Adenosine A2A Receptor Stimulation Reduces Inflammation and Neointimal Growth in a Murine Carotid Ligation Model


Abstract—Endothelial activation and leukocyte recruitment are early events in atherosclerosis and the vascular response to injury. Adenosine has anti-inflammatory effects on leukocytes and endothelial cells mediated through its A2A receptor. We tested the hypothesis that A2A activation would reduce inflammation and neointimal formation in a murine carotid ligation model. Before injury, mice were randomized to a 7-day subcutaneous infusion of a specific A2A receptor agonist (ATL-146e, 0.004 μg/kg per minute), vehicle control, ATL-146e plus ZM241385 (a selective A2A antagonist), or ZM241385 alone. Leukocyte recruitment and adhesion molecule expression were assessed at early time points, and the neointimal area was measured at 14 and 28 days after injury. Compared with control mice, ATL-146e—treated mice had significantly less neutrophil and macrophage recruitment and vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and P-selectin expression in the first 7 days after injury. Neointimal area was markedly and persistently reduced by 80% at 14 and 28 days, despite termination of ATL infusion at 7 days. ATL-146e+ZM241385—treated and ZM241385-treated animals had neointimal areas similar to those of control animals, confirming that the observed effects of ATL-146e were mediated specifically by the A2A receptor. These data demonstrate that novel stimulation of adenosine A2A receptors can inhibit early inflammatory processes that are important in neointimal formation after vascular injury. (Arterioscler Thromb Vasc Biol. 2001;21:791-796.)

Key Words: adenosine □ restenosis □ cell adhesion molecules □ endothelium □ leukocytes

It is increasingly recognized that atherosclerosis is a complex inflammatory disease. Early in this process, leukocytes adhere to the endothelium in areas of turbulent flow and decreased shear stress, where adhesion molecules are preferentially expressed. Acute inflammation, with activation of circulating leukocytes, platelets, and endothelial cells and upregulation of cellular adhesion molecules, is also thought to be important in the early vascular response to mechanical injury, which can result in the formation of a restenotic lesion. Leukocyte recruitment is a multistep process characterized by the expression of multiple adhesion molecules and subsequent leukocyte rolling, firm adhesion, and transmigration across the endothelium. These leukocyte-endothelial interactions cause further cellular activation and increased cytokine and growth factor secretion, which can result in the migration and proliferation of smooth muscle cells (SMCs) and, ultimately, the formation of an arterial lesion.

Adenosine is an important modulator of inflammation and influences the inflammatory responses to various biological insults, such as myocardial ischemia/reperfusion injury. Endogenous adenosine is released locally by injured or activated endothelium, where it can interact locally with widely expressed adenosine receptors. Many of the anti-inflammatory effects of adenosine are attributed to the stimulation of the A2A receptor subtype, which is expressed on most cells involved in inflammation, including neutrophils, macrophages, platelets, SMCs, and endothelial cells. Stimulation of the A2A receptor inhibits oxidative activity and degranulation in neutrophils, inhibits tumor necrosis factor (TNF)-α release by activated monocytes and macrophages, and enhances leukocyte interleukin-10 release, resulting in the diminished secretion of proinflammatory cytokines.

Kumar and colleagues have demonstrated that interruption of blood flow by ligating the left common carotid artery in the mouse results in a marked reduction in luminal area through a combination of neointimal formation and arterial constriction. In this model, the endothelium remains intact proximal to the ligature, and thrombus formation is uncommon. There is evidence of early local inflammation, with leukocytes observed throughout the vessel wall in the first 14 days after ligation and SMC proliferation with the development of an SMC-rich neointimal lesion. It is possible that the severe disturbance in flow and local shear stress created by ligation plays a key role in the observed inflammation and
neointimal growth. Given the importance of early inflammatory events on arterial lesion formation and the various effects of A2A receptor stimulation on leukocyte and endothelial function, we used this novel inflammatory model of vascular injury to test the hypothesis that adenosine A2A receptor activation would significantly reduce acute inflammation and attenuate neointimal formation after carotid ligation.

Methods

Adenosine A2A Receptor Ligands

We have previously characterized ATL-146e (ATL) as a selective and potent agonist of A2A receptors. Selective and 50 times more potent than the most widely used A2A-selective agonist, CGS21680, ZM241385 (ZM), a potent and selective antagonist of A2A receptors, was a gift from S. Zomer, Zeneca Pharmaceuticals, Cheshire, UK.

Mouse Carotid Ligation Model

Female C57BL/6 mice (20 to 25 g) were used for all experiments and were handled in compliance with the Guide for the Care and Use of Laboratory Animals of the NIH. Protocol approval was obtained from the Animal Research Committee of the University of Virginia. All surgical procedures were performed with animals under general anesthesia and by use of a sterile surgical technique with a dissecting microscope (Zeiss, Germany). Through a midline neck incision, the left common carotid artery was exposed. As previously described, the artery was completely ligated just proximal to the carotid bifurcation. The right carotid artery served as a non-injured control artery. After ligation, the incision was closed, and the animals were allowed to recover. At the time of euthanasia, the animals were reanesthetized, and finger palpation was used to assess a pulsation in the ligated vessel. A 27-gauge needle was then placed in the left ventricle to achieve in situ perfusion fixation of the carotid arteries. A pulsation in the ligated vessel was visible. After inflation, the arteries were harvested, dehydrated in ethanol and xylene, and embedded in paraffin.

Treatment Protocol

On the day before carotid ligation, animals were anesthetized as described above and underwent implantation of a 7-day osmotic pump (model 1007D, Alza Corp) placed subcutaneously through a transverse intrascapular incision. To evaluate the effects of ATL on early inflammation after ligation, mice were randomized to treatment with continuous ATL (0.004 mg/kg per minute) or vehicle control (100 μL of 1% dimethyl sulfoxide in saline) for 7 days. All animals (4 at each time point per group, 32 total) were euthanized at 1, 3, 7, and 14 days after ligation. The dose of ATL used in this experiment (0.004 mg/kg per minute) has been shown to effectively attenuate renal ischemia/reperfusion injury and is well below the dose required to produce systemic changes in rat arterial blood pressure or heart rate. The renal-protective effect was completely reversed by the specific A2A inhibitor ZM, which was administered in an equimolar concentration. To evaluate the effects of ATL on neointimal formation and to assess whether the observed effects of ATL were specifically mediated via the A2A receptor, additional mice were randomized into 1 of 4 treatment groups, with infusions for 7 days: group 1, continuous ATL (0.004 μg/kg per minute) plus ZM (ATL/ZM), n = 11; or group 4, continuous ZM (0.003 μg/kg per minute, the molar equivalent dose of ATL), n = 13. Animals in groups 1, 2, and 3 were euthanized at 14 or 28 days after ligation (n = 7 at each time point per group, 51 total) for histomorphometric analysis. Group 4 animals were euthanized only at 28 days.

Histology

Carotid arterial segments were sectioned transversely from 2 mm proximal to the ligature to the aortic arch. Although pulsatile flow was observed in the ligated arteries, a small amount of thrombus was common in the distal 2 mm of the vessel adjacent to the ligature, and this portion was excluded from the analysis. For each vessel, a total of 240 to 300 sections (5 μm) were obtained. Vessels were divided into 5 nearly equal parts, and consecutive sections from each part were selected for analysis, with mean values calculated for each vessel. Paraffin-embedded sections were deparaffinized and used for analysis with the avidin-biotin-peroxidase method (Vector Laboratories). Neutrophils were stained with the use of naphthol AS-D chloroacetate esterase. In each analyzed section, the number of neutrophils attached to the endothelium, within the neointima, and in the media were counted by using an Olympus (BH-2) microscope at ×400 magnification. Neutrophil density was calculated by dividing the number of counted neutrophils by the measured neointimal and medial areas. For macrophage analysis, sections were pretreated with 0.1% trypsin (Sigma Chemical Co) in Tris-HCl with 0.1% calcium chloride (pH 7.8). Staining was performed with an indirect tyramide signal amplification (NEN Life Science Products). Macrophage density was calculated as described above.

For localization of adhesion molecules, microwave treatment with Antigen Unmasking Solution (Vector Laboratories) was used. Negative competition studies on all adhesion molecules for affinity-purified antibodies were run on TNF-treated vessels by using specific peptides (vascular cell adhesion molecule [VCAM-1], intercellular adhesion molecule [ICAM-1], and platelet and endothelial cell adhesion molecule [PECAM-1], Santa Cruz Biotechnology, Inc, and P-selectin, provided by Dr S. Green, University of Virginia, Charlottesville; data not shown). Goat polyclonal antibodies to VCAM-1, ICAM-1, and PECAM-1 (Santa Cruz Biotechnology, Inc) and a rabbit polyclonal anti-P-selectin antibody (provided by Dr S. Green) were used for the analysis. Adhesion molecule expression was determined by microscopic observation of the diaminobenzidine reaction product (Dako Corp) on the analyzed sections. Images were digitized through an Olympus (BH-2) microprojection system with a Dage-MTI DC-330 color camera (Dage-MTI, Inc) and analyzed by using Image-Pro software (Media Cybernetics). For each section, the percent area of positive staining was calculated from the number of positively stained pixels in the endothelium, neointima, and media divided by the total number of pixels in the area of interest.

Histomorphometry

Arterial sections (5 μm) from animals euthanized 14 and 28 days after injury were selected as described above and stained by use of the Russell-Movat pentachrome method. Images were digitized as described above and analyzed with Image-Pro software. The areas of the lumen, internal elastic lamina (IEL), and external elastic lamina (EEL) were determined by computerized planimetry, and the luminal, neointimal, and medial areas were calculated. Neointimal area was calculated by subtracting the luminal area from the IEL area, and medial area was determined by subtracting the EEL area from the IEL area. Arterial size was measured by tracing the circumference of the EEL.

Complete Blood Counts

Three days after pump implantation, animals receiving ATL (n=4) and vehicle (n=4) had blood samples drawn by cardiac puncture into EDTA-containing Microtainer tubes (Becton-Dickinson) for complete blood and differential leucocyte counts.

Statistical Analysis

Data were reported as mean±SEM. Differences between treatment groups were analyzed by using 1-way ANOVA followed by unpaired Student t test to evaluate 2-tailed levels of significance. A value of P<0.05 was considered significant to account for Bonferroni correction when the 3 treatment groups were compared. Otherwise, P<0.05 was accepted as being statistically significant.

Results

Effect of ATL on Early Inflammation After Carotid Ligation

Inflammatory leucocyte recruitment was observed in arterial sections at all early time points (1, 3, 7, and 14 days) after
carotid ligation. Neutrophils were found adjacent to the endothelium primarily at 1 and 3 days after ligation (Figure 1). Compared with control animals, neutrophil density was 71% less in ATL-treated animals at 1 day (55±38 versus 447±134 cells/mm², \( P=0.004 \)) and 72% less at 3 days (169±180 versus 1098±563 cells/mm², \( P=0.04 \)) after ligation (Figure 2).

By day 3, a modest number of macrophages were observed in ATL-treated and control animals. At 7 and 14 days after ligation, a significant number of macrophages were observed in control vessels throughout the developing neointima, media, and adventitia (Figure 3). Compared with control animals, ATL-treated animals had a 77% reduction in macrophage density at 3 days (130±177 versus 574±267 cells/mm², \( P=0.04 \)), a 77% reduction at 7 days (337±246 versus 1459±529 cells/mm², \( P=0.016 \)), and a trend toward fewer macrophages at 14 days (1039±577 versus 1643±116 cells/mm², 37% reduction, \( P=0.12 \)) after ligation (Figure 2).

**Effect of ATL on Cellular Adhesion Molecule Expression**

Immunohistochemical analysis of cellular adhesion molecules was performed at 1, 3, 7, and 14 days after carotid ligation. In control animals, VCAM-1 staining increased by 3 days after ligation, with a marked increase at 7 days. By day 7, staining was observed almost uniformly around the lumen and also in focal areas of the developing neointima and media (Figure I, available online at http://atvb.ahajournals.org). The ATL-treated animals had significantly less VCAM-1 staining at 7 days than did control animals (4±2% versus 28±2%, respectively, \( P<0.0001 \); Figure 4).

Compared with VCAM-1 staining, staining for ICAM-1 was much less intense in all animals (Figure I). However, there was a significantly smaller area of ICAM-1 staining in
the ATL-treated animals at 3 days (0.6±0.1% versus 1.7±0.7%, a reduction of 65%, P=0.05), 7 days (0.8±0.3% versus 2.0±0.7%, a reduction of 60%, P=0.03), and 14 days (1.4±0.5% versus 2.7±0.7%, a reduction of 48%, P=0.03; Figure 4). ICAM-1 staining was observed predominately around the lumen, with only scant staining in the neointima and media in a small number of control vessels.

Staining patterns of P-selectin were similar to those seen with VCAM-1, with peak staining observed 7 days after ligation (Figure 1). By day 7, P-selectin staining was observed not only around the lumen but also in the developing neointima and media. ATL-treated animals had a significantly small staining area at 3 days (9±2% versus 13±1%, a reduction of 31%, P=0.04) and 7 days (12±4% versus 28±5%, a reduction of 57%, P=0.002) than did control animals (Figure 4).

In all arterial sections analyzed after ligation, endothelial integrity was observed, with consistent PECAM-1 staining along the luminal surfaces. Neovascularization in the neointima was seen at 14 days, with PECAM-1 staining in the endothelium of microvessels in the larger lesions (data not shown).

Effect of ATL on Neointimal Formation

At 14 days after ligation, quantitative histomorphometry demonstrated a 76% reduction in neointimal area (0.005±0.004 versus 0.021±0.014 mm², P=0.016) in the ATL-treated animals compared with control animals (Figure 5a). Medial area was similar in the 2 groups. As noted in previous studies,12 neointimal hyperplasia was maximal in the mid-to-distal portion of the vessel and decreased in thickness in the direction of the aortic arch. Between 14 and 28 days, a trend toward an increased neointimal area was seen in the control animals (0.021±0.014 versus 0.037±0.023 mm², P=0.07), whereas the neointimal area did not increase in the ATL-treated animals (0.005±0.004 versus 0.007±0.003 mm², P=0.62). At 28 days, the neointimal area was 81% less in the ATL-treated animals than in the control animals (0.007±0.003 versus 0.037±0.023 mm², P=0.005). Representative histological sections obtained 28 days after ligation are shown in Figure 5b and 5c. Compared with control ratios, the neointimal/media ratio was 80% less in ATL-treated animals at 14 days (0.13±0.07 versus 0.64±0.44, P=0.01) and 28 days (0.23±0.12 versus 1.12±0.65, P=0.01).

Vessel Remodeling

At 28 days, there was a reduction in EEL circumference of the ligated artery compared with the unligated right common carotid artery in all treatment groups (P<0.05). No significant differences in EEL circumference between the ATL-treated and control animals in either the ligated (0.98±0.12 versus 1.0±0.16 mm) or unligated (1.06±0.11 versus 1.12±0.10 mm) arteries were observed. Thus, both groups exhibited a similar degree of vessel constriction in response to carotid ligation.

Specificity of ATL

To confirm that the vascular effects noted in the ATL-treated animals were specifically mediated via the adenosine A2A receptor, the third treatment group received equimolar amounts of ATL and ZM (ATL/ZM), a known A3A receptor antagonist.18 A fourth group received ZM alone. These animals had degrees of neointimal growth similar to those of control animals at 28 days (Figure 5a), confirming that the observed effects of ATL on neointimal growth were mediated specifically through the adenosine A2A receptor.

Complete Blood Counts

No difference in total leukocyte (3.71±1.2×10³/μL versus 3.92±1.6×10³/μL) or in differential count was observed between the ATL and control groups. Platelet counts were also similar (685±72×10³/μL versus 668±189×10³/μL, respectively).

Discussion

The results of the present study demonstrate that stimulation of the adenosine A2A receptor can effectively attenuate the early inflammatory response to murine carotid artery ligation and reduce neointimal formation. Ligation alters the conditions of arterial flow and shear stress, resulting in increased adhesion molecule expression, leukocyte recruitment, and neointimal formation. In this experiment, no effect on medial area or arterial remodeling was observed, and the prolonged systemic administration of an A2A receptor agonist had no demonstrable effect on circulating leukocyte or platelet counts.

Although the present study did not assess the effects of A2A receptor stimulation in a specific model of atherosclerosis or mechanical injury, interruption of flow in the carotid artery did induce an inflammatory response analogous to that seen in the early stages of atherosclerosis and early after vascular injury. This response is characterized by endothelial activation, upregulation of adhesion molecules, and local leukocyte recruitment. The process of neointimal growth in the presence...
of leukocyte infiltration has also been observed in other experimental animal models. One advantage of the carotid ligation model is that an SMC-rich neointima consistently forms by 28 days.

Carotid ligation consistently induced a local inflammatory response manifested by leukocyte recruitment in the vessel wall and upregulation of adhesion molecules. There is growing evidence that the inflammatory effects of turbulent arterial flow and decreased shear stress are important in the development of atherosclerotic lesions. In experimental animal models, localized upregulation of endothelial VCAM-1 occurs in response to decreased shear stress and precedes local leukocyte recruitment. In a mouse model of atherosclerosis, ICAM-1 was preferentially expressed in lesion-prone sites of increased turbulent flow. Conditions of oscillatory flow have also been shown to enhance endothelial expression of cellular adhesion molecules and affect the endothelial redox state. Early inflammation and increased adhesion molecule expression also occur after vascular mechanical injury. Inoue et al demonstrated that expression of the neutrophil adhesion molecule Mac-1 (CD11b) was increased in patients after balloon angioplasty and was more pronounced in patients who later developed restenosis. Tanaka et al have shown that VCAM-1 and ICAM-1 expression in the rabbit aorta is increased and sustained for up to 30 days after balloon withdrawal injury. In addition to the integrins, P-selectin is increasingly recognized as another important mediator in the vascular response to injury. P-selectin inhibition has been shown to reduce leukocyte accumulation and fibrin deposition within Dacron grafts and to reduce intimal hyperplasia after balloon-induced vascular injury in the rabbit. Using the carotid ligation model, Kumar et al demonstrated that local inflammation and neointima formation were markedly reduced in P-selectin-deficient mice. Thus, endothelial activation with increased adhesion molecule expression appears to be an essential element in the development of atherosclerosis and the biological response to mechanical vascular injury.

Consistent with other models of altered arterial flow, in our model we observed a marked increase in VCAM-1 staining in the control group 7 days after ligation, which paralleled the measured increase in macrophage density. A gradual, more modest increase in ICAM-1 staining was observed in control animals in the first 14 days after ligation. In contrast, the pattern of P-selectin staining in vehicle-treated animals was robust and very similar to the pattern of VCAM-1 staining. The interesting finding of P-selectin staining in the developing neointima and media of ligated vessels has been previously described and may represent P-selectin-mediated binding of platelets to leukocytes with transmigration across the endothelium. It is possible that activated platelets, once within the vessel wall, could more effectively stimulate SMCs and amplify neointimal formation.

Our data demonstrate that A2A receptor stimulation markedly reduces early neutrophil and macrophage recruitment and vascular adhesion molecule expression after carotid ligation. Potential mechanisms not addressed in the present in vivo study include A2A receptor stimulation acting directly on endothelial cells to reduce activation and adhesion molecule expression. Increased intracellular cAMP is known to modulate ICAM-1 and VCAM-1 expression by SMCs and to inhibit cytokine-mediated expression of E-selectin, VCAM-1, and tissue factor by endothelial cells. In addition, A2A receptor activation has been reported to induce endothelial cell proliferation independent of cAMP through stimulation of mitogen-activated protein kinase. It is likely that A2A receptor stimulation also indirectly affects endothelial activation through the inhibition of leukocyte-endothelial and platelet-endothelial interactions. In neutrophils, A2A receptor stimulation has been shown to reduce superoxide radical generation and inhibit degranulation. A2A activation also diminishes neutrophil Mac-1 expression, which might result in decreased endothelial adhesion via ICAM-1. Cronstein et al showed that neutrophil adherence to endothelium is inhibited by adenosine A2A receptor activation in vitro, although they did not specifically investigate the role of the A2A receptor. A2A receptor stimulation on activated macrophages has been shown to inhibit TNF-α release, which can act directly on endothelial cells to increase leukocyte adhesion and increase interleukin-10 release, which can independently reduce inflammatory cytokine production. A2A activation with CGS21680 has also recently been shown to reduce platelet P-selectin expression and neutrophil-platelet adhesion in dogs.

In summary, interruption of flow by carotid artery ligation in the mouse induces endothelial activation, upregulation of adhesion molecules, local leukocyte recruitment, and robust neointimal formation at 28 days after ligation. Treatment for 7 days after carotid ligation with a potent and specific A2A receptor agonist, ATL, significantly reduced early inflammation and resulted in a marked and sustained attenuation in neointimal formation. These results suggest that effective blockade of early inflammatory events with a novel adenosine A2A receptor agonist could potentially have therapeutic benefits in inflammation-mediated human cardiovascular diseases.

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