Abstract—Adenosine modulates a variety of cellular functions by interacting with specific cell surface G protein–coupled receptors (A1, A2A, A2B, and A3) and is a potential mediator of angiogenesis through the A2B receptor. The lack of a potent, selective A2B receptor inhibitor has hampered its characterization. Our goal was to design a hammerhead ribozyme that would specifically cleave the A2B receptor mRNA and examine its effect on retinal angiogenesis. Ribozymes specific for the mouse and human A2B receptor mRNAs were designed and cloned in expression plasmids. Human embryonic kidney (HEK) 293 cells were transfected with these plasmids and A2B receptor mRNA levels were determined by quantitative real-time RT-PCR. Human retinal endothelial cells (HRECs) were also transfected and cell migration was examined. The effects of these ribozymes on the levels of preretinal neovascularization were determined using a neonatal mouse model of oxygen-induced retinopathy (OIR). We produced a ribozyme with a Vmax of 515 ± 125 pmol/min and a Kcat of 36.1 ± 8.3 min−1 (P = 1 × 10−3). Transfection of HEK293 cells with the plasmid expressing the ribozyme reduced A2B receptor mRNA levels by 45 ± 4.8% (P = 5.1 × 10−5). Transfection of HRECs reduced NECA-stimulated migration of cells by 47.3 ± 1.2% (P = 7 × 10−5). Intraocular injection of the constructs into the mouse model reduced preretal neovascularization by 53.5 ± 8.2% (P = 4.5 × 10−5). Our results suggest that the A2B receptor ribozyme will provide a tool for the selective inhibition of this receptor and provide further support for the role of A2B receptor in retinal angiogenesis. (Circ Res. 2003;93:500-506.)

Key Words: adenosine receptors ■ neovascularization ■ ribozymes ■ retinopathy

Angiogenesis, the formation of new blood vessels from either preexisting vasculature or circulating stem cells, occurs as part of both normal development and pathologies of the retina. Retinal ischemia stimulates abnormal angiogenesis in conditions such as proliferative diabetic retinopathy (PDR) and retinopathy of prematurity (ROP). Substantial evidence supports a role for adenosine in promoting each step associated with angiogenesis.1,2 Adenosine can act as a mitogen in endothelial cells derived from numerous vascular beds to increase cell number,3–6 DNA synthesis,7 cell migration, and vascularization.8 Endothelial cells are known to have a very active adenosine metabolism.9 Adenosine is a critical mediator of blood flow changes in response to ischemia and is a significant component of the retina’s compensatory hyperemic response to ischemia, hypoxia, and hypoglycemia.10 In the retinal microvasculature, adenosine and adenosine analogues cause concentration-dependent vasodilation.11,12 These observations strongly support a role for endogenously released adenosine as a key mediator of retinal blood flow during conditions of reduced oxygen supply.13

Adenosine interacts with at least four subtypes of G protein–coupled receptors (A1, A2A, A2B, and A3),13,14 which are encoded by distinct genes and are differentiated based on their affinities for adenosine agonists and antagonists.15 The A1 and A2A (high affinity) receptors are activated by submicromolar concentrations of adenosine and the A3 and the A1 receptors (low affinity) are activated when adenosine levels rise to micromolar range.16 The receptors are also differentiated based on their signal transduction pathways.14 A1 and A2A receptors interact with the Gαi and Gαs family to inhibit adenylate cyclase, whereas A2A and A3 receptors interact with Gαi and Gαq to stimulate adenylate cyclase.15 Linden et al16a have shown that overexpression of A2A receptor results in cAMP accumulation and phospholipase C activation. On the other hand, A2B receptor antagonists (eg, enprofylline) inhibit agonist stimulated cAMP accumulation.17 In most cell types and organ systems, adenosine increases oxygen supply by activating the A1 receptors and decreases oxygen demand by activating the A2 receptor, demonstrating that adenosine may be a protective metabolite that rectifies imbalances between oxygen supply and demand.18 A2B receptors are associated with vessels and the A2A and A2B receptors have been implicated in angiogenesis. We have demonstrated that S′-(N-ethylcarboxamido)adenosine
(NECA) interacts with the A2B receptor in human retinal endothelial cells (HRECs) and stimulates cell migration and proliferation.\textsuperscript{19} NECA is a stable analogue of adenosine and activates all four adenosine receptors although with different potencies. Our findings and those of Sexl et al\textsuperscript{20} are in contrast with the work of Van Daele et al,\textsuperscript{21} who reported that adenosine stimulates only DNA synthesis in bovine aortic endothelial cells. These results suggest that endothelial cell populations differ in response to adenosine or adenosine analogues, and these differences may be attributed to differences in both the species and vascular beds studied. Takagi et al\textsuperscript{22} reported that endogenously released adenosine stimulates VEGF expression in bovine retinal endothelial cells and pericytes through stimulation of the A2A receptor. Lutty et al\textsuperscript{23} demonstrated that A2A receptors localized to the edge of the developing vasculature in canine retina. Taomo et al\textsuperscript{24} also demonstrated high levels of A2A receptor immunoreactivity in immature intravitreal neovascular formations in the canine oxygen-induced retinopathy (OIR) model. Our in vitro studies, and others, using human cells, including HRECs,\textsuperscript{6} implicate the A2B receptor in release of VEGF, IL-8, and FGF-2. In addition, the results of our in vivo studies in a mouse model of OIR also demonstrate the role of the A2B receptor in retinal neovascularization.\textsuperscript{25}

Hammerhead ribozymes are catalytic RNA molecules that cleave phosphodiester bonds within RNA nucleotides,\textsuperscript{26} and it has been proposed that ribozymes may act as potential inhibitors of cell proliferation.\textsuperscript{27} Several groups have used antisense RNA to control the expression of the A2B receptor in animals and in tissue culture.\textsuperscript{28-30} These results indicate several sites in the A2B receptor mRNA that are accessible to antisense oligoribonucleotides and, hence, to ribozyme cleavage. Because of their catalytic ability, a lower concentration of ribozyme molecules is required to achieve efficient inhibition of mRNA expression. Therefore, ribozymes should be more effective than antisense RNA in reducing the expression of the A2B receptor.\textsuperscript{31}

Our goal was to target the A2B receptor with a hammerhead ribozyme to further demonstrate its role in angiogenesis. We designed and tested hammerhead ribozymes that specifically cleave the A2B adenosine receptor mRNA of mouse and human (Figure 1). We performed in vitro testing of the reaction kinetics of these ribozymes and determined the effect of these ribozymes on the migration of HRECs, and on the mRNA levels in HRECs and in human embryonic kidney (HEK) 293 cells. We characterized their effects on the level of preretal neovascularization in the mouse model of OIR. Our results demonstrate that A2B receptor ribozymes are effective at reducing the levels of the A2B receptor mRNA and in inhibiting human retinal endothelial cell migration in vitro, as well as in reducing preretal neovascularization in neonatal mice.

Materials and Methods

Synthetic RNA Targets and Ribozymes

We designed the A2B receptor ribozymes based on the mouse sequence of the A2B receptor (PubMed accession No. NM_007413). The sequences of these ribozymes and targets are shown in Figure 1. RNA oligonucleotides for the active and inactive mouse A2B receptor hammerhead ribozymes and mouse and human targets were purchased from Dharamco (Boulder, Colo) and deprotected following the manufacturer’s protocol. RNA oligonucleotides were 5’-end labeled with [γ-32P]-dATP (ICN) using polynucleotide kinase (Promega).

Time Course Analysis of Ribozyme Cleavage and Multiple-Turnover Kinetic Analysis

Time course analysis of ribozyme cleavage and multiple-turnover kinetic analysis were performed using the RNA oligonucleotides as previously described.\textsuperscript{32-34}

Cloning of the Hammerhead Ribozymes Into the rAAV Expression Vector

Two complementary DNA oligonucleotides (Life Technologies) were annealed in order to produce a double-stranded DNA fragment coding for each hammerhead ribozyme. All DNA oligonucleotides were synthesized with 5’-phosphate groups. The DNA oligonucleotides were designed to generate a cut HindIII site at the 5’-end and a cut SpeI site at the 3’-end after annealing. The DNA oligonucleotides were incubated at 65°C for 2 minutes and annealed by slow cooling to room temperature for 30 minutes. The resulting double-stranded DNA fragment was ligated into the HindIII and SpeI sites of the rAAV vector pTRUF-21 (UF Vector Core, http://www.gtc.nfl.edu/gtc-home.htm). A self-cleaving hairpin ribozyme has been cloned downstream of the inserted hammerhead ribozymes into the SpeI and SfiI sites. This vector has the CMV/β-actin chimeric enhancer-promoter and results in the hairpin ribozyme cleaving hairpin ribozyme downstream of the 3’-end of the hammerhead ribozymes. We term the vector with the hairpin ribozyme alone pl21NewHp. We cloned genes for inactive ribozymes (Figure 1), and verified in time course experiments that these ribozymes were inactive (data not shown). The ligated plasmids were transformed into SURE electroporation competent cells (Stratagene) in order to maintain the integrity of the inverted terminal repeats. The ribozyme clones were verified by sequencing.

Transfection of HRECs

HRECs were transfected with plasmids expressing the A2B receptor ribozyme constructs as previously described.\textsuperscript{35,36}

Relative Quantitative RT-PCR

Relative quantitative RT-PCR was performed on RNA isolated from HRECs transfected with plasmids expressing ribozymes (A2B Rx2 active and inactive) and pl21NewHp. RNA was isolated from transfected HRECs using either the GenElute Direct miRNA Miniprep Kit (Sigma) for mRNA or the TRIzol Reagent (Invitrogen) for total RNA. Reverse transcription was accomplished using Super-
script reverse transcriptase and a random hexamer (Invitrogen) according to manufacturer’s protocols.

PCR reactions to determine A2B receptor mRNA levels used gene-specific DNA oligonucleotides synthesized by Invitrogen (5'-GTACGTGGCGCTGGAGCTGG-3' and 5'-CTTGTCTCGGGTCCCCGTGAC-3'). The linear range of the amplification of the A2B receptor RT-PCR product was determined by using a master PCR mix (1 μL RT product/50 μL, 200 μmol/L dNTPs, 1 mmol/L MgCl2, 0.4 μmol/L A2B oligonucleotides, 1X REDTaq DNA polymerase buffer [Sigma], 2 U REDTaq DNA polymerase [Sigma], and 0.5 μCi/50 μL [α32P]-dATP [ICN]). This master mix was separated into eight 0.2 mL tubes, and amplification was performed with an annealing temperature of 61°C. Samples were removed at even-numbered cycles starting at cycle 26. For each PCR sample, 5 μL was removed and 2 μL of formamide dye mix was added. The reaction products were separated on a 6% polyacrylamide-8 mol/L urea gel. Dried gels were analyzed on a Molecular Dynamics PhosphorImager (Amersham) to determine the linear range of amplification. For this oligonucleotide pair, cycle number 34 was determined to be within the linear range of amplification and was used in subsequent experiments.

In the relative quantitative RT-PCR assays the level of A2B receptor mRNA was determined within each sample relative to an internal β-actin standard. β-actin mRNA levels were determined using the QuantumRNA β-actin primer/competimer oligonucleotide set from Ambion. The competitor oligonucleotide primer set anneals to the same target as the primer oligonucleotide pair but they are blocked at their 3'-ends to prevent extension. This primer/competimer oligonucleotide set allowed us to determine the ratio of primer to competitor that yields a β-actin PCR fragment that is approximately equal molar to the A2B receptor PCR product. To determine the ratio of the primer/competimer oligonucleotide set required to achieve this, PCR reactions were performed as described above and amplification proceeded for 34 cycles. The ratio of primer to competitor oligonucleotide was determined to be 10:1 at a final concentration of 0.4 μmol/L for the combined primer/competimer mixture.

Migration Assays on Transfected HRECs
Transfected cells were assayed for their ability to migrate in response to NECA using a modified Boyden chamber assay as previously described.32 Cells (30,000 cells/well) were added to the bottom wells of modified Boyden chambers, which are then fully assembled with a collagen-coated, pyrrolidone- and pyrogen-free porous membrane. The cells adhere to the membrane for 4 hours at 37°C, after which 50 μL of the solution containing NECA was added to the wells. The chambers were then returned to 37°C for 12 hours to allow cell migration. The chambers were then disassembled and the membranes scraped on the side where the cells have adhered. The membranes are then fixed and stained with hematoxylin and eosin. Individual nuclei extending beyond the inner limiting membrane into the vitreous were counted.38 The efficacy of treatment with each plasmid was then calculated as the percent average nuclei per section in the injected eye versus the uninjected contralateral eye.

Results
Time Course Analysis of Ribozymes
Time course experiments indicated that A2B receptor ribozymes rapidly cleaved synthetic targets either under standard in vitro conditions (20 mmol/L MgCl2) or at room temperature in physiological magnesium conditions (Figure 2). Figure 2A is an autoradiograph from a 10% polyacrylamide gel showing the graphical representation of the gel in Figure 2A and of the A2B Rz1 and the mouse target. This analysis was performed to determine the initial velocities for multiple-turnover reactions. This interval is typical when less than
15% of the substrate has been digested. The A2B Rz2 ribozyme exhibited a high rate of cleavage at 37 °C in 20 mmol/L MgCl2 with 15% cleavage occurring at less than one minute. Because this interval was inconvenient for multiple-turnover kinetic analysis, we dropped the reaction temperature to 25 °C in 1 mmol/L MgCl2 (Figure 2C).

Active A2B Rz2 Cleaves Both Mouse and Human Targets

The active A2B Rz2 ribozyme was designed to specifically cleave the mouse target sequence, but also cleaves the human target (Figure 3). As shown in Figure 3B the mouse target has a C at the 3'-end, and this will pair with a G in the ribozyme (Figure 1). The human target has a U at the 3'-end, and this will still form a non-Watson-Crick base pair with the G in the ribozyme and still permit cleavage.

Multiple-Turnover Kinetic Analysis of Ribozymes

Multiple-turnover kinetic analysis was performed on both A2B receptor ribozymes, and the kinetic parameters were determined (Table). Each analysis was performed a minimum of three times (P = 1 × 10^{-3}). Reactions were done at both 37 °C in 20 mmol/L MgCl2 (standard conditions) and at 25 °C in 1 mmol/L MgCl2. The reactions at 37 °C were terminated at 6 minutes for A2B Rz1 and one minute for A2B Rz2. The reactions at 25 °C were terminated at 3 minutes for A2B Rz2. Based on Kcat, A2B Rz2 was 20-fold more active than A2B Rz1 under standard conditions, and retained good activity even in low magnesium and temperature. The A2B Rz2 was as active under these conditions as the naturally occurring hammerhead (from TRSV satellite RNA) under standard conditions. Because this A2B Rz2 was more potent than A2B Rz1, we used it exclusively for experiments in cell culture and in mice.

Effect of A2B Receptor Ribozymes on A2B Receptor mRNA Levels

The active A2B receptor ribozyme reduced A2B receptor mRNA levels in both HRECs and HEK293 cells to 70.5 ± 5.4% (P = 0.003) and 55.1 ± 4.8% (P = 5.1 × 10^{-5}), respectively. Figure 4 shows the results of both relative quantitative RT-PCR and real-time RT-PCR analysis on mRNA isolated from HRECs and HEK293 cells transfected with the active and inactive versions of the A2B receptor ribozyme and with the control plasmid. As expected, the inactive A2B receptor ribozyme did not reduce mRNA levels.

Kinetic Parameters Determined for the Two A2B Hammerhead Ribozymes Using Multiple-Turnover Kinetic Analysis

<table>
<thead>
<tr>
<th>Conditions</th>
<th>A2B Rz1</th>
<th>A2B Rz2</th>
<th>A2B Rz2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vmax, nmol/L per min</td>
<td>27.30 ± 3.2</td>
<td>515 ± 125</td>
<td>16.9 ± 2.8</td>
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<tr>
<td>Km, μmol/L</td>
<td>8.3 ± 0.2</td>
<td>4.3 ± 0.7</td>
<td>14.4 ± 1.1</td>
</tr>
<tr>
<td>Kcat, min^{-1}</td>
<td>1.8 ± 0.06</td>
<td>36.1 ± 8.3</td>
<td>1.1 ± 0.06</td>
</tr>
</tbody>
</table>

For the conditions, 37/20 means that the reactions were carried out at 37 °C and 20 mmol/L MgCl2, and 25/1 means that the reactions were carried out at 25 °C and 1 mmol/L MgCl2.
Effect of A2B Receptor Ribozymes on Migration of HRECs

Plasmids expressing ribozymes or the cloning vector p21NewHp were transfected into HRECs to determine their effect on cell migration in a modified Boyden chamber. Cells transfected with plasmid coding for active A2B Rz2 reduced migration of cells to an average of 52.7% (P<10^-4) when compared with the p21NewHp control at increasing concentrations of NECA (10 and 100 ng/mL), and cells transfected with plasmid coding for the inactive A2B Rz2 reduced migration of cells to an average of 86.8% (P=0.07) (Figure 5).

Effect of A2B Receptor Ribozymes on Neovascularization in the Neonatal Mouse Model of OIR

Plasmids expressing the ribozymes or the cloning vector p21NewHp were injected intravitreally on postnatal day one in the right eye of mouse pups, with no injection in the left eye. The pups and their dams were taken through the OIR. The extent of retinal angiogenesis was determined and the results are shown in Figure 6. Whereas p21NewHp had no effect on retinal angiogenesis, the active A2B Rz2 reduced the average number of nuclei per section on average by 53.5±8.2% (P=4.5×10^-5). The inactive A2B Rz2 reduced the average number of nuclei per section on average by 10.5±7.4% (P=0.3). Figure 7 shows representative sections through a left-uninjected eye and a right eye injected with the active A2B Rz2 in the same mouse pup.

Discussion

We have developed a hammerhead ribozyme that specifically cleaves the mouse and human adenosine A2B receptor mRNAs (Figures 2 and 3). We have demonstrated that this ribozyme reduces the expression of the A2B receptor mRNA and the function of the A2B receptor in HEK293 cells and HRECs, respectively (Figures 4 and 5). We found a 30% reduction in the A2B receptor mRNA signal in HRECs and a 45% reduction in the A2B receptor mRNA signal in HEK293 cells transfected with the active ribozyme compared with...
cells transfected with the control plasmid. The difference in the ability of the active ribozyme to reduce receptor mRNA levels between the two cell types most likely results from differences in transfection efficiency. This reduction in expression of the A2B receptor mRNA correlated with a reduction in A2B receptor function that we found in transfected HRECs. The chemotactic migration of HRECs across a porous membrane toward solutions containing increasing concentrations of NECA is dependent on the presence of A2B receptors on the cell surface.\(^3\)\(^{19}\) We found that the number of migrating cells diminished to 47% in cells expressing the active ribozyme relative to cells transfected with the empty vector. The reduction in migration of HRECs transfected with a plasmid expressing the active A2B receptor ribozyme suggests that cell surface levels of the A2B receptor protein have been reduced due to inhibition of expression of the A2B receptor mRNA by the ribozyme. These results provide strong evidence that cleavage of the A2B receptor mRNA reduces expression of the protein in cultured cells to a level that significantly inhibits the cellular function of the A2B receptor.

We also demonstrate that the active ribozyme inhibits preretinal neovascularization in vivo in a mouse model of OIR (Figure 6). Preretinal neovascularization was reduced 53% in eyes injected with the plasmid expressing the active ribozyme. These results suggest that this reduction in neovascularization is a result of ribozyme inhibition of the expression of the A2B receptor and demonstrate that this ribozyme could be useful for in vivo studies of A2B receptor function in retinopathies. By selectively reducing the expression of the A2B receptor mRNA, we are able to inhibit preretinal neovascularization in the mouse model of OIR and provide further evidence for the involvement of the A2B receptor in retinal angiogenesis.

One result of this study was to separate the inhibitory effects of the active ribozyme into its catalytic and antisense effects. Antisense DNA oligonucleotides have been shown to inhibit the physiological activity of the A2B receptor in cultured cells.\(^29\) We attribute the reduced migration after treatment with the catalytically inert inactive ribozyme to be caused by a minor antisense effect (Figure 5) rather than RNA reduction. Injection of the inactive ribozyme into the mouse model demonstrates that only a small portion of this reduction can be attributed to an antisense effect. Therefore, there is, at most, a minor antisense contribution in the reduction of preretinal neovascularization in the animal model. We conclude that ribozyme genes delivered as naked DNA lead to a substantial reduction in neovascularization in this model due to the catalytic activity of the ribozyme.

Finally, this study demonstrates the utility of using ribozymes to study complex physiological pathways. We successfully transfected human cells and an in vivo mouse model with a plasmid expressing a hammerhead ribozyme that reduced the expression of a single target mRNA. We were able to quantify the effects of the ribozyme through functional assays of the target. We will be able to use routine plasmid transfection of cells with vectors expressing the active ribozyme to study how inhibition of A2B receptor expression affects other components of this pathway. We also demonstrate that injection of the plasmid expressing the A2B receptor ribozyme into the mouse model reduces preretinal neovascularization. This model will allow us to further dissect the role of the A2B receptor in angiogenesis.

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Reduction in Preretinal Neovascularization by Ribozymes That Cleave the $A_2B$ Adenosine Receptor mRNA

A. Afzal, L.C. Shaw, S. Caballero, P.E. Spoerri, A.S. Lewin, D. Zeng, L. Belardinelli and M.B. Grant

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