonist. As

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**Figure 3.** Effect of EHNA, dipyridamole, and XAC on adenosine-induced apoptosis of ASMCs. A, Effect of EHNA, an inhibitor of adenosine degradation. Percentages of apoptotic ASMCs were determined after a 24-hour incubation in SFM (control), SFM with 100 μmol/L adenosine, SFM with 10 μmol/L EHNA, or SFM with 100 μmol/L adenosine plus 10 μmol/L EHNA (n=3). B, Effect of dipyridamole, an inhibitor of adenosine uptake. Percentages of apoptotic ASMCs were determined after a 24-hour treatment in SFM (control), SFM with 100 μmol/L adenosine, SFM with 1 μmol/L dipyridamole (DIP), or SFM with 100 μmol/L adenosine plus 1 μmol/L dipyridamole (n=3). C, Effect of XAC, a nonspecific inhibitor of adenosine receptors. Percentages of apoptotic ASMCs were determined after a 24-hour exposure to 100 μmol/L adenosine (black bar), to increasing concentrations of XAC (white bar), or to increasing concentrations of XAC plus 100 μmol/L adenosine (gray bar). Control in SFM corresponds to the white bar without XAC. XAC (10 μmol/L) completely inhibited adenosine-induced ASMC apoptosis. NS indicates no significant difference from the adenosine 100 μmol/L–treated cell value (P>0.01). “Significant difference from the adenosine 100 μmol/L–treated cell value (P<0.01).

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**Figure 4.** Effects of partially selective adenosine receptor agonists. Line graph shows the concentration-response relationships for the induction of ASMC apoptosis by a 24-hour incubation with CPA (A1-adenosine receptor agonist), with CGS-21680 (A2a-adenosine receptor agonist), and with IB-MECA, an A3-adenosine receptor agonist. As

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apoptosis may involve mediation via the A2bR, given that A2bR, A2aR and A1R-receptor antagonists did not block the proapoptotic effect of adenosine, whereas nonspecific A2bR antagonists inhibited this effect.

Involvement of cAMP-Dependent Pathway in Adenosine-Induced ASMC Apoptosis

Activation of A2bRs, which are positively coupled with adenylate cyclase, results in a significant increase in intracellular cAMP levels. In contrast, stimulation of A1R-receptor induces a cAMP decrease. Therefore, to confirm the role of A2bR in adenosine-induced apoptosis, we examined the role of the cAMP-dependent cell-signaling pathway in this process.

First, we found that adenosine concentrations from 1 to 100 μmol/L triggered a dose-dependent increase in ASMC cAMP levels (Table 3). The SMC cAMP content was increased by 3.4-fold after 100 μmol/L adenosine treatment.

Second, we demonstrated that the cAMP elevation induced ASMC apoptosis by the following 3 experimental approaches: (1) using a stable cAMP analogue, (2) by stimulation of adenylate cyclase, and (3) by inhibition of cAMP-dependent phosphodiesterase. As shown in Figure 6A, the administration of dibutyl cAMP, a membrane-permeable cAMP analogue, induced ASMC apoptosis in a manner similar to that observed with adenosine. The apoptotic effects of adenosine on ASMC were also mimicked in a concentration-dependent manner by forskolin38 (Figure 6B), an activator of adenylate cyclase, and by rolipram39 (data not shown), an inhibitor of cAMP-dependent phosphodiesterase. Forskolin at 5 μmol/L induced a cAMP increase comparable with that triggered by 100 μmol/L adenosine (Figure 6C). In both cases, the percentage of apoptotic cells was in the range of 20%.

Because the increase in cAMP levels triggered ASMC death, we tested the hypothesis that adenosine-induced apoptosis is mediated by a cAMP-dependent pathway. Therefore, we investigated the effect of blocking cAMP accumulation by inhibition of adenylate cyclase. The adenylate cyclase inhibitor SQ 22536 with adenosine (100 μmol/L) significantly abolished adenosine-induced ASMC apoptosis (Figure 7A). Furthermore, we tested the hypothesis that the induction of ASMC apoptosis by adenosine was related to the cAMP-dependent activation of cAMP-dependent protein kinase. The
cAMP-dependent protein kinase inhibitor PKI 5-24 alone failed to trigger ASMC apoptosis (Figure 7B). However, coincubation of PKI 5-24 with adenosine (100 \( \mu \text{mol/L} \)) blocked adenosine-induced ASMC apoptosis. These findings confirm the role of A2bR in adenosine-induced apoptosis and suggest that this induction is essentially mediated by a cAMP-dependent pathway.

**A2b Expression in Cultured ASMCs**

Western analysis of cultured ASMCs using an anti-A2bR antibody identified a protein band of \( \approx 52 \text{ kDa} \) (Figure 8), corresponding to the molecular mass expected for this receptor (50 to 55 kDa). To confirm that the 52-kDa band was due to the A2bR, we preincubated the anti-A2bR antibody with a 40-fold excess of the A2bR competitor peptide. In this condition, the 52-kDa immunoreactive band was not detected; nor was it detectable when blots were developed with secondary antibody alone (data not shown).

**Discussion**

The present study tests the potentiality of extracellular adenosine to trigger ASMC death by apoptosis. The addition of adenosine to the culture medium of human ASMCs causes an extensive cell death due mainly to apoptosis, as suggested by chromatin condensation and internucleosomal DNA fragmentation. An ASMC apoptosis increase was detected with a 1 \( \mu \text{mol/L} \) adenosine concentration, and a maximal value was reached at 1 mmol/L. However, high adenosine concentrations (up to 500 \( \mu \text{mol/L} \)) could also trigger ASMC death by oncosis, but only in a low percentage of dying cells.
Among the various mechanisms that could be involved in adenosine-induced apoptosis (ie, activation of adenosine membrane receptors, intracellular action of adenosine, or generation of an active adenosine catabolic product), it appears that adenosine acts through its binding to a specific adenosine membrane receptor of ASMCs. Indeed, neither the blockade of adenosine catabolism nor that of adenosine intracellular uptake inhibited adenosine-induced apoptosis. Consequently, it seems that these apoptotic effects of adenosine result from an extracellular action that is presumably receptor mediated. As a confirmation of this hypothesis, complete inhibition of adenosine-induced apoptosis was achieved by XAC, an antagonist of A1- and A2-adenosine receptors. Moreover, apoptosis induced by adenosine was entirely inhibited by 10 μmol/L alloxazine or enprofylline. At this concentration, alloxazine presents a higher specificity for A1- and A2-adenosine receptors but was not effective on A2-adenosine receptor, which has been demonstrated to mediate adenosine-induced apoptosis in other cell types. Similarly, enprofylline was identified as a selective antagonist of recombinant A2B receptor. The involvement of A2B receptors is suggested by the fact that alloxazine and enprofylline inhibited adenosine-induced ASMC apoptosis, whereas DPCPX, CSC, and MRS 1191, the respective antagonists of A1-, A2-, and A3-receptors, were unable to block it.

Furthermore, adenosine activation led to an increase in cellular cAMP content, a characteristic of the A2B receptor that stimulates adenylate cyclase via G protein activation. Consequently, we hypothesize that adenosine-induced apoptosis is mediated, at least in part, via a cAMP increase. We observed that the apoptotic effect of adenosine was mimicked by a membrane-permeable cAMP analogue or by an increase in intracellular cAMP, by stimulating adenylate cyclase or inhibiting phosphodiesterase. The role of the cAMP-dependent cell-signaling pathway in adenosine-induced ASMC apoptosis was evidenced, demonstrating that the apoptotic effects of adenosine were almost entirely abolished both when cAMP elevation was blocked by adenylate cyclase inhibition and when protein kinase A activity was specifically inhibited. Moreover, a clear parallelism was demonstrated between the cAMP level increase and the percentage of apoptotic cells after SMC treatment with adenosine concentrations ranging from 1 to 100 μmol/L, ie, for concentrations around the EC50 value found for adenosine-induced ASMC apoptosis (14 μmol/L) and for adenosine-induced cAMP level (7 μmol/L) in ASMCs and in cells expressing the recombinant adenosine low-affinity A2B receptor (10 μmol/L). Taken together, these findings provide the first evidence that extracellular adenosine may trigger ASMC apoptosis via the A2B receptor and suggest that this nucleoside induces ASMC apoptosis by stimulation of the cAMP/PKA signal transduction pathway. The role of A2B is strengthened by the demonstration of A2B receptor on the surface membrane of cultured human ASMCs. These results confirm the role of adenosine in cell death but also the variability of the mechanisms involved in adenosine-induced apoptosis according to the cell type. Indeed, it has been shown that adenosine may induce apoptosis via its internalization or by binding to specific P1 receptor(s). In this case, the intracellular mechanisms are poorly understood, although cAMP seems to be involved in some cases. Our study clearly demonstrates that the apoptotic effect of adenosine on ASMC depends on binding on the A2B receptor and on the cAMP pathway.

Extracellular adenosine might thus be involved in the ASMC apoptosis arising in atherosclerotic and restenotic intimal lesions and consequently might play an important role both in the control of hyperplasia and in plaque weakening. Moreover, extracellular adenosine has been described as an inhibitor of ASMC proliferation in vitro and in vivo. The A2B receptor seems particularly involved both in adenosine-induced apoptosis (this study) and in adenosine-mediated inhibition of proliferation of ASMCs, probably by increasing the cAMP concentration. However, other intracellular pathways might be involved in adenosine-inhibited ASMC growth. Indeed, adenosine enhances the NO or the cytokine-induced NO synthesis in rat aortic ASMCs and in vivo. It seems that NO, which may be generated during adenosine treatment, does not intervene significantly in apoptosis induction by adenosine, given that NO-donor d-arginine (NO synthase inhibitor) did not block adenosine-induced apoptosis (data not shown). Moreover, although adenosine induces NO production, this activation does not depend on the cAMP/PKA pathway, whereas adenosine-induced apoptosis is greatly dependent on it.

The minimal concentration of adenosine required to induce ASMC apoptosis was 1 μmol/L. Adenosine concentrations of 1 to 100 μmol/L increase the percentage of apoptosis in the range of 2% to 13% of apoptotic cells, a value compatible with the apoptosis rate found in atherosclerotic plaque or in restenotic lesions. The adenosine concentration in the interstitial space within the arterial space or intimal thickening has not yet been evaluated. However, interstitial adenosine concentrations have been evaluated in some tissues. In skeletal muscle, this concentration was 0.44 μmol/L during normoxia and 0.85 to 1.03 μmol/L during hypoxia. The 0.2 to 1 μmol/L basal concentration found in myocardial interstitial fluid in-
creases to 0.7 to 6 μmol/L during ischemia.52–55 For the arterial wall, several in vitro studies demonstrate that both endothelial cells and ASMCs release adenosine and its precursor ATP on stimulation by various physicochemical agents such as hypoxia, shear stress, or free radicals,56,57 thus suggesting that extracellular adenosine concentrations comparable with those found in other tissues may occur. Extracellular concentrations of ATP and adenosine released by endothelial cells or ASMCs are dependent of the volume of the local extracellular compartment. However, recent studies have demonstrated that the concentration of released ATP could be higher in the vicinity of the releasing cells because of a concentration gradient58 or the release in membrane structures also containing the effector system, particularly purinergic receptors.59 It is also noticeable that ASMCs can metabolize cAMP to generate releasable adenosine via the cAMP-adenosine pathway.25 Therefore, the cAMP increase induced by A2bR stimulation not only directly triggers ASMC apoptosis, but also promotes the regeneration of adenosine, hence allowing the maintenance of the adenosine proapoptotic effect. In addition to the release from intact cells, adenosine and its precursors can be released in large quantities from dying cells found within intimal thickenings.5–10 So, adenosine concentrations in the range 1 to 10 μmol/L could be generated within the intimal thickenings and participate in the ASMC apoptosis found in these lesions.

Therefore, adenosine could play a dual role in the evolution of intimal thickening. First, it could restrict intimal hyperplasia not only via its antiproliferative45,46 but also by promoting re-endothelialization via its mitogenic effect on endothelial cells.60 Secondly, it could play an important role in the formation of the necrotic core in advanced atherosclerotic lesions by triggering not only the death of ASMCs but also that of macrophages via the NO pathway.61,62 Indeed, an initial event such as hypoxia63 could induce a first wave of cell death due to adenosine release. Adenosine generated from ATP64 released from dead cells might in turn lead to the lysis of surrounding cells and, by a cascade of events, to necrotic core spread. Then, in synergy with other events such as matrix degradation, this might lead to plaque rupture. Furthermore, adenosine could also participate in the decrease in the number of cells in the fibrous cap and thus promote plaque weakening.

Therefore, according to the status of arterial intimal lesions, adenosine can be considered either as a beneficial factor controlling intimal thickening formation and even regression14 or as a deleterious one leading to plaque rupture and its dramatic clinical consequences. Consequently, if these opposing effects were confirmed in vivo, different strategies favoring the role of adenosine during intimal lesion development, or in contrast inhibiting its effects in advanced plaques, might be considered.

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References


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