A₁ Adenosine Receptor Activation Promotes Angiogenesis and Release of VEGF From Monocytes

Adam N. Clark, Rebecca Youkey, Xiaoping Liu, Liguo Jia, Rebecca Blatt, Yuan-Ji Day, Gail W. Sullivan, Joel Linden, Amy L. Tucker

Abstract—Adenosine is a proangiogenic purine nucleoside released from ischemic and hypoxic tissues. Of the 4 adenosine receptor (AR) subtypes (A₁, A₂A, A₂B, and A₃), the A₂ and A₃ have been previously linked to the modulation of angiogenesis. We used the chicken chorioallantoic membrane (CAM) model to determine whether A₁ AR activation affects angiogenesis. We cloned and pharmacologically characterized chicken AR subtypes to evaluate the selectivity of various agonists and antagonists. Application of the A₁ AR-selective agonist N⁶-cyclopentyladenosine (CPA; 100 nmol/L) to the CAM resulted in a 40% increase in blood vessel number (P<0.01), which was blocked by the A₁ AR-selective antagonist C⁹-(N-methylisopropyl)-amino-N⁴-(5'-endoxyhydroxy)-endonorbornan-2-yl-9-methyladenine (WRC-0571; 1 μmol/L). Selective A²A AR agonists did not stimulate angiogenesis in the CAM. In an ex vivo rat aortic ring model of angiogenesis that includes cocultured endothelial cells, fibroblasts, and smooth muscle cells, 50 nmol/L CPA did not directly stimulate capillary formation; however, medium from human mononuclear cells pretreated with CPA, but not vehicle, increased capillary formation by 48% (P<0.05). This effect was blocked by WRC-0571 (1.5 μmol/L) or anti-VEGF antibody (1 μg/mL). CPA (5 nmol/L) stimulated a 1.7-fold increase in VEGF release from the mononuclear cells. This is the first study to show that A₁ AR activation induces angiogenesis. Stimulation of A₂ ARs on endothelial cells results in proliferation and tube formation, and A₂ and A₃ ARs on inflammatory cells modulate release of angiogenic factors. We conclude that adenosine promotes a coordinated angiogenic response through its interactions with multiple receptors on multiple cell types. (Circ Res. 2007;101:0-0.)

Key Words: angiogenesis ■ receptor pharmacology ■ growth factors/cytokines

Adenosine is a purine nucleoside released from ischemic and hypoxic tissues, where it acts via 4 subtypes (A₁, A₂A, A₂B, A₃) of G protein–coupled cell surface receptors to restore homeostasis by increasing blood supply and decreasing energy demand. Adenosine increases blood flow through vasodilation of preexisting vasculature and by stimulating angiogenesis. Adenosine-modulated angiogenesis, suggested by experiments on skeletal muscle in the early 1980’s,¹ was first demonstrated in 1986, through application of adenosine to the chicken chorioallantoic membrane (CAM) using Elvax pellets.² Hypoxia-induced angiogenesis in the CAM was reduced 66% by methyl-isobutyl-xanthine, a cAMP phosphodiesterase inhibitor and nonselective competitive adenosine receptor (AR) antagonist.³ In vitro studies have convincingly established direct proangiogenic mechanisms for A₂A and A₃ ARs, which promote endothelial cell (EC) proliferation and VEGF release,⁴⁵ and indirect roles for A₂A, A₂B, and A₃ ARs which stimulate release of proangiogenic growth factors and cytokines from smooth muscle⁶ and inflammatory cells.⁷⁸ A₁ AR-mediated modulation of angiogenesis has not been well-characterized.

Effects of A₁ AR activation on ECs remain speculative. A₁ AR message can be detected in ECs; however, the functional response to A₁ AR activation in ECs is not mitogenic, but related to thrombosis¹⁰ and inflammation.¹¹ Although one study has suggested that A₁ ARs may promote EC tube formation,¹² the bulk of in vitro experimental data does not support a proangiogenic mechanism of direct EC activation by the A₁ AR.

Activation of A₂A, A₂B, and A₃ ARs has been shown to cause release of proangiogenic growth factors and cytokines from mast cells and monocytes.⁷⁸ To our knowledge, no studies on human and rodent mast cells have identified A₁ AR expression or function; however, monocytes possess A₁ ARs, activation of which stimulates multi-nucleated giant cell formation and phagocytosis.¹³¹⁴ A₁ AR activation also promotes chemotaxis in a subset of proangiogenic mononuclearoid cells, plasmacytoid dendritic cells, that concentrate in some tumors.¹⁵

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We used the CAM model to test our hypothesis that A₁ AR activation stimulates angiogenesis in vivo. Angiogenesis after application of the relatively A₁ AR-selective agonist, CPA, is completely blocked by the highly A₁ AR-selective antagonist, WRC 0571. We used rat aortic rings as an ex vivo coculture system (157.8 ± 10.0 vessels vs 127.3 ± 6.8 control, P < 0.01, Bonferroni test; between 2 means were analyzed using Student t test; between multiple means using ANOVA with Bonferroni post-hoc analysis comparing each mean with control).

Materials and Methods
See online Expanded Materials and Methods, available online at http://circres.ahajournals.org.

Chorioallantoic Membrane Angiogenesis Assay
Assays were performed using a modification of the method described by Dusseau.²

Chicken A₁, A₂B, and A₃ AR Cloning and Characterization
Chicken A₁, A₂B, and A₃ ARs were cloned from chicken RNA by RT-PCR using published sequences.¹⁶⁻²⁰ The cDNAs were sequenced and expressed in HEK293 cells. Membranes from cells expressing recombinant receptors were harvested as previously described.²¹ For saturation isotherms on A₁ and A₃ ARs, serial dilutions from a maximum concentration of 1.5 nmol/L [³H]-ABA + 10 nmol/L I-ABA in HE buffer, containing 10 μg/mL ADA and 4.9 mmol/L MgCl₂, were incubated with 10 to 20 μg membrane protein at 25°C for 2 to 3 hours. NECA (150 μmol/L) was used to define nonspecific binding. Saturation isotherms on A₂B receptors were done similarly using serial dilutions from a maximum concentration of 1.0 nmol/L [³H]-LABOPX + 80 nmol/L I-ABOPX with 100 μmol/L NECA defining nonspecific binding. Reactions were terminated by dilution and rapid filtration through GF/C Whatman filters. Competition curves were performed by incubating 0.3 nmol/L [³H]-I-ABA or 0.5 nmol/L [³H]-I-ABOPX with 15 to 25 μg membrane protein in HE buffer containing 10 μg/mL ADA and 4.9 mmol/L MgCl₂ ± competing ligands for 2 hours at 25°C. Nonspecific binding was determined in the presence of 150 μmol/L NECA. Reactions were terminated by rapid filtration.

Isolation of Human Monocytes
Sixty ml of human blood was obtained from each consenting subject according to the guidelines of the University of Virginia Institutional Review Board and Human Investigational Committee. Human peripheral mononuclear cells were isolated according to the Histopaque-1077 protocol outlined by the manufacturer. Mononuclear cells were enriched for monocytes using the Dynal Biotech system according to the manufacturer’s instructions. The enriched population was 65% monocytes as quantified by CD14 FITC labeling and fluorescence activated cell sorter (FACS) analysis, the other 35% being predominantly lymphocytes. These cells were further enriched for monocytes (>90%) by plating with subsequent removal of nonadherent cells.

Human Peripheral Monocyte Culture
Enriched monocytes were cultured at 0.5 × 10⁶ per mL in DMEM/F12, 365 mg/L L-glutamine, and 100 U/mL Penicillin/Streptomycin (see online Expanded Materials and Methods). Human peripheral mononuclear cells were isolated according to the guidelines of the University of Virginia Institutional Review Board and Human Investigational Committee. Human peripheral mononuclear cells were isolated according to the manufacturer’s instructions. The enriched population was 65% monocytes as quantified by CD14 FITC labeling and fluorescence activated cell sorter (FACS) analysis, the other 35% being predominantly lymphocytes. These cells were further enriched for monocytes (>90%) by plating with subsequent removal of nonadherent cells.

Rat Thoracic Aortic Ring Angiogenesis Assay
All procedures are approved by the University of Virginia Animal Care and Use Committee. Rat thoracic aorta cultures were prepared as described by Nicosia¹⁷ using rings embedded in collagen matrix prepared according to Elsdale and Bard.²³

Statistical Analysis
Radioligand binding data were analyzed using GraphPad Prism 4.0.

Results
Adenosine Stimulates Angiogenesis in the CAM
Dusseau et al reported previously that adenosine delivered to CAMs via Elvax pellets stimulated angiogenesis; however, Barnhill et al were unable to confirm this.²⁴ Using Elvax pellets impregnated with adenosine (3 mg) or vehicle we show that adenosine is proangiogenic in the CAM. Adenosine-containing pellets caused a 36% increase in the number of vessels intersecting the pellet margin compared with vehicle-impregnated control pellets (Figure 1A).

Figure 1. A, Adenosine stimulates angiogenesis in the CAM. Elvax pellets containing 3 mg adenosine or vehicle were applied to CAMs. Mean vessel number for vehicle-treated CAMs, 141.0 ± 14.0 (n = 12); adenosine-treated CAMs, 219.1 ± 17.2 (n = 15, **P < 0.01). B, Subtype-selective AR ligands stimulate angiogenesis in the CAM. Each bar represents the mean ± SEM number of blood vessels from 13 to 19 CAMs with the exception of XAC, which represents 4 CAMs. The A₁ AR agonist CPA (179.4 ± 10.0 vessels vs 127.3 ± 6.8 control, P < 0.01, Bonferroni analysis after ANOVA), the nonselective agonist NECA (186.1 ± 11.0, P < 0.001), and the A₂B AR agonist IB-MECA (191.5 ± 14.8, P < 0.001) elicited an angiogenic response. The A₃ AR agonist CGS 21680 (135.5 ± 9.7, P < 0.05) did not. The nonselective AR antagonist XAC did not inhibit baseline angiogenesis (157.8 ± 12.8, P > 0.05).
The A₁ AR Agonist CPA Stimulates Angiogenesis in the CAM

AR subtype-selective ligands in solution (100 nmol/L) were applied to filter paper discs placed on CAMs daily for 7 days (Figure 1B). The concentration was selected to be low enough to retain subtype selectivity, not necessarily to elicit a maximal response. Dose-dependent proangiogenic effects were observed in response to CPA with maximal effects seen at micromolar (μmol/L) concentrations. Twenty nanomolar (nmol/L), 200 nmol/L, and 2 μmol/L CPA (data not shown) were associated with 13%, 20%, and 31% increases in vessel number, respectively. By comparison, application of 0.5 μg of VEGF, known to be a potent modulator of angiogenesis in the CAM, resulted in a 25% increase in vessel number (Figure 2). The nonselective AR agonist NECA was used as a positive control, because a proangiogenic effect was expected based on prior in vivo and in vitro studies on A₂ ARs. There is no selective A₂B AR agonist; functional responses were attributed to this subtype if they were stimulated by NECA and blocked by the A₂B AR-selective antagonist, MRS-1754. Surprisingly, neither of the A₂A AR-selective agonists, 2-[4-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680) or ATL-14625 (data not shown), elicited angiogenesis in the CAM, despite the fact that CGS21680 is a high affinity agonist for chicken A₂A ARs as demonstrated in studies showing it to be neuroprotective in chicken embryos.²⁶ The A₃ AR-selective ligand N⁶-(2-iodo)benzyl-5'-N-methylcarboxamidoadenosine (IB-MECA) stimulated vessel growth in the CAM. The nonselective AR antagonist, xanthine amine congener (XAC), did not block baseline angiogenesis.

Because there are significant species differences in pharmacology among A₁, A₂B, and A₃ ARs, the subtypes that potentially mediate angiogenesis in the CAM, we cloned and pharmacologically characterized these chicken ARs to confirm that our ligand concentrations would differentiate among subtypes. Saturation equilibrium binding to recombinant receptors revealed adequate expression and high affinity binding for each (Table). CPA was not selective for the chicken A₁ versus A₃ AR at 100 nmol/L, but was selective for the A₁ or A₃AR compared with the A₂B receptor. Because A₂A AR agonists had no effect, CPA-stimulated angiogenesis in the CAM model was thought not to be A₂A AR-mediated and this receptor was not investigated further. Inhibition by WRC-0571, an A₁ AR antagonist with 700-fold selectivity for the chicken A₁ over the A₃ AR (Table), was used to confirm that angiogenesis in the CAM in response to CPA was not A₃ AR-mediated. Figure 2 shows inhibition of the angiogenic response to 50 nmol/L CPA to below control levels by 1.0 μmol/L WRC-0571, a concentration which would not be expected to completely antagonize effects of CPA on the chicken A₁ AR. Based on our radioligand binding data, 100 nmol/L CPA should not activate the A₂B AR (Kᵢ >10 000). We believe that A₂B ARs in our system are well-coupled because our binding affinities for human A₂B ARs (data not shown) correlate well with published EC₅₀s

### Table. Pharmacologic Characterization of Chicken A₁, A₂B, and A₃ ARs

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Radioligand Kᵦ (nmol/L)</th>
<th>Bmax (pmol/mg protein)</th>
<th>CPA Kᵦ (nmol/L)</th>
<th>WRC-0571 Kᵦ (nmol/L)</th>
<th>IB-MECA Kᵦ (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td>0.6 ± 0.10</td>
<td>603 ± 25</td>
<td>0.2 ± 0.03</td>
<td>4.1 ± 0.9</td>
<td>2420 ± 1190</td>
</tr>
<tr>
<td>A₂B</td>
<td>65 ± 22</td>
<td>20 946 ± 5614</td>
<td>10 900 ± 2730</td>
<td>90 ± 12</td>
<td>&gt;10 000</td>
</tr>
<tr>
<td>A₃</td>
<td>3.1 ± 0.3</td>
<td>2726 ± 70</td>
<td>1.6 ± 0.8</td>
<td>2940 ± 255</td>
<td>92.45 ± 2.85</td>
</tr>
</tbody>
</table>

Kᵦ values were derived from saturation equilibrium binding assays; Kᵦ values calculated from IC₅₀ values derived from competition curves.²² Data are averages from 2 to 4 experiments, each performed in triplicate.
from functional assays using NECA and CPA, and compared with human, chicken A\textsubscript{2B} ARs in our system have comparable or higher affinities for agonists tested. Our results suggest that the angiogenic response to 100 nmol/L CPA in the CAM is A\textsubscript{1} AR-mediated. A\textsubscript{3} AR-activation may also stimulate angiogenesis in the CAM, as suggested by the response to IB-MECA, which, at 100 nmol/L, should be selective for the chicken A\textsubscript{3} AR (see Table).

**CPA Does Not Promote Tube Formation in Ex Vivo Aortic Rings**

We used the rat thoracic aortic ring model to test whether CPA would stimulate angiogenesis through direct effects on vascular cells. This ex vivo coculture model contains ECs, fibroblasts, and smooth muscle cells, but in the absence of injury is largely devoid of inflammatory cells. Adenosine stimulates proliferation in ECs and fibroblasts, but inhibits proliferation from smooth muscle cells from most vascular beds, via interactions with A\textsubscript{3A} or A\textsubscript{2B} ARs. In our hands, CPA failed to affect proliferation in human microvascular endothelial cells (HMECs) or in rat aortic smooth muscle cells (data not shown). CPA (50 nmol/L) did not increase endothelial sprouting when applied directly to aortic rings (Figure 3). NECA, used as a positive control, increased tube formation 1.9-fold over vehicle (P<0.05), a response \(\approx 60\%\) of that observed to VEGF (10 ng/mL).

**Conditioned Medium From CPA-Treated Monocytes Increases Endothelial Tube Formation in Aortic Rings**

Given that CPA did not stimulate angiogenesis through direct interactions with vascular cells, we hypothesized that it may stimulate inflammatory cells to release proangiogenic factors. The 2 inflammatory cells most closely linked to angiogenesis, the mast cell and the monocyte, each possess multiple functional AR subtypes, but A\textsubscript{1} AR mRNA has not been identified in mast cells and has been in monocytes. In our hands, A\textsubscript{1} AR activation did not cause mast cell degranulation (data not shown).

We investigated the effects of A\textsubscript{1} AR stimulation on human monocytes isolated from peripheral blood. Our isolated monocytes express message for all 4 AR subtypes (data not shown). By FACS, there was some lymphocytic contamination, but only a small fraction of lymphocytes, if any,
possess A1 ARs. There was very little to no contamination with polymorphonuclear leukocytes, which express functional A1 ARs.

Conditioned medium from CPA-stimulated (50 nmol/L) human monocytes increased the number of endothelial tube sprouts from aortic rings by 48% compared with control rings treated with medium from vehicle-exposed cells (135.4 ± 12.4 tubes with CPA versus 91.0 ± 9.2 with vehicle, P < 0.05; Figure 4). Tube number was reduced to control levels in the presence of 50 nmol/L CPA and 1.5 μmol/L WRC (98.0 ± 10.4, n = 9; 10 ng/mL VEGF 158 ± 29, n = 6; *P < 0.05 vs conditioned medium control, ANOVA with Bonferroni post-hoc analysis, CPA + WRC P > 0.05 vs control).

**Figure 4.** Conditioned medium from CPA-treated monocytes stimulates endothelial tube formation in aortic rings. A, Representative photomicrographs of aortic rings (20×). B, Computerized calculation of tube number. Bars represent mean ± SEM of data compiled from all experiments. Control vessels, 91.0 ± 9.2, n = 12; 50 nmol/L CPA 135 ± 12, n = 12; 50 nmol/L CPA + 1.5 μmol/L WRC, 98.0 ± 10.4, n = 9; 10 ng/mL VEGF 158 ± 29, n = 6; *P < 0.05 vs conditioned medium control, ANOVA with Bonferroni post-hoc analysis, CPA + WRC P > 0.05 vs control.

**Discussion**

Ours is the first study demonstrating A1 AR-mediated angiogenesis in vivo and A1 AR-mediated release of a proangiogenic factor (VEGF) from peripherally-derived monocytes. We screened subtype-selective AR ligands using the CAM model because it is a highly vascular surface in vivo that is well-characterized, inexpensive, convenient, and easy to image. Drawbacks are that the CAM is not mammalian, is not currently amenable to transgenic experimentation, and there is limited pharmacologic characterization of chicken receptors. Stimulation of angiogenesis by A25,33,34 and A3 ARs8 was expected based on previous investigations, but discovering A1 AR-modulated angiogenesis was surprising. We cloned and pharmacologically characterized the chicken A1, A2B, and A3ARs to show that the ligand concentrations used in the CAM were selective for the chicken A1 AR. We were not successful in cloning the chicken A2A AR, but angiogenesis in the CAM was not stimulated by CSG21680, an agonist of known high affinity for the chicken A2A AR, or to ATL-146e, another potent A2A AR agonist. The CAM is a good in vivo screening model, but does not lend itself easily to mechanistic studies, so we used ex vivo and in vitro mammalian systems for these.

Whereas activation of A2A and A2B ARs12,33,34 on ECs promotes angiogenesis, our evidence pointed against a direct angiogenic effect for the A1 AR on ECs. A1 AR activation stimulates blood vessel formation in the CAM model, which includes both vascular and inflammatory cells, but not in the aortic ring assay, a model largely devoid of inflammatory cells. A1 AR expression in ECs has been reported,35,36 but the mitogenic properties of adenosine in ECs have been attributed to A2 AR activation,5,33 with the role of the A1 AR being

VEGF Release From A1 AR-Stimulated Human Monocytes

CPA (5 nmol/L) resulted in a 1.7-fold increase in VEGF release from human monocytes, from 581 ± 277 to 802 ± 355 pg/mL (P < 0.05; Figure 6). This was blocked by the A1 AR-selective antagonists WRC-0571 (200 nmol/L, P > 0.05 versus control) and 1,3-dipropyl-8-cyclopentylxanthine (CPX, 300 nmol/L, P > 0.05 versus control), but not by the A2A AR-selective antagonist ZM241385 (50 nmol/L, P < 0.05 versus control). These ligand concentrations were chosen to identify A1 AR-mediated effects based on our previous pharmacologic characterization of human ARs.32 Experiments were performed on unactivated monocytes. CPA did not cause aFGF or bFGF release from human monocytes (data not shown).
more obscure. Grant et al have shown that adenosine directly stimulates proliferation, chemotaxis, tube formation, and VEGF release in human retinal endothelial cells (HRECs), predominantly through A2B AR activation; however, their data suggest that the A1 AR may in some way modulate the angiogenic effects of adenosine on ECs because adenosine-stimulated HREC proliferation is partly mediated through ERK activation, which is blocked by A1 AR antagonists. Work by Lutty et al in canine retinal ECs suggests that A1AR activation may stimulate migration and tube formation, but not proliferation. In our hands, CPA failed to stimulate proliferation of cultured HMECs. Further, our ex vivo results in the thoracic aortic ring suggest that activation of A1 ARs in the rat aorta does not promote angiogenesis under the conditions we used. This having been said, expression and function of A1 ARs in endothelium and smooth muscle may vary with the vascular bed studied, and with the physiologic milieu, including factors such as shear stress, hypoxia, reperfusion, and concentrations of glucose or lipid. In cultured cells, confluence may also influence A1 AR expression and function. Finally, the role of A1 AR in vascular cells may vary among species. We cannot exclude a role for the A1 AR in modulating mitogenic effects on ECs from vascular beds other than the aorta, in species other than those we used, or in the presence of physiologic stressors not yet investigated.

Our observation that conditioned medium from CPA-stimulated human monocytes promotes angiogenesis is consistent with the hypothesis that the A1 AR can modulate angiogenesis through an indirect mechanism involving stimulation of inflammatory cells. Our data, in combination with other studies to date, are most consistent with the hypothesis that the A1 AR-modulated response is monocyte-dependent. ARs are expressed on a variety of inflammatory cells, including neutrophils, lymphocytes, mast cells, and monocytes/macrophages. Of these, AR activation on mast cells and monocytes/macrophages has been most convincingly associated with angiogenesis. These effects have previously been attributable to A2 or A3 AR subtypes. We did
not observe mast cell degranulation in response to A1 AR agonists. Although monocytes used for our studies are selected in a 2-step process using cell surface markers and substrate adherence, there are some residual contaminating lymphocytes. We do not observe significant contamination with neutrophils, making it unlikely that they are responsible for the angiogenic effects of CPA-conditioned medium. Conceivably A1 AR-mediated angiogenic effects could involve contaminating lymphocytes, but others have shown that only a small fraction of circulating lymphocytes express the A1 AR.\textsuperscript{31} Functional A\textsubscript{2A} ARs are present on monocyte/macrophages, modulating phagocytosis, chemotaxis, and multinucleate giant cell formation.\textsuperscript{13–15} We show that one possible mechanism for A1 AR-mediated angiogenesis in vivo is via effects on monocytes. The response may depend on interactions between more than one subpopulation of inflammatory cells, of which the monocyte is one. The A1 AR may also have direct proangiogenic effects on vascular cells in vivo.

On the surface, our results might appear to contradict observations by Leibovich et al that murine peritoneal macrophages do not release VEGF in response to A1 AR agonist.\textsuperscript{7} Several explanations may reconcile the apparent discrepancy. First, as in mast cells,\textsuperscript{39} there may be differences among species in the expression and function of AR subtypes in monocytes/macrophages. We used human monocytes derived from peripheral blood, whereas Leibovich et al used tissue-resident intraperitoneal murine macrophages. Second, A1 AR expression in monocytes and macrophages changes with culture conditions and cellular differentiation.\textsuperscript{30,40} The tissue resident macrophages studied by Leibovich et al would be expected to have significant phenotypic differences from our cultured peripheral blood monocytes. We confirmed the presence of message for the AR subtypes in our monocytes using RT-PCR and sequencing (data not shown), obtaining results similar to those previously reported by Theile et al.\textsuperscript{30} Characterization of the role of the A1 AR in subpopulations of monocytes and macrophages is not as well-developed as for the A\textsubscript{2A} AR, but there is evidence that the A1 AR is functionally significant in subsets of monocyctic cells. Haskò et al have described differential chemokine release in response to activation of A1 and A2 AR subtypes in the RAW 264.7 macrophage cell line, suggesting that these receptors modulate distinct effects in these cells.\textsuperscript{41} Further, adenosine stimulates chemotaxis of plasmacytoid dendritic cells isolated from peripheral blood via an A1 AR-mediated mechanism.\textsuperscript{15} This subpopulation of dendritic cells has been observed in high numbers in some forms of malignancy, where they induce angiogenesis.\textsuperscript{42}

Finally, it is possible that A1 ARs in monocytes/macrophages are upregulated in response to specific physiologic stresses. Our studies were performed in systems unperturbed by hypoxic, oxidative, inflammatory, or glycemic stresses. There is evidence that alterations in expression of A1 ARs on monocyte/macrophage cells are associated with some types of inflammatory diseases, including multiple sclerosis\textsuperscript{43} and pulmonary damage associated with adenosine deaminase deficiency.\textsuperscript{44}

To our knowledge, this study is the first description of A1 AR-mediated VEGF release, although A3\textsubscript{A}, A3\textsubscript{B}, and A1 ARs have each been shown to regulate VEGF release in a context-specific fashion.\textsuperscript{4,6,45,46} In previous studies the effects of adenosine on VEGF release have depended on the AR subtype activated, the species, the tissue, and the presence of specific physiologic stresses. In our cultured monocytes, the magnitude of VEGF release is modest, perhaps because our cultured cells were not exposed to another activating stimulus, which has been shown to be important for A2 AR-mediated VEGF release, although A2A, A2B, and A3 ARs have each been shown to regulate VEGF expression in a context-specific fashion.\textsuperscript{4,6,45,46} The tissue resident macrophages studied by Leibovich et al would be expected to have significant phenotypic differences from our cultured peripheral blood monocytes. We confirmed the presence of message for the AR subtypes in our monocytes using RT-PCR and sequencing (data not shown), obtaining results similar to those previously reported by Theile et al.\textsuperscript{30} Characterization of the role of the A1 AR in subpopulations of monocytes and macrophages is not as well-developed as for the A\textsubscript{2A} AR, but there is evidence that the A1 AR is

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure7}
\caption{Overview of proangiogenic effects of AR subtypes. On monocytes/macrophages, adenosine (Ado) activation of A1 (this report) and A2A ARs\textsuperscript{7} promotes VEGF release. LPS and the A2B AR synergistically amplify VEGF release from macrophages.\textsuperscript{7} On mast cells, A\textsubscript{3B} AR activation causes release of VEGF and IL-8; and A2 AR, angioptietin 2 (Ang2).\textsuperscript{9} A\textsubscript{2A} ARs on smooth muscle cells (SMCs)\textsuperscript{47} and some ECs\textsuperscript{48,49} modulate VEGF release. A\textsubscript{3A} ARs inhibit thrombospondin 1 (Tsp1) production by ECs.\textsuperscript{47} A\textsubscript{3B} ARs on ECs are upregulated by hypoxia and promote release of VEGF and IL-8.\textsuperscript{5,48,49}}
\end{figure}
taxis, whereas A_{2A} AR activation decreased phagocytosis and inhibited oxidative burst.\textsuperscript{37} We have not yet explored stimuli potentially synergistic with A_{1} AR agonists for VEGF release in the monocyte or the macrophage. It is also possible that A_{1} AR activation on monocytes and macrophages promotes release of angiogenic factors other than VEGF, but we have not yet identified them. Figure 7 is an overview of proangiogenic effects associated with AR activation on vascular and inflammatory cells.

In conclusion, we have demonstrated that A_{1} AR activation elicits an angiogenic response in vivo and promotes VEGF-release from cultured monocytes.

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**Disclosures**

Drs Linden, Sullivan, and Tucker have significant interest in, and are on the Advisory Board for, Adenosine Therapeutics, LLC.

**References**


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Expanded Materials and Methods

Reagents. Histopaque 1077 was obtained from Sigma-Aldrich (St. Louis, MO). Human bFGF, aFGF, and VEGF ELISA kits, human VEGF, and anti-VEGF antibody were obtained from R & D Systems (Minneapolis, MN). CD19 and CD2-coated magnetic beads were from Dynal Biotech Inc. (Lake Success, NY). CD14 FITC-labeled antibody was obtained from Immunotech. (Marseille, France). White Leghorn chicken eggs were acquired from CBT Farms (Chestertown, Md). CPA and 5'-N-ethylcarboxamido-adenosine (NECA) were purchased from Sigma-Aldrich (St. Louis, MO). 1,3-dipropyl-8-cyclopentylxanthine (CPX) was from Research Biochemical International (Natick, MA, U.S.A.). 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]-ethyl)phenol (ZM241385)\(^1\) was a gift from Simon Poucher (Astra-Zeneca Pharmaceuticals, Cheshire, UK). WRC 0571 was a gift from Dr. Pauline Martin and N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)-phenoxy]acetamide (MRS-1754) was a gift from Dr. Ken Jacobsen (Molecular Recognition Section, National Institutes of Health, Bethesda, Maryland). \(^{125}\text{I}-N^6\)-iodoaminobenzyladenosine \((^{125}\text{I}-\text{ABA})\)\(^2\) and \(^{125}\text{I}\)-3-(4-amino-3-iodobenzyl)-8-oxyacetate-1-propyl-xanthine \((^{125}\text{I}-\text{ABOPX})\) were synthesized as previously described\(^3\). Adenosine deaminase (ADA) was from Boehringer Mannheim.

Chorioallantoic membrane (CAM) angiogenesis assay. One day fertilized White Leghorn chicken eggs were incubated at 37ºC with rotation for 7 days prior to being windowed. Eggs were cleaned with alcohol and betadine, following which a 1x1 cm window was etched into the shell with a razor blade. The shell and underlying membranes were removed, with care taken not to drop eggshell on the CAM. Elvax
polymer pellets each containing 3 mg of adenosine or solutions of subtype-selective ligands applied to a 38 mm² Whatman 3MM filter paper disc were placed on the windowed 7 day old CAMs, and the windows sealed using a plastic coverslip and cellophane. On day 14, CAMs were harvested and imaged.

*Rat thoracic aortic ring assay.* Two month old Fischer 344 male rats were sacrificed and the thoracic aorta was removed and cleaned of fibroadipose tissue with microdissection forceps. The aorta was incised into 1 mm rings and washed x6 in EBM-2. Aortic arch segments were not used. Collagen was purified from rat tail according to the method of Elsdale and Bard and was prepared for use by mixing on ice eight volumes of 1.25 mg/ml collagen in 0.1X Minimum Essential Medium (MEM), pH 4.0, with a freshly made solution containing one volume of 10XMEM and one volume of 23.4 mg/ml Na HCO₃. Four drops of collagen solution were placed on the bottom of each well of a 48-well culture plate using a transfer pipette. The culture dish was then transferred into a humidified incubator for 5 min at 37 °C. After the bottom collagen was gelled, an aortic ring was placed at the bottom of each well, after which 6 drops of collagen were placed on top. Rings were positioned on the bottom collagen gel so that the two cut edges of the explant were clearly visible to allow viewing of the endothelial tubes in their entirety as they grow from the vessel lumen. Growth medium ± adenosine ligands or conditioned media were added to the rings and changed three times a week starting from day 3 of culture. DMSO concentration was kept ≤ 0.5%. Aortic explants were photographed at day 7 utilizing a Leitz inverted microscope under Brightfield with the iris diaphragm of the condenser closed at 20X magnification. The angiogenic response
was quantified by computer-assisted image analysis adapted from the method of Nissanov to ImagePro Plus software $^{5,6}$. All analysis is done in a single blinded fashion.

References


