Mast Cell–Mediated Stimulation of Angiogenesis
Cooperative Interaction Between A_{2B} and A_3 Adenosine Receptors

Igor Feoktistov, Sergey Ryzhov, Anna E. Goldstein, Italo Biaggioni

Abstract—Adenosine is released during tissue injury, ischemia and tumor growth, and promotes angiogenesis. Because mast cells accumulate in the proximity of new blood vessel development, we examined if they may contribute to adenosine-induced angiogenesis. We found that HMC-1 human mast cells express A_{2A}, A_{2B}, and A_3 adenosine receptors. The adenosine agonist NECA (100 μmol/L) increased interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and angiopoietin-2 mRNA expression. NECA-induced secretion of IL-8 and VEGF was verified by ELISA. A_{2B} receptors mediate VEGF and IL-8 secretion because neither CGS21680 (selective A_{2A} agonist) nor IB-MECA (selective A_3 agonist) produced this effect, and it was inhibited by the selective A_{2B} antagonist IPDX but not by the selective A_{2A} antagonist SCH58261 or the selective A_3 antagonist MRS1191. In contrast, the selective A_3 agonist IB-MECA (EC_{50} 1 nmol/L) stimulated angiopoietin-2 expression. Conditioned media from NECA-activated HMC-1 stimulated human umbilical vein endothelial cell proliferation and migration, and induced capillary tube formation. Capillary formation induced by mast cell–conditioned media was maximal if both HMC-1 A_{2B} and A_3 receptors were activated, whereas activation of A_{2B} receptor alone was less effective. Thus, adenosine A_{2B} and A_3 receptors act in a functional cooperative fashion to promote angiogenesis by a paracrine mechanism involving the differential expression and secretion of angiogenic factors from human mast cells. **(*Circ Res*. 2003;92:485-492.)**

Key Words: adenosine receptors ■ mast cells ■ angiogenesis ■ angiopoietin-2 ■ vascular endothelial growth factor

The purine nucleoside adenosine is an intermediate product of adenine nucleotide metabolism. In many organs adenosine serves as a "retaliatory metabolite" in situations when oxygen supply is decreased or energy consumption is increased. Under these conditions, adenosine is released into the extracellular space and signals to restore the balance between energy supply and local energy requirements. Energy supply to the affected tissue can be modulated acutely by regulation of vascular tone or chronically by formation of new capillaries from preexisting blood vessels, by the process known as angiogenesis. Adenosine, produced in high concentrations during tissue injury, ischemia, or tumor growth, has been implicated in promoting angiogenesis.[1–4]

There is also growing evidence that mast cells, because of their ubiquitous distribution and location in proximity to blood vessels, can be involved in regulation of angiogenesis. Their number is increased in situations associated with angiogenesis and remodeling, such as arteriosclerosis,[5] asthma,[6] psoriasis,[7] wound repair,[8] and tumor growth (see review[9]). Accumulation of mast cells at the periphery of carcinomatous nodules occurs before the onset of angiogenesis.[10] Mast cells can serve as a rich source of cytokines and growth factors that induce or modulate angiogenesis. A widely used human mast cell line HMC-1 has been reported to produce the potent proangiogenic factors interleukin-8 (IL-8)[11] and vascular endothelial growth factor (VEGF)[12] in response to nonspecific stimulation with phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore A23187.

We have previously shown that HMC-1 express functional A_{2A} and A_{2B} receptors, but only A_{2B} receptors induced the production of IL-8.[13] In the present study, we determined that, in addition to A_{2A} and A_{2B} receptors, human mast cells express also A_3 adenosine receptors. Further experimentation revealed that activation of A_{2B} adenosine receptors stimulates the synthesis of the proangiogenic factors VEGF and IL-8, whereas activation of A_3 receptor induces the expression of angiopoietin-2. Stimulation of both A_{2B} and A_3 receptors was essential for inducing angiogenesis in a human umbilical vein endothelial cell (HUVEC) model in vitro. We propose that adenosine-induced release of proangiogenic factors from mast cells may represent a novel paracrine mechanism of regulation of angiogenesis in various physiological and pathological situations associated with elevated extracellular concentrations of adenosine. This phenomenon takes advantage of a novel cooperative interaction between A_{2B} and A_3 adenosine receptors, which are coexpressed in human mast cells.

Materials and Methods

Cell Cultures

Human mast cells (HMC-1), a generous gift from Dr J.H. Butterfield (Mayo Clinic, Rochester, Minn), were maintained in suspension

Original received October 17, 2002; revision received January 16, 2003; accepted January 30, 2003.

From the Divisions of Cardiovascular Medicine (I.F., A.E.G.) and Clinical Pharmacology (S.R., I.B.), Departments of Medicine (I.F., S.R., A.E.G., I.B.) and Pharmacology (I.F., I.B.), Vanderbilt University, Nashville, Tenn.

Correspondence to Igor Feoktistov, PhD, 360 PRB, Vanderbilt University, Nashville, TN 37232-6300. E-mail igor.feoktistov@mcmail.vanderbilt.edu

Circulation Research **is available at http://www.circresaha.org** **DOI: 10.1161/01.RES.0000061572.10929.2D**

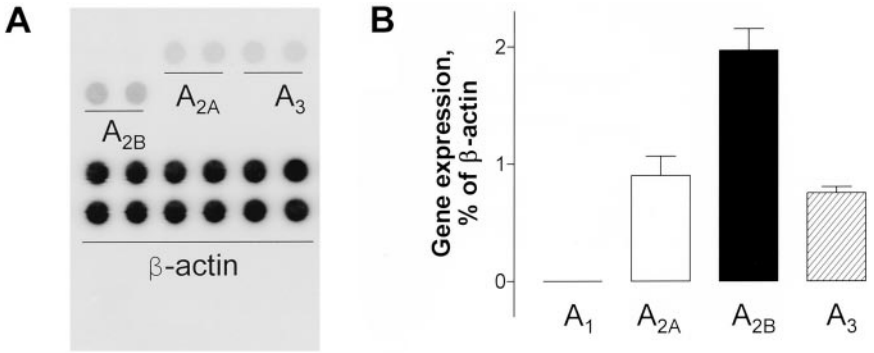

Figure 1. Expression of adenosine receptors in HMC-1. A, Representative gene expression array analysis of adenosine receptor mRNA in HMC-1. Adenosine receptor mRNA expression is shown in the two top lanes and is compared with β-actin mRNA (two bottom lanes). B, Levels of adenosine receptor mRNA expression calculated from gene expression array data and expressed as a percentage of β-actin mRNA expression. Values are expressed as mean±SEM (n=3).

culture at a density between 3 and 9×10^5 cells/mL by dilution with Iscove's medium supplemented with 10% (vol/vol) FBS, 2 mmol/L glutamine, antibiotics, and 1.2 mmol/L α-thioglycerol.

HUVECs propagated from pooled primary cultures of human umbilical veins were kindly provided by Dr D.E. Vaughan, Vanderbilt University (Nashville, Tenn). HUVECs were maintained in M-199 medium supplemented with 15% (vol/vol) FBS, 1× antibiotic-antimycotic mixture (Gibco BRL catalog No. 15240-062), and 0.3 μg/mL bovine hypothalamus endothelial mitogen (Biomedical Technologies, Stoughton, Mass). All cells were kept under humidified atmosphere of air/CO_2 (19:1) at 37°C.

Chemicals

CGS21680 (4-((*N*-ethyl-5′-carbamoyladenos-2-yl)-aminoethyl)-phenylpropionic acid), NECA (5′-*N*-ethylcarboxamidoadenosine), and MRS1191 (3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate) were purchased from Research Biochemicals (Natick, Mass). IB-MECA (N^6-(3-iodobenzyl)-*N*-methyl-5′-carbamoyladenosine) was obtained from Tocris Cookson (Ellisville, Mo). [^{125}I]AB-MECA (N^6-(4-amino-3-iodobenzyl)-adenosine-5′-(*N*-methyluronamide), 2200 Ci/mmol, was purchased from NEN Lifescience Products (Boston, Mass). SCH58261 (5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo-[1,5-c]-pyrimidine) was a generous gift from Drs C. Zocchi and E. Ongini (Schering Plough Research Institute, Milan, Italy). IPDX (3-isobutyl-8-pyrrolidinoxanthine) was synthesized as previously described.[14]

Gene Expression Assay

Total RNA was isolated using RNeasy mini kit (Qiagen). Expression of angiogenic factors was evaluated using gene expression arrays (Super Array). Human adenosine receptors gene expression array was custom designed by Super Array. The assay was performed according to manufacturer's instructions.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

VEGF and angiopoietin-2 mRNA expression was determined with dual-quantitative RT-PCR kits (Maxim Biotech) in accordance with manufacturer's instructions.

Determination of IL-8 and VEGF Levels in Conditioned Media

IL-8 and VEGF concentrations were measured using ELISA kits (R&D Systems) as previously described.[13]

Western Blot Analysis of Angiopoietin-2 Expression

HMC-1 lysates were separated on 4% to 12% gradient SDS-PAGE gel and processed for Western blotting. Rabbit polyclonal anti-human angiopoietin-2 antibody (Zymed Laboratories, South San Francisco, Calif), 1 μg/mL, was used as a primary antibody, and horseradish peroxidase–conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa) was used as a second antibody at a dilution 1:25 000.

Capillary Tube Formation Assay

To determine morphogenic effects of HMC-1 conditioned media, we used the In Vitro Angiogenesis assay kit (Chemicon). Plates (96-well) were coated with ECMatrix according to the manufacturer's recommendations. HUVECs were harvested and resuspended in HMC-1 conditioned media. The same media, incubated in the absence of HMC-1, was used for control purposes. HUVECs were seeded at a density of 10^4 cells/well and incubated for 4 hours under humidified atmosphere of air/CO_2 (19:1) at 37°C. Tube formation was inspected under inverted phase-contrast light Olympus IX-70 microscope equipped with a digital camera.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Expression of Adenosine Receptors in Human Mast Cells

Gene expression array results indicate that HMC-1 express mRNA encoding A_{2A}, A_{2B}, and A_3 receptors (Figure 1A). No A_1 receptor mRNA was detected. Expression levels of A_{2A}, A_{2B}, and A_3 receptors were 0.9±0.16% (mean±SEM), 1.97±0.18%, and 0.75±0.05% of β-actin expression, respectively (Figure 1B). These data agree well with previous results of functional assays indicating the presence of A_{2A} and A_{2B} receptors in HMC-1.[13,15] In this study, we found that HMC-1 express A_3 receptor mRNA in addition to A_{2A} and A_{2B} receptor mRNA. Therefore, we thought it important to confirm the expression of A_3 receptor protein.

We used [^{125}I]AB-MECA binding to membranes isolated from HMC-1 to determine the expression of A_3 receptor protein (online Figure 1A). Nonlinear regression analysis revealed a K_d of 0.55 nmol/L for [^{125}I]AB-MECA, in agreement with previously published data for human A_3 receptors.[16] Western analysis of A_3 receptor expression in HMC-1 membranes (online Figure 1B) detected immunoreactive bands with apparent molecular masses of 37 and 40 kDa (36 184.6, theoretical M_r for the unglycosylated A_3 receptor).

We also tested if A_3 receptors are linked to the same intracellular signaling pathways regulated by A_{2A} and A_{2B} adenosine receptors in HMC-1. Online Figure 2A shows that the nonselective adenosine agonist NECA, acting on both A_{2A} and A_{2B} adenosine receptors, stimulates adenylate cyclase more efficaciously than the selective A_{2A} agonist CGS21680, in agreement with previous reports.[13,15] The specific A_3 agonist IB-MECA failed to inhibit forskolin-stimulated ade-

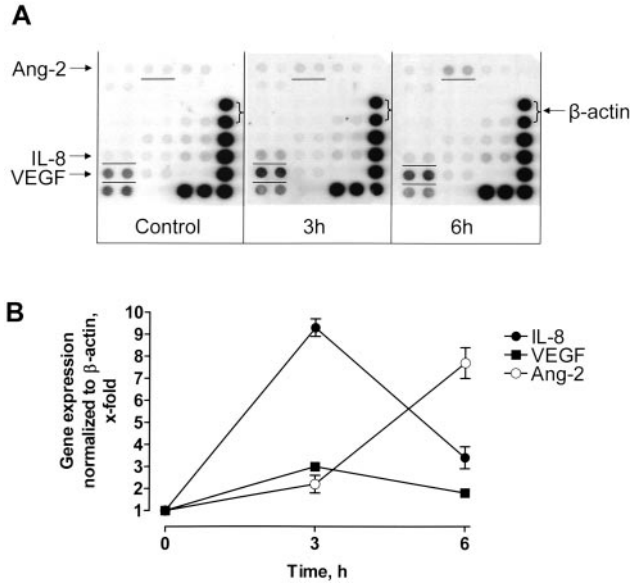

Figure 2. Modulation of angiogenic factor expression by adenosine. A, Representative gene expression array analysis of angiogenic factors in unstimulated HMC-1 (control) and in cells stimulated with 100 μmol/L NECA for 3 (3h) and 6 hours (6h). Arrows indicate positions of underlined dots in arrays, which represent expression of angiopoietin-2 (Ang-2), IL-8, and VEGF mRNA. Expression of angiogenic factors was compared with the expression of β-actin, indicated by brackets in each individual array. B, Time course of adenosine-induced changes in mRNA levels of IL-8, VEGF, and angiopoietin-2 in HMC-1. Data were calculated from gene expression array analysis and expressed as mean±SEM (n=3).

nylate cyclase at concentrations (10^{-9} to 10^{-7} mol/L) that selectively activate A_3 receptors (online Figure 2B). Therefore, we found no evidence for functional coupling of A_3 receptors to adenylate cyclase in HMC-1. Similarly, only the nonselective adenosine agonist NECA stimulated phosphoinositol hydrolysis, whereas the selective A_{2A} agonist CGS21680 and the selective A_3 agonist IB-MECA had no effect (online Figure 3). These data confirm that only A_{2B} receptors are functionally coupled to stimulation of phospholipase Cβ in HMC-1.

Adenosine-Induced Expression of Angiogenic Factors in Human Mast Cells

Incubation of HMC-1 in the presence of 100 μmol/L NECA and 1 U/mL adenosine deaminase for 3 hours increased mRNA expression of the angiogenic factors IL-8, VEGF, and angiopoietin-2 by 9.3±0.4-, 3.0±0.1-, and 2.2±0.4-fold, respectively, compared with cells incubated with vehicle and 1 U/mL adenosine deaminase. After 6 hours of incubation with NECA, the increase in IL-8 and VEGF mRNA expression was on the decline (3.4±0.5- and 1.8±0.2-fold), whereas the expression of angiopoietin-2 increased further, to 7.7±0.7-fold (Figure 2A). Figure 2B illustrates this differential kinetics of adenosine-induced expression of angiogenic factors. The expression of other genes in our array was either negligible (<0.1% of β-actin expression) or was not changed (<2-fold) on stimulation with NECA. The list of genes included in this assay can be found in the expanded Materials and Methods section of the online supplement.

Adenosine Stimulates IL-8 Production via A_{2B} Receptors

Measurements of IL-8 protein levels in conditioned media confirmed the results of gene array analysis. NECA produced a dose-dependent increase in IL-8 levels in conditioned media after 6 hours of incubation with HMC-1, from 6.1±1.8 to 97.8±3.8 pg/mL. The selective A_{2B} antagonist IPDX competitively inhibited NECA-induced IL-8 secretion, indicating that this process is mediated via A_{2B} receptors (Figure 3A). We showed previously that stimulation of A_{2A} adenosine receptors had no effect on IL-8 production in HMC-1.[13] As seen in Figure 3B, the selective A_3 agonist IB-MECA did not stimulate IL-8 production and the selective A_3 antagonist MRS1191 did not inhibit the IL-8 production induced by NECA.

Adenosine Increases VEGF Expression via A_{2B} Receptors

We used dual-quantitative RT-PCR to confirm adenosine-dependent increase in VEGF mRNA expression and also to determine the effect of adenosine on the expression of the major VEGF isoforms $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$. Amplification of cDNA using a specific set of primers common to all three VEGF isoforms showed the increase in their expression in HMC-1 stimulated with 100 μmol/L NECA for 3 hours compared with unstimulated cells (Figures 4A and 4B). The increase ranged from 1.23-fold for $VEGF_{189}$ to 3.2-fold for $VEGF_{121}$.

HMC-1 tonically release VEGF (Figure 4C). VEGF secretion was increased in the presence of NECA. VEGF concen-

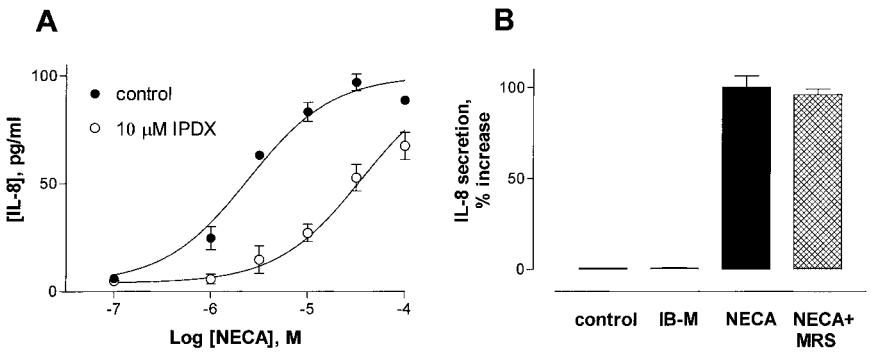

Figure 3. Release of IL-8 from HMC-1. A, Concentration-response curves for IL-8 secretion induced by NECA in the absence (●) or in the presence of the selective A_{2B} antagonist IPDX 10 μmol/L (○). Values are expressed as mean±SEM (n=4). B, Pharmacological analysis of the potential involvement of A_3 adenosine receptors in the modulation of IL-8 secretion. The selective A_3 agonist IB-MECA 10 nmol/L (IB-M) did not induce IL-8 secretion. IL-8 secretion stimulated by 10 μmol/L NECA (NECA) was not inhibited by the selective A_3 antagonist MRS1191 100 nmol/L (NECA+MRS). Values are mean±SEM (n=4). Results are expressed as a percentage of NECA-stimulated IL-8 secretion normalized to spontaneous IL-8 release in the presence of vehicle.

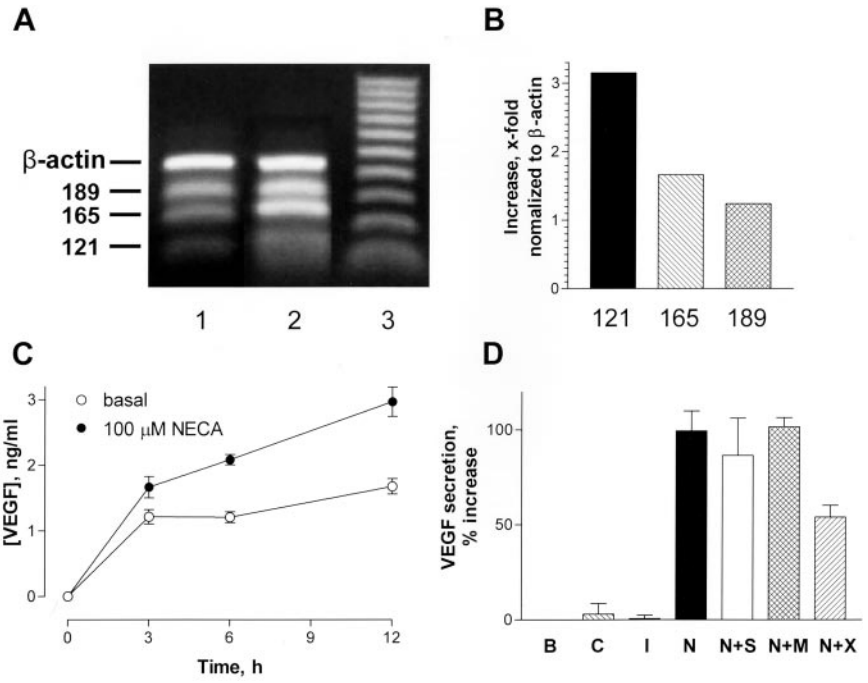

Figure 4. Effect of adenosine on VEGF expression in HMC-1. A, Dual-quantitative RT-PCR analysis of VEGF splice variant isoforms in HMC-1 incubated in the absence (lane 1) or in the presence of 100 μmol/L NECA for 3 hours (lane 2). Positions of amplified products corresponding to β-actin and VEGF isoforms 121, 165, and 189 on an agarose gel are shown on the left side. Lane 3 represents a DNA size marker ladder (100- to 1 000-bp range). B, NECA-induced increase in VEGF splice variant mRNA expression calculated from RT-PCR data in panel A and expressed as a percentage of β-actin mRNA expression. Increase in $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ mRNA expression is indicated as 121, 165, and 189, respectively. C, Time course of VEGF release into culture media from HMC-1 incubated in the absence or in the presence of 100 μmol/L NECA. D, Pharmacological analysis of adenosine receptors regulating VEGF secretion. Effects of adenosine agonists 1 μmol/L CGS21680 (A_{2A} selective, C), 100 nmol/L IB-MECA (A_3 selective, I), and 100 μmol/L NECA (nonselective, N) on basal (B) VEGF secretion and effect of adenosine antagonists 10 nmol/L SCH58261 (A_{2A} selective, N+S), 100 nmol/L MRS1191 (A_3 selective, N+M), and 10 μmol/L IPDX (A_{2B} selective, N+X) on NECA-stimulated VEGF secretion. Values are mean±SEM (n=4). Results are expressed as a percentage of NECA-stimulated VEGF secretion normalized to spontaneous VEGF release in the presence of vehicle.

trations in conditioned media from HMC-1 incubated for 6 hours in the absence or in the presence of 100 μmol/L NECA were 1.21±0.09 and 2.09±0.08 ng/mL, respectively. As seen in Figure 4D, only the nonselective agonist NECA stimulated VEGF production. Neither the selective A_{2A} agonist CGS21680 nor the selective A_3 agonist IB-MECA increased VEGF levels. Furthermore, only the selective A_{2B} antagonist IPDX, but not the selective A_{2A} antagonist SCH58261 or the selective A_3 antagonist MRS1191, inhibited the NECA-stimulated VEGF secretion. Based on these data, we concluded that A_{2B} receptors mediate VEGF increase in HMC-1.

Adenosine Induces Angiopoietin-2 Expression via A_3 Receptors

As shown above, NECA stimulated angiopoietin-2 expression with kinetics different from those for stimulation of IL-8 and VEGF (Figure 2B). Angiopoietin-2 expression also was different from IL-8 and VEGF by its sensitivity to the A_3 selective adenosine agonist IB-MECA. As seen in Figures 5A and 5B, IB-MECA increased angiopoietin-2 mRNA expression with an EC_{50}, 1.2 nmol/L, that agrees with its reported affinity at human A_3 receptors.[16] Furthermore, only the selective A_3 antagonist MRS1191, but not the selective A_{2A} antagonist SCH58261 or the selective A_{2B} antagonist IPDX, inhibited the NECA-stimulated angiopoietin-2 expression (online Figure 4).

Angiopoietin-2 protein expression was demonstrated in lysates of HMC-1 stimulated with 100 μmol/L NECA for 12 hours. Figure 5C shows angiopoietin-2 immunoreactivity detected in lysates of stimulated HMC-1 (lanes 6 and 7) but not in lysates of unstimulated cells (lanes 4 and 5).

Effects of HMC-1 Conditioned Media on Angiogenesis In Vitro

Conditioned media, collected from HMC-1 exposed to 10 μmol/L NECA for 48 hours, stimulated HUVEC proliferation by 25±9% (n=6) (online Figure 5) and migration by 103±5% (n=4) (online Figure 6), compared with conditioned media from unstimulated HMC-1.

Conditioned media collected from NECA-stimulated HMC-1 promoted capillary tube formation of HUVECs incubated on ECMatrix for 4 hours. HUVECs, incubated with conditioned media from unstimulated HMC-1, showed only few cells aligned in an organized manner, with the majority of them remaining unassociated (Figures 6A and 6C). In contrast, conditioned media from NECA-stimulated HMC-1 induced the formation of a characteristic tube network (Figures 6B and 6D). In parallel experiments, we found that nonconditioned medium containing 10 μmol/L NECA did not have a direct angiogenic action on HUVECs. The effects of conditioned media on HUVEC tube formation were inhibited when HMC-1 were incubated with NECA in the presence of the selective A_{2B} antagonist IPDX 10 μmol/L (Figure 6E) and, to a lesser extent, in the presence of the selective A_3 antagonist MRS1191 100 nmol/L (Figure 6F). In ancillary studies, we demonstrated that this effect cannot be explained by cytotoxic effects of adenosine antagonists, because nonconditioned media containing 10 μmol/L IPDX or 100 nmol/L MRS1191 did not affect viability of HUVECs (data not shown). Furthermore, conditioned media from HMC-1 stimulated with the selective A_3 agonist IB-MECA did not induce HUVEC capillary formation (Figure 7) indicating that stimulation of A_3 receptor alone is not sufficient to induce angiogenesis in vitro.

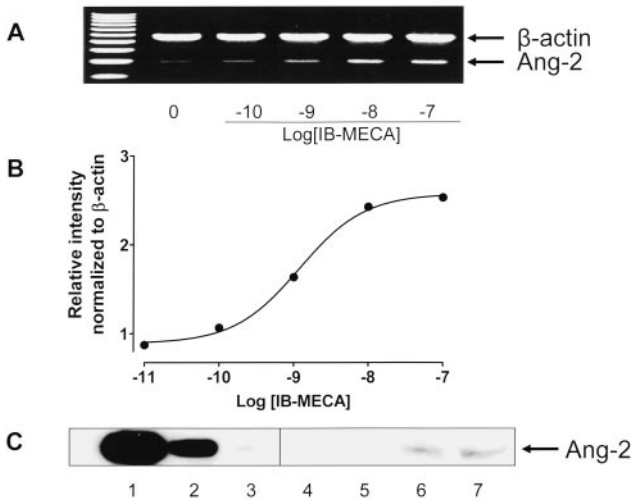

Figure 5. Stimulation of angiopoietin-2 expression mediated by A_3 adenosine receptors. A, Dual-quantitative RT-PCR analysis of angiopoietin-2 expression in HMC-1 incubated in the absence (0) or in the presence of increasing concentrations of IB-MECA for 6 hours. Positions of amplified products corresponding to β-actin and angiopoietin-2 on agarose gel are shown on the right side. The first lane on the gel from the left represents a DNA size marker ladder (100- to 1 000-bp range). B, Concentration-response curve for angiopoietin-2 mRNA expression induced by IB-MECA. Angiopoietin-2 expression levels were calculated from the RT-PCR data in panel A and expressed as a percentage of β-actin mRNA expression. C, Western immunoblotting of HMC-1 lysates incubated for 12 hours in the absence (lanes 4 and 5) or in the presence of 100 μmol/L NECA (lanes 6 and 7). Lanes 1, 2, and 3 on the gel were loaded with 10, 1, and 0.1 μg/mL human recombinant angiopoietin-2, respectively, for calibration purposes.

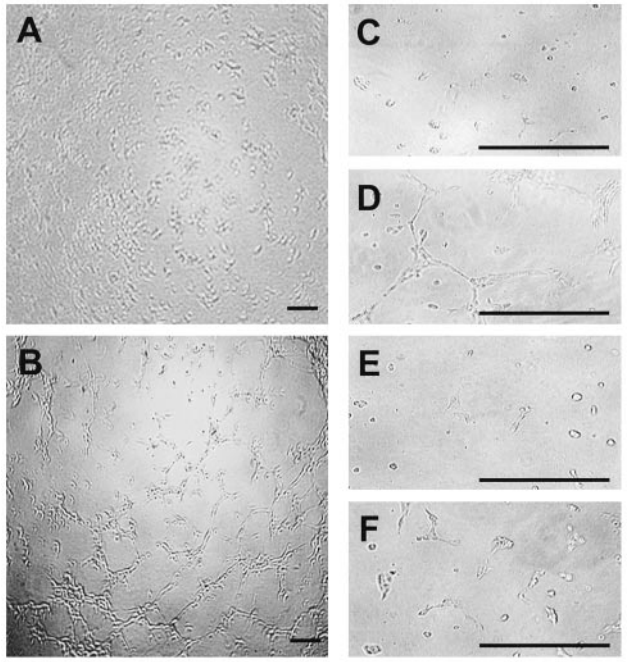

Figure 6. Effect of HMC-1 conditioned media on capillary tube formation. Tube formation was inspected after incubation of HUVECs on basement membrane matrix for 4 hours in media conditioned by incubating HMC-1 for 48 hours in the absence (panels A and C) or in the presence of 10 μmol/L NECA, in the absence (panels B and D) or in the presence of the selective A_{2B} antagonist 10 μmol/L IPDX (panel E) or the selective A_3 antagonist 100 nmol/L MRS1191 (panel F). Representative images shown were obtained at magnifications $\times$40 for panels A and B, and $\times$200 for panels C through F. Bar=200 μm.

Treatment of conditioned media from NECA-stimulated HMC-1 with neutralizing antibodies specific for VEGF abolished its ability to induce HUVEC tube formation, indicating the importance of VGEF in our model. Antibodies against IL-8 failed to inhibit HUVEC tube formation, probably reflecting the relatively low concentrations of IL-8 achieved in our model (online Figure 7).

Discussion

The evidence pointing to an association between mast cells and angiogenesis has been growing for more than a hundred years. Shortly after the discovery of mast cells, it was recognized that they tend to concentrate around blood vessels in inflammatory and neoplastic foci.[17] It was later demonstrated that mast cells accumulate in the proximity of tumors before the onset of tumor-associated angiogenesis.[10] Their numbers also rise in other physiological and pathological events associated with an increase in angiogenic activity.[5,6,8] However, it is unclear which stimuli would signal mast cells to induce neovascularization. The events leading to angiogenesis are often accompanied by tissue hypoxia and the subsequent increase in extracellular adenosine. For example, adenosine is released into extracellular space at high concentrations at early stages of solid tumor growth due to severe hypoxia and necrosis. Tissue injury, ischemia, and inflammation are also accompanied by an increase in extracellular adenosine concentrations. We and others have shown that adenosine stimulates the secretion of the angiogenic factors IL-8, VEGF, and basic fibroblast growth factor (bFGF) from microvascular endothelial cells.[1–4] In this study, we tested the hypothesis that extracellular adenosine can promote angiogenesis by triggering the synthesis and release of angiogenic factors from mast cells.

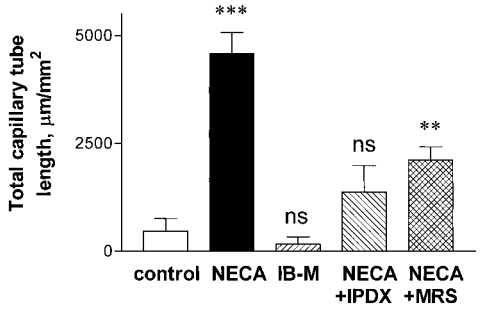

Figure 7. Morphogenic activity of HMC-1 conditioned media. Capillary tube formation was estimated by measuring their total length from low-magnification images using ImageJ software and normalized per 1 mm^2 growth area. Capillary tube formation was measured after incubation of HUVECs on basement membrane matrix for 4 hours in media conditioned by incubating HMC-1 for 48 hours in the presence of vehicle (control), in the presence of 10 nmol/L IB-MECA (IB-M) or in the presence of 10 μmol/L NECA, in the absence (NECA) or in the presence of the selective A_{2B} antagonist 10 μmol/L IPDX (NECA+IPDX), or the selective A_3 antagonist 100 nmol/L MRS1191 (NECA+MRS). Data are expressed as mean±SEM (n=4). Asterisks indicate significant differences from control (**P<0.01; ***P<0.001; ns indicates nonsignificant).

We have previously reported the presence of functional A_{2A} and A_{2B} adenosine receptors in the human mast cell line HMC-1.[13] In the present study, we found that HMC-1 express also A_3 receptor mRNA in addition to A_{2A} and A_{2B} transcripts. Radioligand binding experiments and Western blot analysis confirmed the expression of A_3 receptor protein on the surface of HMC-1. A_3 receptors are not linked to adenylate cyclase or phospholipase Cβ, the intracellular signaling systems regulated by A_{2B} receptors in HMC-1. Although coupling of A_3 to adenylate cyclase via α_i and to phospholipase Cβ via $\beta\gamma$ subunits has been shown in some cells, recent studies in ventricular myocytes demonstrated that A_3 receptors are coupled to RhoA and phospholipase D.[18,19] The intracellular signaling pathways linked to A_3 receptors in HMC-1 remain to be elucidated.

There is growing evidence that IL-8 plays an important and specific role in promoting angiogenesis.[20–22] IL-8 is elevated in wounds and enhances wound healing.[23] IL-8, secreted by inflammatory and neoplastic cells, stimulates angiogenesis in various solid tumors.[20–22] We have previously reported that A_{2B}, but not A_{2A}, receptors stimulate IL-8.[13] In this study, we found that A_3 receptors are not involved in IL-8 secretion either; the selective A_3 agonist IB-MECA failed to stimulate IL-8 secretion, and NECA-induced IL-8 secretion was competitively inhibited by the selective A_{2B} antagonist IPDX but not by the selective A_3 antagonist MRS1191. Of interest, while this article was in preparation, Meade et al[24] reported the presence of A_3 receptors on HMC-1 and the failure of these receptors to stimulate IL-8 release. Furthermore, they found that A_{2B}-mediated IL-8 release could be greatly potentiated by stem cell factor. Taken together, these results confirm that A_{2B} receptors are solely responsible for adenosine-induced secretion of IL-8.

In this study, we found that adenosine A_{2B} receptors also stimulate the production of another potent angiogenic factor, VEGF. Mast cells were known to produce VEGF,[25–28] and HMC-1 were found to constitutively produce and secrete VEGF in vitro.[12] We demonstrate, for the first time, that VEGF mRNA expression and protein secretion are specifically stimulated by adenosine in human mast cells. Adenosine preferentially increases mRNA expression of $VEGF_{121}$ and $VEGF_{165}$, the diffusible isoforms that are secreted in the medium. Adenosine increased to a lesser extent mRNA expression of $VEGF_{189}$, the isoform that is mostly bound to heparin-containing proteoglycans of the extracellular matrix. This pattern of stimulation of VEGF isoforms by adenosine correlates with the increase in VEGF secretion. NECA stimulated VEGF secretion from HMC-1 by ≈1.7-fold over its constitutive secretion. Using selective agonists and antagonists, we determined that A_{2B} adenosine receptors regulate VEGF production in human mast cells, in addition to IL-8. Our results, therefore, suggest that angiogenesis is an important function of adenosine A_{2B} receptors in mast cells.

In addition to upregulation of IL-8 and VEGF secretion, we also found that adenosine stimulates the expression of angiopoietin-2 in human mast cells. The difference between the time course of stimulation of mRNA expression of angiopoietin-2, and that of IL-8 and VEGF, led us to explore the possibility that their expression is regulated by distinct

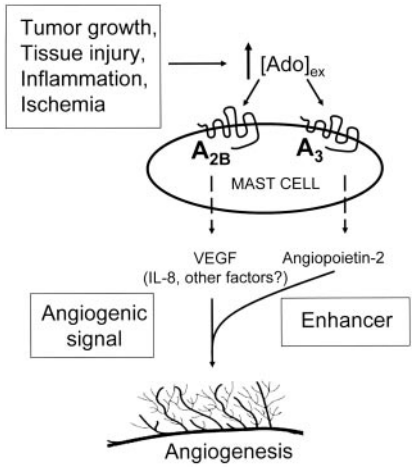

Figure 8. Proposed model of regulation of mast cell–mediated angiogenesis by adenosine. During tumor growth, tissue injury, ischemia, or inflammation, adenosine is released into the extracellular space. Activation of mast cell A_{2B} receptors leads to the secretion of VEGF, IL-8, and possibly other angiogenic factors. The effect of these factors on new capillary formation is facilitated by the concomitant stimulation of mast cell A_3 receptors that induce the expression of angiopoietin-2. These, and potentially other, factors released by mast cells act synergistically, and in a paracrine fashion, on endothelial cells to induce angiogenesis.

receptors. Indeed, our results demonstrate that, whereas A_{2B} receptors mediate the expression of IL-8 and VEGF, angiopoietin-2 expression is mediated by A_3 adenosine receptors. To the best of our knowledge, adenosine has not been previously shown to release angiopoietin-2. Our results raise the possibility that adenosine can contribute to angiogenesis not only by stimulation of VEGF and IL-8 secretion via A_{2B} receptors but also by stimulation of angiopoietin-2 secretion via A_3 receptors.

Localization of angiopoietin-2 primarily at sites of vascular remodeling suggests its role in neovascularization. It has been proposed that angiopoietin-2 participates in angiogenesis by blocking the stabilizing action of angiopoietin-1 on endothelial cells. According to this generally accepted model, angiopoietin-2 is viewed as an enhancer of angiogenic signals. Angiopoietin-2 does not induce angiogenesis on its own, but primes blood vessels for angiogenesis and facilitates the effects of VEGF.[29] Based on our observations, we suggested that such a model of cooperative regulation of angiogenic response could be applied to regulation of mast cell–mediated angiogenesis by adenosine. In this model (Figure 8), A_3 receptors on mast cells would play the role of an "enhancer" of angiogenic signals mediated by A_{2B} receptors. During tumor growth, tissue injury, ischemia, or inflammation, adenosine is released into the extracellular space. Binding of adenosine to mast cell A_{2B} receptors stimulates the secretion of VEGF, IL-8, and possibly other angiogenic factors. The effect of VEGF on new capillary formation is facilitated by the concomitant stimulation of mast cell A_3 receptors that induce the expression of angiopoietin-2. This model was validated in experiments with HUVEC tube formation. Our results indicate that A_{2B} and A_3 receptors interact in a functional cooperative fashion to promote angiogenesis. This

conclusion is based on the observation that tube formation was substantially greater when human endothelial cells were exposed to conditioned media from human mast cells if both A_{2B} and A_3 receptors were simultaneously activated compared with the selective activation of A_{2B} receptor alone. Such cooperation between adenosine receptors is a novel finding. Adenosine A_{2B} receptors appear to be ubiquitously expressed, but in virtually every cell they are coexpressed with other adenosine receptor subtypes. The functional relevance of this coexpression had not previously been investigated. There is currently no evidence that a similar cooperation exists between A_{2B} and A_3 receptors in the modulation of mast cell–mediated inflammatory processes, but such a possibility should be explored.

It should be noted that our experiments were done in an established mast cell line HMC-1 with phenotypic characteristics resembling MC_T-type human mast cells.[30] Mast cells are heterogeneous, and not all mast cell lines may express the same adenosine receptor subtypes as HMC-1. Similarly, we chose HUVECs as a model to study paracrine regulation of angiogenesis because these cells do not secrete VEGF, IL-8, or angiopoietin-2 in response to adenosine.[4] However, endothelial cells are also heterogeneous. We have previously shown that A_{2B} receptors can directly induce release of VEGF and IL-8 from microvascular endothelial cells.[2,4] It is possible that in some vascular beds adenosine provides autocrine stimulation of angiogenesis by acting directly on endothelial A_{2B} receptors, in addition to paracrine modulation mediated by mast cell A_{2B} and A_3 receptors. Finally, we cannot exclude that, in addition to VEGF, IL-8, and angiopoietin-2, there are also other factors involved in the angiogenic response induced by adenosine. For example, mast cells were shown to stimulate angiogenesis by releasing tryptase, chymase, and matrix metalloproteinase-9 (MMP-9).[31–33] In this study, we screened only a limited number of angiogenesis-related genes in human mast cells. Nonetheless, our findings that adenosine stimulates secretion of such angiogenic factors as VEGF and IL-8 via A_{2B} receptors, and angiopoietin-2 via A_3 receptors, demonstrate for the first time an example of functional cooperation between different adenosine receptor subtypes in promoting an angiogenic response.

In summary, we have shown the presence of A_{2B} and A_3 receptors in human mast cells. Activation of A_{2B} receptors leads to the secretion of VEGF and IL-8, whereas activation of A_3 receptors upregulates the expression of angiopoietin-2. These, and potentially other factors released by mast cells, can act synergistically, and in a paracrine fashion, on endothelial cells to induce angiogenesis.

Acknowledgments

This work was supported by NIH grants R29 HL55596 and R01 HL70073 (to I.F.) and R01 HL67232 (to I.B.).

References

1. Fischer S, Sharma HS, Karliczek GF, Schaper W. Expression of vascular permeability factor/vascular endothelial growth factor in pig cerebral microvascular endothelial cells and its upregulation by adenosine. *Brain Res*. 1995;28:141–148.
2. Grant MB, Tarnuzzer RW, Caballero S, Ozeck MJ, Davis MI, Spoerri PE, Feoktistov I, Biaggioni I, Shryock JC, Belardinelli L. Adenosine receptor activation induces vascular endothelial growth factor in human retinal endothelial cells. *Circ Res*. 1999;85:699–706.
3. Grant MB, Davis MI, Caballero S, Feoktistov I, Biaggioni I, Belardinelli L. Proliferation, migration, and ERK activation in human retinal endothelial cells through A_{2B} adenosine receptor stimulation. *Invest Ophthalmol Vis Sci*. 2001;42:2068–2073.
4. Feoktistov I, Goldstein AE, Ryzhov S, Zeng D, Belardinelli L, Voyno-Yasenetskaya T, Biaggioni I. Differential expression of adenosine receptors in human endothelial cells: role of A_{2B} receptors in angiogenic factor regulation. *Circ Res*. 2002;90:531–538.
5. Atkinson JB, Harlan CW, Harlan GC, Virmani R. The association of mast cells and atherosclerosis: a morphologic study of early atherosclerotic lesions in young people. *Hum Pathol*. 1994;25:154–159.
6. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med*. 2002;346:1699–1705.
7. Schubert C, Christophers E. Mast cells and macrophages in early relapsing psoriasis. *Arch Dermatol Res*. 1985;277:352–358.
8. Trautmann A, Toksoy A, Engelhardt E, Brocker EB, Gillitzer R. Mast cell involvement in normal human skin wound healing: expression of monocyte chemoattractant protein-1 is correlated with recruitment of mast cells which synthesize interleukin-4 in vivo. *J Pathol*. 2000;190:100–106.
9. Ribatti D, Vacca A, Nico B, Crivellato E, Roncali L, Dammacco F. The role of mast cells in tumour angiogenesis. *Br J Haematol*. 2001;115:514–521.
10. Dabbous MK, Walker R, Haney L, Carter LM, Nicolson GL, Woolley DE. Mast cells and matrix degradation at sites of tumour invasion in rat mammary adenocarcinoma. *Br J Cancer*. 1986;54:459–465.
11. Moller A, Lippert U, Lessmann D, Kolde G, Hamann K, Welker P, Schadendorf D, Rosenbach T, Luger T, Czarnetzki BM. Human mast cells produce IL-8. *J Immunol*. 1993;151:3261–3266.
12. Grutzkau A, Kruger-Krasagakes S, Baumeister H, Schwarz C, Kogel H, Welker P, Lippert U, Henz BM, Moller A. Synthesis, storage, and release of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) by human mast cells: implications for the biological significance of VEGF. *Mol Biol Cell*. 1998;206:9:875–884.
13. Feoktistov I, Biaggioni I. Adenosine A_{2b} receptors evoke interleukin-8 secretion in human mast cells: an enprofylline-sensitive mechanism with implications for asthma. *J Clin Invest*. 1995;96:1979–1986.
14. Feoktistov I, Garland E, Goldstein AE, Zeng D, Belardinelli L, Wells JN, Biaggioni I. Inhibition of human mast cell activation with the novel selective adenosine A_{2B} receptor antagonist 3-isobutyl-8-pyrrolidinoxanthine (IPDX). *Biochem Pharmacol*. 2001;62:1163–1173.
15. Feoktistov I, Biaggioni I. Pharmacological characterization of adenosine A_{2B} receptors: studies in human mast cells co-expressing A_{2A} and A_{2B} adenosine receptor subtypes. *Biochem Pharmacol*. 1998;55:627–633.
16. Klotz KN, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB, Lohse MJ. Comparative pharmacology of human adenosine receptor subtypes: characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol*. 1998;357:1–9.
17. Selye H. *The Mast Cells*. Washington, DC: Butterworth Publications; 1965.
18. Parsons M, Young L, Lee JE, Jacobson KA, Liang BT. Distinct cardioprotective effects of adenosine mediated by differential coupling of receptor subtypes to phospholipases C and D. *FASEB J*. 2000;14:1423–1431.
19. Lee JE, Bokoch G, Liang BT. A novel cardioprotective role of RhoA: new signaling mechanism for adenosine. *FASEB J*. 2001;15:1886–1894.
20. Rofstad EK, Halsor EF. Vascular endothelial growth factor, interleukin-8, platelet-derived endothelial cell growth factor, and basic fibroblast growth factor promote angiogenesis and metastasis in human melanoma xenografts. *Cancer Res*. 2000;60:4932–4938.
21. Westphal JR, Van't Hullenaar R, Peek R, Willems RW, Crickard K, Crickard U, Askaa J, Clemmensen I, Ruiter DJ, De Waal RM. Angiogenic balance in human melanoma: expression of VEGF, bFGF, IL-8, PDGF and angiostatin in relation to vascular density of xenografts in vivo. *Int J Cancer*. 2000;86:768–776.
22. Inoue K, Slaton JW, Eve BY, Kim SJ, Perrotte P, Balbay MD, Yano S, Bar-Eli M, Radinsky R, Pettaway CA, Dinney CP. Interleukin-8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. *Clin Cancer Res*. 2000;6:2104–2119.
23. Rennekampff HO, Hansbrough JF, Kiessig V, Dore C, Sticherling M, Schroder JM. Bioactive interleukin-8 is expressed in wounds and enhances wound healing. *J Surg Res*. 2000;93:41–54.

24. Meade CJ, Worrall L, Hayes D, Protin U. Induction of interleukin-8 release from the HMC-1 mast cell line: synergy between stem cell factor and activators of the adenosine A_{2b} receptor. *Biochem Pharmacol*. 2002; 64:317–325.
25. Boesiger J, Tsai M, Maurer M, Yamaguchi M, Brown LF, Claffey KP, Dvorak HF, Galli SJ. Mast cells can secrete vascular permeability factor/ vascular endothelial cell growth factor and exhibit enhanced release after immunoglobulin E-dependent upregulation of $Fc_{\epsilon}R1$ expression. *J Exp Med*. 1998;188:1135–1145.
26. Sawatsubashi M, Yamada T, Fukushima N, Mizokami H, Tokunaga O, Shin T. Association of vascular endothelial growth factor and mast cells with angiogenesis in laryngeal squamous cell carcinoma. *Virchows Arch*. 2000;436:243–248.
27. Toth-Jakatics R, Jimi S, Takebayashi S, Kawamoto N. Cutaneous malignant melanoma: correlation between neovascularization and peritumor accumulation of mast cells overexpressing vascular endothelial growth factor. *Hum Pathol*. 2000;31:955–960.
28. Fan L, Iseki S. Immunohistochemical localization of vascular endothelial growth factor in the globule leukocyte/mucosal mast cell of the rat respiratory and digestive tracts. *Histochem Cell Biol*. 1999;111:13–21.
29. Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science*. 1999;284:1994–1998.
30. Nilsson G, Blom T, Kusche-Gullberg M, Kjellen I, Butterfield JH, Sundstrom C, Nilsson K, Hellman L. Phenotypic characterization of the human mast-cell line HMC-1. *Scand J Immunol*. 1994;39: 489–498.
31. Blair RJ, Meng H, Marchese MJ, Ren S, Schwartz LB, Tonnesen MG, Gruber BL. Human mast cells stimulate vascular tube formation: tryptase is a novel, potent angiogenic factor. *J Clin Invest*. 1997;99: 2691–2700.
32. Coussens LM, Raymond WW, Bergers G, Laig-Webster M, Behrendtsen O, Werb Z, Caughey GH, Hanahan D. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. *Genes Dev*. 1999;13:1382–1397.
33. Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell*. 2000;103: 481–490.