Growth regulation of the vascular system: an emerging role for adenosine

Thomas H. Adair
Angiogenesis Research Laboratories, Department of Physiology and Biophysics, Center for Excellence in Cardiovascular-Renal Research, University of Mississippi Medical Center, Jackson, Mississippi

Adair, Thomas H. Growth regulation of the vascular system: an emerging role for adenosine. Am J Physiol Regul Integr Comp Physiol 289: R283–R296, 2005; doi:10.1152/ajpregu.00840.2004.—The importance of metabolic factors in the regulation of angiogenesis is well understood. An increase in metabolic activity leads to a decrease in tissue oxygenation causing tissues to become hypoxic. The hypoxia initiates a variety of signals that stimulate angiogenesis, and the increase in vascularity that follows promotes oxygen delivery to the tissues. When the tissues receive adequate amounts of oxygen, the intermediate effectors return to normal levels, and angiogenesis ceases. An emerging concept is that adenosine released from hypoxic tissues has an important role in driving the angiogenesis. The following feedback control hypothesis is proposed: AMP is dephosphorylated by ecto-5′-nucleotidase, producing adenosine under hypoxic conditions in the extracellular space adjacent to a parenchymal cell (e.g., cardiomyocyte, skeletal muscle fiber, hepatocyte, etc.). Extracellular adenosine activates A2 receptors, which stimulates the release of vascular endothelial growth factor (VEGF) from the parenchymal cell. VEGF binds to its receptor (VEGF receptor 2) on endothelial cells, stimulating their proliferation and migration. Adenosine can also stimulate endothelial cell proliferation independently of VEGF, which probably involves modulation of other proangiogenic and antiangiogenic growth factors and perhaps an intracellular mechanism. In addition, hemodynamic factors associated with adenosine-induced vasodilation may have a role in the development and remodeling of the vasculature. Once a new capillary network has been established, and the diffusion/perfusion capabilities of the vasculature are sufficient to supply the parenchymal cells with adequate amounts of oxygen, adenosine and VEGF as well as other proangiogenic and antiangiogenic growth factors return to near-normal levels, thus closing the negative feedback loop. The available data indicate that adenosine might be an essential mediator for up to 50–70% of the hypoxia-induced angiogenesis in some situations; however, additional studies in intact animals will be required to fully understand the quantitative importance of adenosine.

vascular endothelial growth factor; angiogenic growth factors; angiogenesis; adenosine receptors; negative feedback

ADENOSINE IS A UBQUITOUS NUCLEOSIDE produced by stepwise dephosphorylation of ATP. A role for adenosine in the regulation of cardiovascular function was first considered 75 years ago, when Drury and Szent-Gyorgyi (41) found that extracts from the heart and other tissues produced vasodilation, hypotension, bradycardia, and a decrease in atrioventricular conduction velocity. Berne and associates (19, 20) later proposed a retaliatory metabolite concept in which hypoxic tissues produce adenosine from ATP, and the adenosine in turn functions to restore balance between oxygen demand and oxygen supply. Adenosine increases oxygen supply by causing vasodilation and increased blood flow in the heart, skeletal muscle, brain, and other tissues (19, 20, 101), and the antiadrenergic effects of adenosine decrease oxygen demand in the heart (17, 40, 108, 126). Adenosine thus serves as a negative feedback signal to maintain tissue oxygenation within a normal range.

An emerging concept is that adenosine also has a long-term role in maintaining tissue oxygenation by stimulating the growth of blood vessels. Physiological concentrations of adenosine produced under hypoxic conditions stimulate a concentration-dependent proliferation and migration of endothelial cells (ECs) obtained from both large and small blood vessels (46, 60, 61, 64, 94, 104, 133, 149). EC proliferation and migration are key steps in the angiogenesis process necessary for establishing capillary sprouts in the microenvironment of a hypoxic tissue where adenosine levels are highest. Numerous studies have shown that adenosine or adenosine uptake inhibitors can stimulate blood vessel growth in vivo (5, 7, 18, 32, 44, 100, 112, 153–155, 165, 169).

Although the mechanism by which adenosine stimulates angiogenesis is poorly understood, many studies have shown that the administration of adenosine ( 50–52, 54, 61, 63, 69, 145) as well as the upregulation of endogenous adenosine (64) can induce the expression of vascular endothelial growth factor (VEGF) in a variety of cell types. VEGF is a vital mediator of angiogenesis released from hypoxic tissues in both physiological and pathological settings and is a target for clinical therapy in several pathological conditions (53). Adenosine can also stimulate EC proliferation independently of VEGF, which may involve the modulation of other proangiogenic and antiangiogenic growth factors; negative feedback.
Adenosine production and consumption. The concentration of adenosine in the interstitial fluids may be an important determinant of the angiogenic activity in a tissue because adenosine can induce angiogenic growth factors and initiate hemodynamic events that lead to vascular growth. The major pathway for adenosine formation in most tissues is stepwise dephosphorylation of ATP. Adenosine accumulates in the heart and other tissues when oxygen demand exceeds oxygen supply, e.g., during exercise, vascular deficiencies, and other conditions associated with tissue hypoxia/ischemia and/or increased ATP turnover (38, 77, 137). An increase in ATP turnover in tissues with relatively high metabolic rates may increase AMP levels and subsequently also adenosine levels independent of hypoxia (13, 83, 110, 161), i.e., tissues with high metabolic rates are expected to have high adenosine concentrations even under normoxic conditions, as discussed later.

At least four enzymes and a membrane carrier are involved in controlling the interstitial adenosine concentration, as shown in Fig. 1. Adenosine is produced by dephosphorylation of AMP and hydrolysis of S-adenosylhomocysteine (SAH). The hydrolysis of SAH to adenosine (and homocysteine) by SAH hydrolase is thought to be a constitutive pathway that contributes marginally to adenosine production (83, 161). Dephosphorylation of AMP is the major source of adenosine under hypoxic/ischemic conditions; this reaction occurs intracellularly by cytosolic-5'-nucleotidase and extracellularly by cd73/ecto-5'-nucleotidase. The relative contribution of the cytosolic and ecto pathways for adenosine production has been the subject of much debate (22, 39, 70, 86, 128, 131); however, recent studies in mice show that targeted disruption of cd73/ecto-5'-nucleotidase can modulate basal coronary vascular tone as well as other adenosine-mediated events, suggesting an important role for adenosine produced extracellularly (82). This latter finding is consistent with the notion that physiologically significant amounts of adenosine are produced extracellularly by cardiomyocytes (36, 39) and skeletal muscle fibers (97) and that both cell types are a sink rather than a source for adenosine. Histochemical and/or functional studies have demonstrated the existence of ecto-5'-nucleotidase on many different cell types including cardiomyocytes (127), skeletal muscle fibers (71), Muller cells of the retina (95), astrocytes (171), various cell types in the kidney (87), and fibroblasts (87, 109, 129). Two enzymes can utilize adenosine and thus decrease its concentration. Adenosine is either deaminated to form inosine via adenosine deaminase or rephosphorylated into AMP via adenosine kinase, using ATP as the phosphate donor. The nucleoside transporter shown in Fig. 1 represents a bidirectional equilibrative nucleoside transporter (ENT1) that translocates adenosine down its concentration gradient by facilitated diffusion (16, 30).

Regulation of adenosine metabolism under hypoxic conditions. The adenosine concentration in interstitial fluids might be expected to increase when 1) the activities of adenosine-producing enzymes (nucleotidases) are increased, 2) the activities of adenosine-utilizing enzymes (adenosine kinase, adenosine deaminase) are decreased, 3) the availability of the primary substrate for adenosine formation (AMP) is increased, and/or 4) ENT is inhibited.

AMP hydrolysis via nucleotidase is the dominant pathway for adenosine production under normoxic conditions, and more than 90% of the adenosine produced under normoxic conditions is thought to be rephosphorylated to AMP via adenosine kinase (this is called the salvage pathway) (13, 83, 161). Because of the large flux through this AMP-adenosine substrate cycle, small changes in the activities of adenosine kinase or nucleotidase can produce large changes in adenosine concentration. For example, Gu and associates (64) have shown that pharmacological inhibition of adenosine kinase can raise adenosine levels sufficiently to induce VEGF mRNA and protein expression in rat myocardial myoblasts. Modulation of adenosine deaminase activity, on the other hand, is expected to have a minimal effect on adenosine concentration under basal conditions because the Michaelis-Menten constant of the deaminase is far greater compared with that of the kinase (13).

Recent studies indicate that exposing cells to a hypoxic environment can increase the activity of nucleotidase and decrease the activity of adenosine kinase, thereby causing a net increase in the production of adenosine and hence an increase in the interstitial adenosine concentration (90). Deeking and associates (35) perfused isolated guinea pig hearts with hypoxic perfusate and used a mathematical model to determine that hypoxia decreased adenosine kinase activity to 6% of basal levels. Although the mechanism by which hypoxia inhibits adenosine kinase activity is poorly understood, studies by Gorman et al. (59) indicate that cytosolic levels of inorganic phosphate achieved under hypoxic conditions in the heart are capable of inhibiting adenosine kinase activity. Other studies

Intracellular and extracellular metabolism of adenosine. SAH, S-adenosylhomocysteine; 5'N, 5'-nucleotidase.
(86) have shown that exposing aortic ECs to an anoxic environment (0% oxygen, 18 h) induced a twofold increase in cd73/ecto-5'-nucleotidase activity and increased cell surface expression of the enzyme but had no effect on its synthesis. The hypoxic induction of cd73/ecto-5'-nucleotidase activity can also occur in the ischemic heart (107) and brain (24) of intact animals, possibly by way of a hypoxia inducible factor-1 (HIF-1)-dependent regulatory pathway (143). Hypoxia can also downregulate (albeit transiently) the gene expression of a dipyridamole-sensitive ENT (mENT1) in mouse cardiomyocytes and thereby decrease [3H]adenosine uptake by cells (31). The hydrolysis of SAH to adenosine (and homocysteine) by SAH hydrolase is probably not upregulated under hypoxic conditions (37, 81, 161).

Hypoxia not only regulates the activity of enzymes and transporters but may also increase the availability of the primary substrate for adenosine, AMP. Hellsten and associates (72) found that knee extensor exercise in humans increased interstitial AMP levels by ~20-fold. Others (110) have confirmed that muscle contraction can increase interstitial AMP levels in perfused dog skeletal muscle; however, interstitial levels of AMP did not increase when resting muscles were perfused under hypoxic conditions. Using NMR spectroscopy, Pucar and associates (123) showed that hypoxia induced a 2.5-fold increase in AMP levels in the Langendorff-perfused rat heart. Also, Kuzmin and associates (84) found that ischemia increased interstitial ATP levels by ~10-fold in the Langendorff-perfused rat heart and that adenine nucleotides were sequentially dephosphorylated in the interstitial space by a chain of separate ectoenzymes. Therefore, it appears that hypoxia/ischemia can increase AMP levels in the interstitial fluid.

Hypoxic modulation of nucleoside transporters and enzymes of adenosine metabolism may require hours to days for full adaptation. Kobayashi and associates (81) found that prolonged exposure (but not acute exposure) of rat pheochromocytoma (PC12) cells to a hypoxic environment (5% oxygen, 48 h) caused the cells to shift toward an adenosine-producing phenotype. The adaptations consisted of decreased gene expression of the rENT1/nucleoside transporter, downregulation of adenosine-metabolizing enzymes (adenosine kinase, adenosine deaminase), and upregulation of adenosine-producing enzymes (cytosolic and cd73/ecto-5'-nucleotidase). It is likely that prolonged exposure to a hypoxic environment will prove necessary to determine the quantitative importance of adenosine in the angiogenesis process.

**Interstitial adenosine concentrations under normoxic and hypoxic/ischemic conditions.** Adenosine can increase, decrease, or have no effect on the proliferation and migration of ECs, depending on the concentration to which the cells are exposed. For this reason, it is important to determine the full range of interstitial adenosine concentrations that occur under normoxic and hypoxic/ischemic conditions in the intact animal. Interstitial fluid is typically collected using a flow-through microdialysis technique (157, 158) in which microdialysis tubing with a low-molecular-weight cutoff is implanted into a tissue and then perfused at a slow rate with a physiological electrolyte solution. The adenosine concentration in the effluent is then measured using HPLC. Adenosine measured in the blood greatly underestimates the interstitial concentration because of rapid metabolism by cellular elements in the blood and also because the vascular endothelium has a high capacity to metabolize adenosine (111, 114).

The interstitial adenosine concentration measured using the microdialysis technique typically ranges from ~0.5 to 2 μM under basal conditions and from 10 to 15 μM under ischemic conditions in the left ventricle of dogs (158), swine (66, 130), and rats (118). In skeletal muscle, the interstitial adenosine concentration typically ranges from ~0.1 to 0.4 μM under basal conditions and from 0.5 to 1 μM after hypoxic-hypoxia, exercise, or muscle stimulation in humans (72, 99) and rats (91). In the frontal cortex of newborn piglets, the interstitial adenosine concentration was ~0.7 μM under basal conditions and ~1.6 μM after hypoxic-hypoxia (117). Adenosine increased from 2 to ~40 μM in the rat striatum after 15 min of ischemia (65). Adenosine concentrations in the extracellular fluids of solid tumors in mice ranged from 0.2 to 2.4 μM (mean, 0.5 μM), which is 10- to 20-fold higher compared with the concentrations in adjacent subcutaneous tissues (21). Adenosine levels in tumors increased to as high as 13 μM (mean, ~9 μM) after simultaneous treatment with inhibitors of adenosine deaminase and adenosine kinase (21).

The possibility that adenosine is generated in close proximity to its receptors would limit the usefulness of interstitial adenosine concentrations to determine physiological relevance because interstitial values would underestimate the local concentration at the receptor (26). However, based on the available data, the upper limit of interstitial adenosine concentration under steady-state conditions would appear to be much less than 50 μM, even in severely ischemic tissues. It is important to understand the limits of interstitial adenosine concentrations in living animals to distinguish between physiological/pathological mechanisms and pharmacological effects.

**EVIDENCE FOR ADENOSINE-INDUCED ANGIOGENESIS**

**Adenosine induces EC proliferation and migration in vitro.** The proliferation and migration of ECs are key steps in the angiogenesis process. Many investigators have shown that adenosine or adenosine analogs/agonists can stimulate EC proliferation and/or migration at physiological concentrations (42, 46, 47, 60, 61, 64, 94, 104, 133, 149).

The results of EC proliferation studies in which cells were actually counted are summarized in Fig. 2. Ethier and associates (46) found that adenosine caused a concentration-dependent increase in human umbilical vein EC (HUVEC) proliferation over a physiological range of 1 nM to 10 μM (Fig. 2). Proliferation was blocked by the nonselective adenosine receptor antagonist 8-phenyltheophylline (8-PT), indicating mediation by an external membrane receptor. Physiological concentrations of adenosine had no effect on the proliferation of human embryonic lung fibroblasts in the Ethier et al. study (46). Meininger and associates (104) found that adenosine was chemotactic and mitogenic for bovine aortic and coronary venular ECs. Adenosine caused a concentration-dependent proliferation of coronary venular ECs that could be blocked with 8-PT, again indicating mediation by an external membrane receptor. Grant and associates (61) found that the nonselective adenosine agonist 5’-N-ethylcarboxamidoadenosine (NECA) caused a concentration-dependent proliferation of human retinal ECs mediated by A2b receptors. In other studies, they found a concentration-dependent stimulation of EC mi-
Adenosine or adenosine agonists cause a concentration-dependent stimulation of endothelial cell (EC) proliferation. HUVEC, human umbilical vein ECs; BCVEC, bovine coronary venular ECs; PCAEC, porcine coronary artery ECs; HREC, human retinal ECs; CREC, canine retinal ECs. The shaded area indicates the physiological/pathological range of adenosine concentrations in vivo.

Fig. 2. Adenosine or adenosine agonists cause a concentration-dependent stimulation of endothelial cell (EC) proliferation. HUVEC, human umbilical vein ECs; BCVEC, bovine coronary venular ECs; PCAEC, porcine coronary artery ECs; HREC, human retinal ECs; CREC, canine retinal ECs. The shaded area indicates the physiological/pathological range of adenosine concentrations in vivo.

Adenosine induces blood vessel growth in vivo. Numerous studies have shown that adenosine, adenosine agonists, and/or adenosine transport inhibitors can stimulate blood vessel growth in mammalian skeletal muscle (7, 8, 151, 152, 169), the mammalian heart (18, 100, 102, 153–155, 169), in myocardial capillary density with no change in the heart-to-body weight ratio, suggesting that adenosine stimulated angiogenesis in the heart. Adenosine also caused a 26% increase in the capillary-to-muscle fiber ratio in the skeletal muscles of the same animals, indicating that adenosine stimulated capillary growth (169). Wothe and associates (165) found that adenosine alone caused the CAM vasculature to proliferate after only 2 wk of treatment in some studies. Dipyridamole inhibits ENT1, which translocates adenosine down its concentration gradient by facilitated diffusion (16, 30), and thus raises adenosine levels in the interstitial fluid and blood during both normoxic and hypoxic conditions (80, 115, 163). Definitive proof that diprydamole-induced angiogenesis is actually mediated by adenosine has not been forthcoming. Nevertheless, the adenosine antagonist 8-PT can inhibit dipyridamole-induced vasodilation in perfused guinea pig hearts (159), and theophylline can antagonize cardiovascular responses to dipyridamole in humans, e.g., changes in heart rate and blood pressure (136), indicating that acute cardiovascular responses to dipyridamole are mediated by adenosine. The possibility must also be considered that dipyridamole stimulates angiogenesis by its phosphodiesterase-inhibiting effect (103, 150), which can enhance the proangiogenic effects of the nitric oxide/cGMP system (113, 167, 170).

Interestingly, dipyridamole stimulated blood vessel growth in the body of the chick embryo (5) but not in the CAM (44). Yet, adenosine alone caused the CAM vasculature to proliferate, and the angiogenic effects of adenosine in the CAM were potentiated by dipyridamole and attenuated by the adenosine antagonist isobutylmethylxanthine (44). These results indicate that adenosine receptors are present in the CAM vasculature but that adenosine levels are low in CAM tissues under basal conditions. This could be expected because the CAM is a respiratory organ that facilitates oxygen delivery to the chick embryo body rather than local membrane tissues.

Prolonged administration of adenosine itself stimulates blood vessel growth in the intact animal. Ziada and associates (169) found that a continuous intravenous infusion of adenosine (42 μmol/h) for 3–5 wk in rabbits caused a 27% increase in myocardial capillary density with no change in the heart-to-body weight ratio, suggesting that adenosine stimulated angiogenesis in the heart. Adenosine also caused a 26% increase in the capillary-to-muscle fiber ratio in the skeletal muscles of the same animals, indicating that adenosine stimulated capillary growth (169). Wothe and associates (165) found that adenosine caused an approximately twofold increase in structural coronary conductance (i.e., the conductance of the maximally dilated vasculature) when adenosine was infused (12 h/day) into the circumflex coronary artery of near-term fetal sheep for 4 days, suggesting adenosine-induced growth of the coronary vasculature.

Dusseau et al. (44) found that adenosine applied locally to the chick CAM caused a concentration-dependent stimulation of angiogenesis that was inhibited with the adenosine antagonist isobutylmethylxanthine. Adair and associates (5) found that prolonged administration of adenosine into the air space of the chick egg caused a concentration-dependent increase in the structural vascular conductance in the embryo. Dipyridamole caused a similar concentration-dependent increase in vascularity, and the normal increase in vascularity that occurs in the developing embryo was attenuated in a concentration-dependent manner by the adenosine antagonist aminophylline. These latter findings suggest that adenosine could have a physiological role in the development of the vasculature (5).
MECHANISM OF ADENOSINE-INDUCED ANGIOGENESIS

Although the mechanism by which adenosine induces angiogenesis is poorly understood, many studies have shown that the administration of adenosine or adenosine agonists/analogs (50–52, 54, 56, 61, 63, 69, 145) as well as the upregulation of endogenous adenosine (64) can modulate the expression of VEGF and other angiogenic factors (50, 51, 61, 112) in a variety of cell types.

Adenosine receptors. Adenosine exerts most of its physiological actions by way of cell surface G protein-coupled receptors termed adenosine A1, A2A, A2B, and A3. All four receptors have been cloned and characterized pharmacologically using a variety of agonists and antagonists in several mammalian species (58). A1 and A2A are high-affinity receptors activated by submicromolar concentrations of adenosine, whereas A2B and A3 are low-affinity receptors activated when adenosine levels rise to micromolar concentrations (27, 49, 58). Adenosine receptors are also differentiated by signal transduction pathways. A2A and A2B receptors interact with the Gs and Gi protein families to stimulate adenylyl cyclase, whereas A1 and A3 receptors interact with the Gi and Go protein families to inhibit adenylyl cyclase (57, 58). A2 receptor activation is classically known to increase oxygen supply, whereas A1 receptor activation (and possibly A3 receptor activation) (15) is known to decrease oxygen demand. For example, in the cardiovascular system, A1 receptors mediate the antiadrenergic actions of adenosine, whereas A2 receptors mediate coronary vasodilation (26, 134). The physiological role of A2 receptor activation to induce angiogenesis is a major focus of this review.

Adenosine induces VEGF and other angiogenic growth factors in vitro. VEGF is an EC-specific mitogen in vitro and an angiogenic inducer in a variety of animal models (53). As a key mediator of angiogenesis, VEGF is released from hypoxic tissues in both physiological and pathological settings (53) and is a target for clinical therapy in several pathological conditions (53). VEGF has a major physiological role to induce angiogenesis in exercising muscle (25, 28, 67, 125, 162) and is a survival factor (or maintenance factor) for capillary ECs in resting skeletal muscle (147) as well as in other tissues (53). Also, it is likely that VEGF is subject to negative feedback regulation in the exercising muscles of intact animals (67).

Many cell types respond to hypoxia by increasing VEGF release (53, 62, 85, 135), which stimulates angiogenesis, ensuring adequate oxygenation. Hypoxia induces both increased transcription and decreased degradation of VEGF mRNA. Transcriptional regulation is mediated by HIF-1, which accumulates under hypoxic conditions and activates VEGF transcription by binding to specific promoter sequences (56, 132). In addition to the stabilization of mRNA (76, 146), hypoxia can induce VEGF mRNA by other HIF-1-independent pathways (73, 121, 164). The mechanism by which adenosine induces VEGF is poorly understood, but recent studies indicate mediation by a HIF-1-independent pathway (52).

Adenosine or adenosine agonists/analogs cause a concentration-dependent increase in VEGF expression (Fig. 3) (50, 61, 63, 64, 69, 88, 124, 145). Gu and associates (63) found that as little as 1 μM adenosine caused a significant increase in VEGF protein release from dog myocardial vascular smooth muscle cells; high, pharmacological concentrations of adenosine suppressed VEGF expression (Fig. 3). Grant and associates (61) found that the adenosine analog NECA increased VEGF protein release by nearly ~16-fold in human retinal ECs when the cells were cultured in serum-free media. NECA, 5′-N-ethylcarboxamidoadenosine: NECA-SF, NECA in serum-free media; CGS, 2-p-carboxyethylpiperazine-5′-N-ethylcarboxamidoadenosine (CGS-21680). The shaded area indicates the physiological/pathological range of adenosine concentrations in vivo.

Adenosine can also modulate the expression of VEGF receptors (VEGFRs). Takagi and associates (144) found that activation of A2 receptors mediated an acute response to hypoxia (3 h) consisting of a decline in VEGFR-2 (formerly called KDR/Flik) mRNA expression and VEGF binding sites in bovine retinal ECs. Chronic hypoxia lasting for 72 h increased VEGF binding sites. As pointed out by the authors (144), this biphasic receptor response in the face of elevated VEGF levels in the tissues could have an important in vivo function to minimize angiogenesis during brief episodes of hypoxia and enhance angiogenesis during chronic oxygen deficits when angiogenesis is a more appropriate response.

Recent reports from several laboratories indicate that adenosine can modulate the expression of other angiogenic growth factors. The adenosine analog NECA increased the expression of the proangiogenic factors insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF) in a time-dependent manner in human retinal ECs (61). NECA also increased the expression of the proangiogenic factors interleukin-8 (IL-8) and angiopoietin-2 (Ang-2) mRNA in human mast
Adenosine induces VEGF expression in the chick embryo heart and skeletal muscle

Table 1. Adenosine induces VEGF expression in the chick embryo heart and skeletal muscle

<table>
<thead>
<tr>
<th>Relative VEGF mRNA</th>
<th>Skeletal muscle</th>
<th>Cardiac muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>3.47 ± 1.24*</td>
</tr>
<tr>
<td>Adenosine</td>
<td>4.03 ± 1.12</td>
<td>10.89 ± 2.85*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 4. Total RNA was isolated from the chick embryo hindlimb and heart after embryos were treated with adenosine (4 μmol/day) or vehicle for 5 days. VEGF mRNA expression was determined in 14-day-old embryos by Northern blot analysis. The VEGF cDNA probe is a 390-bp KpnI-SacI fragment of quail VEGF cDNA cloned into the pBluescript plasmid (kindly provided by Dr. Ingo Flamme of the Max-Planck Institute). 28S rRNA was used to verify the relative amount of total RNA for each sample. *P < 0.05.

Adenosine increases plasma levels of VEGF in humans

Table 2. Adenosine increases plasma levels of VEGF in humans

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Plasma VEGF, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20.3 ± 2.0</td>
</tr>
<tr>
<td>1</td>
<td>62.7 ± 18.1*</td>
</tr>
<tr>
<td>2</td>
<td>23.3 ± 3.4</td>
</tr>
<tr>
<td>8</td>
<td>24.3 ± 7.0</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 5. Five outpatients 49.3 ± 6.7 yr of age weighing 88.2 ± 8.5 kg were given a 6-min intravenous infusion of adenosine (0.14 mg·kg⁻¹·min⁻¹ for 6 min) caused a threefold increase in plasma levels of VEGF 1 h after the infusion. The possibility that adenosine can induce VEGF expression in humans could provide a basis for development of adenosine-based angiogenic therapies for the future.
Table 3. Adenosine induces VEGF expression in many cell types

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>VEGF Response</th>
<th>Receptor Subtype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocardial myoblasts</td>
<td>Rat</td>
<td>GP-515 + ADA</td>
<td>U, A2A abolished</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Adenosine</td>
<td>U</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary artery</td>
<td>Dog</td>
<td>Adenosine + CSC</td>
<td>U, CSC blocked</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>Dog</td>
<td>DPMA</td>
<td>U</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>Dog</td>
<td>R-PIA</td>
<td>N</td>
</tr>
<tr>
<td>Aorta</td>
<td>Rat</td>
<td>Adenosine</td>
<td>U</td>
</tr>
<tr>
<td>Bronchiole</td>
<td>Human</td>
<td>NECA + hypoxia-conditioned cells</td>
<td>U, only after preconditioning</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebral microvascular</td>
<td>Pig</td>
<td>R-PIA</td>
<td>U</td>
</tr>
<tr>
<td>Umbilical vein</td>
<td>Human</td>
<td>NECA</td>
<td>N</td>
</tr>
<tr>
<td>Umbilical vein</td>
<td>Human</td>
<td>NECA + hypoxia-conditioned cells</td>
<td>U, only after preconditioning</td>
</tr>
<tr>
<td>Skin microvascular</td>
<td>Human</td>
<td>NECA</td>
<td>U</td>
</tr>
<tr>
<td>Skin microvascular</td>
<td>Human</td>
<td>CGS-21680</td>
<td>N</td>
</tr>
<tr>
<td>Retinal</td>
<td>Human</td>
<td>NECA + SCH-58261 or CPX</td>
<td>U, not blocked by SCH58261 or CPX</td>
</tr>
<tr>
<td>Retinal</td>
<td>Human</td>
<td>NECA + A2B AS-ODN</td>
<td>U, blocked by A2B AS-ODN</td>
</tr>
<tr>
<td>Miscellaneous cell types</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mast cells</td>
<td>Human</td>
<td>NECA + IPDA or SCH-58261</td>
<td>U, IPDA blocked, SCH58261 did not</td>
</tr>
<tr>
<td>PC12 pheochromocytoma cells</td>
<td>Rat</td>
<td>NECA or CGS-21680</td>
<td>D</td>
</tr>
<tr>
<td>Macrophage U937 cells</td>
<td>Human</td>
<td>NECA + DMPPX</td>
<td>U, DMPPX blocked</td>
</tr>
<tr>
<td>Macrophage cells</td>
<td>Mouse</td>
<td>NECA or CGS-21680 (A2A-deficient mice)</td>
<td>U, eliminated in A2A-/- mice</td>
</tr>
<tr>
<td>Retinal pericytes</td>
<td>Bovine</td>
<td>NECA or DMPPX</td>
<td>U</td>
</tr>
<tr>
<td>Retinal pericytes</td>
<td>Bovine</td>
<td>CGS-21680</td>
<td>U</td>
</tr>
</tbody>
</table>

*According to conclusions of authors.

(50) found that only A2B receptors mediated adenosine-induced transcription of IL-8 and VEGF genes in human microvascular ECs after cotransfection of IL-8 and VEGF reporters with adenosine receptors (50). Overexpression of A2A receptors attenuated the stimulation of IL-8 and VEGF mediated by native A2B receptors (50). Other studies have shown that A2A receptor activation downregulated IL-8 in HUVECs (23) as well as VEGF in rat pheochromocytoma PC12 cells (116). But again, both pharmacological (145) and gene manipulation (88) studies provide strong evidence that A2A receptor activation can induce VEGF expression in some cell types. Also, Taomoto and associates (148) used immunohistochemical techniques to show that A2A receptors were upregulated at the edge of the proliferating vasculature in a canine model of oxygen-induced retinopathy (in which angiogenesis is induced in hypoxic retina after hyperoxic obliteration of retinal blood vessels), implicating the importance of these receptors in the angiogenesis process. The possibility that different cell types within a given species can modulate the expression of VEGF and other angiogenic growth factors via different receptor subtypes has not been confirmed nor refuted at the writing of this review.

**Hypoxic induction of an angiogenic phenotype by modulating adenosine A2 receptor expression.** Prolonged exposure to a hypoxic environment can create an angiogenic phenotype that promotes adenosine-induced VEGF expression. Feoktistov and associates (52) found that exposure to a hypoxic environment upregulates A2B receptors and downregulates A2A receptors in HUVECs and human smooth muscle cells. Both cell types normally express A2A and A2B receptors, but A2A receptors predominate under normoxic conditions both in terms of mRNA expression and functional coupling to agonists. Because adenosine-induced VEGF expression is mediated by A2B receptors in both cell types, exposure to hypoxia was found to amplify the VEGF response to adenosine. NECA did not stimulate VEGF release from either cell type under normoxic conditions; however, NECA caused a 0.5- to 2.5-fold increase in VEGF release from cells preconditioned in a hypoxic environment, indicating that A2B receptors were functionally coupled to the upregulation of VEGF.

Studies from other laboratories confirm that exposure to hypoxia (45, 168) or simulation of hypoxia by cobalt ions (166) can upregulate A2B receptor expression. Treatment with cobalt ions caused a time-dependent increase in A2B receptor mRNA expression in U87MG human glioma cells, increasing mRNA levels by more than threefold at 24 h (166). Eltischig and associates (45) found that exposing confluent human microvascular ECs to a hypoxic environment (P02 = 20 mmHg) caused a four- to six-fold increase in A2B receptor mRNA expression after 6–24 h of exposure as well as a ~75% reduction in A2A receptor expression. Other studies suggest that cerebral ischemia can upregulate A2B receptor expression in rats (168). Even so, Gu and associates (63) found that an adenosine A2 antagonist could not block more than ~20% of the VEGF expression induced by exposing myocardial vascular smooth muscle cells to a hypoxic environment, suggesting a relatively minor role for adenosine under hypoxic conditions. Other studies (56) indicate that VEGF mRNA is induced only
slightly under hypoxic conditions in mutant mouse hepatoma cells that do not express the HIF-1α (ARNT) subunit, which also suggests a minor role for adenosine independent of any HIF-1-mediated pathway.

Nevertheless, it could be that adenosine mediates a level of compensatory angiogenesis in hypoxic tissues that cannot be predicted from studies performed under normoxic conditions, especially because adenosine can stimulate EC proliferation independently of VEGF, as discussed in Can adenosine induce angiogenesis independently of VEGF?. Also, adenosine is a potent vasodilator in many vascular beds, and mechanical events associated with vasodilation of larger blood vessels and increased shear rate in capillaries is thought to stimulate angiogenesis as well as vascular remodeling (2, 75, 78, 169).

Interestingly, A2B receptors are more abundant in oxidative fibers (type I) compared with glycolytic fibers (type II) in human skeletal muscle (96), and the activation of adenylyl cyclase by adenosine in rat skeletal muscle is mediated mainly by the A2B receptor (98). Given that VEGF-induced angiogenesis occurs in hypoxic tissues and given that oxidative fibers have a more extensive capillary network compared with glycolytic fibers (2, 74, 75), one can speculate that hypoxia in the microenvironment of an oxidative fiber can induce the expression of A2B receptors. This increase in A2B receptor expression would promote angiogenesis in the vicinity of the fiber by adenosine induction of VEGF expression. In other words, the A2B receptors could have an important role in stimulating the release of extra amounts of VEGF from the oxidative fiber, not only to maintain the relatively extensive capillary network associated with the fiber but also to stimulate the growth of new capillaries in the vicinity of the fiber when metabolic demand increases. This speculation is supported by the findings that oxidative skeletal muscle fibers have higher levels of VEGF protein in the adjacent interstitial spaces (12) as well as higher VEGF mRNA levels within the fibers themselves during basal conditions (28) compared with glycolytic fibers. The possibility that adenosine is a vascular maintenance factor is discussed in the next major section.

Can adenosine induce angiogenesis independently of VEGF? The actions of adenosine to induce VEGF and stimulate EC proliferation and migration have been attributed to the activation of A2A or A2B receptors; however, it is possible that adenosine can stimulate EC proliferation by mechanisms independent of VEGF. For example, adenosine caused a concentration-dependent proliferation of HUVECs under normoxic conditions (Fig. 2) (46), but the adenosine analog NECA did not induce VEGF in HUVECs under normoxic conditions (Table 3) (50). The induction of VEGF by NECA in HUVECs preconditioned in a hypoxic environment can be attributed to upregulation of the A2B receptor subtype (52), as discussed previously.

Ethier and Dobson (47) found that adenosine stimulation of [3H]thymidine uptake by HUVECs cultured in serum-free medium could not be mimicked by adenosine receptor agonists or inhibited by adenosine receptor antagonists, suggesting a mechanism independent of A1, A2, and A3 receptors. Yet, the high-molecular-weight nontransportable adenosine analog Poly(A) also increased [3H]thymidine uptake in a concentration-dependent manner, suggesting that extracellular (not intracellular) adenosine stimulated DNA synthesis. The stimulation of DNA synthesis did not appear to involve the cAMP, PKC, or tyrosine kinase signaling pathways. Inhibition of Na+/H+ exchange and phospholipase A2 quelled the effect, suggesting that cell alkalinization and arachidonic acid metabolites may contribute to an adenosine-induced signaling pathway leading to DNA synthesis. Others have shown that adenosine stimulation of DNA synthesis in bovine aortic ECs cultured in serum-free medium was neither inhibited by the adenosine antagonist 8-PT nor mimicked by A1- or A2-selective agonists (156).

It is important to point out that neither adenosine nor adenosine agonists/analogs can stimulate HUVEC proliferation in serum-free medium (47) and that the adenosine-induced stimulation of HUVEC proliferation (which occurs in the presence of fetal calf serum) could be inhibited by 8-PT in other studies (46), implicating a receptor-dependent mechanism. In any case, the Ethier and Dobson study (47) provides another example in which [3H]thymidine uptake may not provide a reasonable estimate of cell proliferation.

Gu and associates (64) found that the adenosine kinase inhibitor GP-515 caused a greater increase in cell proliferation and DNA synthesis in HUVECs compared with equimolar amounts of exogenous adenosine even though exogenous adenosine was more effective than GP-515 on an equimolar basis in stimulating VEGF release from rat myocardial myoblasts. It is conceivable that adenosine kinase inhibition raises intracellular levels of adenosine to a greater extent than can be achieved by exogenous adenosine and that the high intracellular concentration of adenosine somehow stimulated EC growth. Although it is not clear whether adenosine can induce EC growth by way of an intracellular mechanism, the available data indicate that adenosine can stimulate the proliferation of HUVECs without first stimulating the release of VEGF from cells.

What is the quantitative importance of adenosine-induced angiogenesis under hypoxic conditions? Few studies have assessed quantitatively the importance of adenosine as a mediator of hypoxia-induced angiogenesis in the intact animal. Dusseau and Hutchins (43) found that hypoxia-induced angiogenesis in the chick CAM could be attenuated by 50–70% when adenosine receptors were blocked with methylisobutylxanthine. Adair and associates (3–5) found that the maximum increase in structural vascular conductance in chick embryos caused by administration of adenosine was 50% of that achieved with prolonged exposure to a hypoxic environment. Neovascularization was reduced by 50% in the retina of the eye after intraocular administration of an anti-A2B receptor ribozyme in a mouse model of oxygen-induced retinopathy (10). Therefore, the available data indicate that adenosine might be an essential mediator for 50–70% of the hypoxia-induced angiogenesis in some situations; however, additional studies in intact animals will be required to fully understand the quantitative importance of adenosine.

Mechanism of adenosine-induced angiogenesis. Figure 4 shows a first approximation mechanism of adenosine-induced angiogenesis under hypoxic conditions. AMP is dephosphorylated by ecto-5′-nucleotidase, producing adenosine in the extracellular space adjacent to a parenchymal cell, which is the major important source of adenosine under hypoxic/ischemic conditions. The parenchymal cell could be a cardiomyocyte, skeletal muscle fiber, hepatocyte, or any other functional element of an organ or tissue that is subjected to a hypoxic environment. Extracellular adenosine then stimulates the re-
lease of VEGF from the parenchymal cell by activating A2A or A2B receptors. The receptor subtype (A2A or A2B) that leads to the induction of VEGF may depend on species and cell type; however, the available data suggest that the A2B receptor is a likely mediator in human cells. VEGF released from parenchyma cells binds to its receptor (VEGFR-2) on ECs, stimulating EC proliferation and migration. VEGF is also a survival factor or maintenance factor for ECs that may be regulated by adenosine under basal conditions, as discussed in DOES ADENOSINE PROVIDE A MAINTENANCE FACTOR FUNCTION FOR THE VASCULARATURE?

Does Adenosine Provide a Maintenance Factor Function for the Vasculature?

Adenosine can stimulate EC proliferation independently of VEGF, which probably involves modulation of other proangiogenic and antiangiogenic growth factors or perhaps an intracellular mechanism. In addition, hemodynamic factors associated with adenosine-induced vasodilation may have a role in development and remodeling of the capillaries as well as larger blood vessels. Once a new capillary network has been established and the diffusion/perfusion capabilities of the vasculature can supply the parenchymal cells with adequate amounts of oxygen, adenosine and VEGF as well as other proangiogenic and antiangiogenic growth factors return to near-normal levels, thus closing the negative feedback loop.

Does Adenosine Provide a Maintenance Factor Function for the Vasculature?

An interesting possibility is that adenosine stimulation of VEGF expression provides a maintenance factor function for the vasculature. The concept of a vascular maintenance factor (also called a survival factor) is based on the knowledge that the length density of a capillary network (i.e., capillary length per unit tissue volume) is dependent on the oxidative capacity of the tissues that it serves (2). An increase in metabolic activity (e.g., exercise) induces VEGF expression and stimulates angiogenesis in skeletal muscle, whereas factors that decrease metabolic activity (e.g., tenotomy, muscle paralysis, denervation) downregulate VEGF, and capillary rarefaction follows (2, 11, 75, 89, 160). These latter findings indicate that basal levels of VEGF (necessary for maintaining vascular integrity) are dependent on the metabolic activity of the muscle. Moreover, VEGF expression remains elevated in skeletal muscle after exercise-induced angiogenesis, suggesting a proportional relationship between VEGF concentration and muscle capillarity (12, 67).

That a basal level of VEGF is essential for maintaining capillary integrity has been demonstrated by Tang and associates (147). Targeted skeletal muscle inhibition of VEGF expression in VEGFloxP mice through viral delivery of cre recombinase caused a ~70% decrease in both capillary density and the capillary-to-fiber ratio in the VEGF-inactivated regions of the muscle. Furthermore, capillary regression was accompanied by Tdt-mediated dUTP nick-end labeling (TUNEL)-positive apoptotic ECs. Additional evidence that VEGF is a vascular maintenance factor is provided in a recent review (53).

The possibility that adenosine is important for establishing basal levels of VEGF under normoxic conditions and therefore provides an essential maintenance factor function for the vasculature is partially based on the following findings: 1) >60% of the VEGF released from cultured myocardial vascular smooth muscle cells can be inhibited by adenosine A2 antagonist during normoxic conditions (63), and 2) adenosine deaminase (which metabolizes adenosine to inosine) can decrease basal levels of VEGF by ~60% in the media of myocardial myoblasts (64). Also, oxidative muscle has a relatively high metabolic rate, high basal level of adenosine, high basal level of VEGF, and high capillarity compared with glycolytic muscle (2, 12, 28), and, when a glycolytic muscle is converted to an oxidative muscle by prolonged electrical stimulation of a motor nerve, an approximately twofold increase in capillarity (34, 74) is associated with an approximately twofold increase in basal VEGF expression when full adaptation has occurred (67).

Is HIF-1 important for establishing basal levels of VEGF?

HIF-1α is detectable in normoxic tissues (106, 140), and HIF-1α levels increase under hypoxic conditions. However, it is not clear whether HIF-1α levels are proportional to the basal metabolic activity of a tissue under normoxic conditions, which would appear necessary to account for differences in the basal levels of VEGF. Stroka and associates (140) found that basal levels of HIF-1α were higher in skeletal muscle com-
pared with the heart, which argues against a maintenance factor role for HIF-1 considering that basal levels of VEGF are higher in the heart compared with skeletal muscle. Additional studies will be required to determine the possible role of HIF-1 in establishing the basal levels of VEGF.

For these reasons, it is conceivable that adenosine plays an important role in establishing the basal levels of VEGF in a tissue under normoxic conditions. If this is true, and if adenosine serves to “fine-tune” the levels of VEGF in a tissue in accordance with the metabolic requirements of the tissue, then adenosine can be considered a vascular maintenance factor.

ADENOSINE MODULATION OF ANGIOGENESIS COULD HAVE THERAPEUTIC VALUE

Mounting evidence suggests that modulating adenosine levels in the tissues or adenosine receptor function could provide a basis for the treatment of cancer (105, 122, 138, 139, 166), wounds (32, 88, 112, 120), vasoproliferative retinopathies (10, 60, 61, 94, 148), coronary artery disease (9, 18, 92, 119, 142), and other angiogenic diseases characterized by too much or too little angiogenesis. Chronic treatment with dipyridamole (which increases interstitial adenosine levels) can decrease myocardial infarct size in rats (9) and improve coronary collateralization and left ventricular systolic performance in humans (18). This latter finding suggests that the angiogenic actions of dipyridamole may have clinical relevance (119). Also, dipyridamole can increase the survival of experimental skin flaps in rats (14). Several laboratories seek to prevent inappropriate angiogenesis in the retina of the eye through modulation of adenosine and its receptors (60, 61, 94, 148). In particular, Afzal and associates (10) found that ribozymes that cleave A$_{2B}$ receptor mRNA caused a substantial reduction in preretinal neovascularization in a neonatal mouse model of oxygen-induced retinopathy.

Cronstein and associates (32, 105, 112) provide convincing evidence that adenosine can promote neovascularization in wounds. Recent studies (112) indicate that topical application of the adenosine A$_{2A}$ agonist CGS-21680 increases neovascularization in full-thickness excisional wounds in mice by increasing both local vessel sprouting and recruitment of endothelial progenitor cells from the bone marrow. Clinical studies are currently underway to determine the utility of topical adenosine A$_{2A}$ receptor agonists in the therapy of diabetic foot ulcers (32).

Adenosine stimulation of angiogenesis is a natural phenomenon that occurs in the microenvironment of tissues in accordance with local metabolic needs. Amplification of this natural process through modulation of the enzymes of adenosine metabolism or genetic or pharmacological induction of A$_{2A}$ or A$_{2B}$ receptors could, theoretically, lead to stimulation of angiogenesis in those areas of a tissue where hypoxia is most severe and adenosine levels are highest. This type of physiological therapy can be viewed as a departure from classical attempts to stimulate angiogenesis in a global manner, in which many or most cells of an organ or tissue are made to produce greater amounts of VEGF. In contrast to adenosine-based therapies, global induction of VEGF may not be an effective means to build capillary networks in the microenvironment of a tissue where hypoxia is most severe.

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