Stimulation of Angiogenesis by Adenosine on the Chick Chorioallantoic Membrane

JERRY W. DUSSEAU, PHILLIP M. HUTCHINS, AND DANIELLE S. MALBASA

The effect of adenosine on the vascular density of the chick chorioallantoic membrane was studied. Elvax polymer pellets containing 0.2–3.0 mg of adenosine were placed on the chorioallantoic membrane of 10-day embryos. Control pellets containing mannitol were placed at least 1 cm away. After 4 days the membrane was formalin-fixed and removed. A thin plastic coverslip, inscribed with concentric circles (4–8 mm in diameter), was placed over the pellet. A vascular density index was estimated at 20 × by counting the number of intercepts between vessels and the inscribed circles. Adenosine stimulated a dose-dependent increase (p < 0.01) in the vascular density index with the 3-mg pellets inducing a 15% increase. Inosine, a major metabolite of adenosine, did not cause a change in the number of intercepts counted. The adenosine-stimulated increase in vascularity was blocked with 110 μg of methyl-isobutyl-xanthine injected daily into the albumen. Partial inhibition was observed with 55 μg/day. Methyl-isobutyl-xanthine by itself did not affect the vascular density index. Dipyridamole enhanced adenosine’s stimulation of vascular growth an additional 52%. Given alone, however, it had no effect on the membrane’s vascularity. These data support an angiogenic role for adenosine. The modest, but consistent, increase in the vascular density index stimulated by adenosine, and the fact that it may be released during tissue hypoxia, is consistent with an hypothesis that this nucleoside plays a modulatory role in vessel proliferation accompanying conditions of long-term hypoxia. (Circulation Research 1986;59:163–170)

NUMEROUS studies have demonstrated that the tissue oxygen tension can influence vascular tone and blood flow.1-4 It is generally agreed that these vascular responses to acute changes in oxygen tension are autoregulatory in nature, directed toward matching blood flow with local metabolic requirements. Over extended periods, a proliferation or involution of blood vessels is also closely related to changes in local tissue perfusion and metabolic needs. For example, increased vascularity often follows the increased oxygen availability associated with chronic exercise,5 high altitude adaptation,6 and wound repair.7 Conversely, a hyperoxic environment can result in a reduced vascular density.8,9 While the role of oxygen is not understood, its availability to satisfy chronic metabolic demands appears to be an integral component of the signal initiating a change in vessel number.

During vascular proliferation, locally dilated vessels and capillaries characteristically give rise to the new vascular sprouts.10,11 It is well established that microvascular dilation occurs in response to a decrease in oxygen tension.1-3 Whether this change in vessel caliber (perhaps via changes induced in local blood flow) provides an adequate stimulus for neovascularization is not known. However, Lombard and Duling12 dissociated the oxygen tension from the time course of vasodilation during reactive hyperemia, suggesting that at least one other locally produced substance was required to maintain the vasodilated state. Locally produced vasodilator metabolites may play such a role.13

Significantly, several vasoactive metabolites produced with tissue hypoxia have been implicated in the angiogenesis process. Among these are the prostaglandins,14-16 ADP,17 and lactic acid.18 In addition, pharmacological studies suggest a role for adenosine. Dipyridamole, a vasodilator that inhibits the activity of adenosine deaminase and adenosine re-uptake, was shown to elicit a neovascular response in rat cardiac and skeletal muscle.19-21

As various vasoactive metabolites function acutely to match oxygen supply to oxygen need, they may also, when produced over extended periods, perform an analogous role of maintaining metabolic equilibrium via promoting local vascular growth. In this experiment vasoproliferative activity for adenosine, a vasodilator produced in response to tissue hypoxia, is demonstrated using the chick chorioallantoic membrane. An established model for angiogenesis, the chorioallantoic membrane exhibits a vasoproliferative response to selected stimuli. Furthermore, studies employing altered environmental oxygen tensions have reported changes of this membrane’s vascular function,4 as well as various oxygen transport parameters.22,23

Materials and Methods

Fertile eggs were obtained locally and incubated at 38°C and 55% relative humidity for 7 days. The eggs were rotated twice daily. On Day 7 of incubation a 1 × 1.5 cm window was placed over the chorioallantoic membrane (CAM). The surface of the egg and all instruments were disinfected with 70% isopropyl alcohol. The window was closed with clear cellophane tape.

From the Department of Physiology and Pharmacology, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina.

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Address for reprints: Dr. Jerry W. Dusseau, Department of Physiology and Pharmacology, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27103.

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and the incubation continued without rotation until Day 10. In placing the window, it was particularly important to avoid shell dust or fragments falling onto the CAM, as they can stimulate a local inflammatory response. The experimental manipulations were started on Day 10 and continued through Day 14. At this time the experiments were terminated by fixing the CAM in situ with 10% buffered formalin. A thin piece of glass was slid under the fixed CAM and the membrane removed and stored in formalin until the vascular density was estimated, usually within a week.

**Pellet Preparation**

Ten percent Elvax-40 polymer pellets (ethylene-vinyl acetate copolymer, 40% vinyl acetate content) for the sustained release of the experimental materials were prepared using a simple modification of the method described by Langer. The polymer was dissolved in methylene chloride (1:9 wt:vol). When completely dissolved, the test material was added to achieve the desired dose and the mixture thoroughly vortexed. Ten-microliter aliquots were quickly pipetted onto a sterile 50 x 75 mm glass slide to form individual pellets. The pellets were polymerized at -15°C for 24-48 hours and then placed in a vacuum dessicator at 3°C for an additional 24-36 hours to remove residual methylene chloride. The pellets were stored in a small air-tight plastic box at -15°C until used. All preparative materials and instruments were sterile.

Each substance was applied to the CAM via such Elvax pellets except the methyl-isobutyl-xanthine (MIX). MIX, at doses of 55 and 110 μg in 50 and 100 μl of Normosol-R, respectively, was injected daily in 50 and 100 μl of Normosol-R, and the incubation continued without rotation until the experiments were terminated by fixing the CAM in situ.

**Quantitation of Vascular Density**

A vascular density index (VDI) was obtained to estimate CAM vascularity. An adaptation of a method described by Harris-Hooker and coworkers was used. A thin plastic coverslip inscribed with 4-, 5-, 6-, and 8-mm-diameter concentric circles was centered over the Elvax pellet on the formalin-fixed CAM. Vascular density was estimated, usually within a week. The positioning of the pellet relative to the large and small end of the egg was randomized. All quantitation was done without knowledge of the experimental group. All data are presented as the mean ± SEM.

**Acute in Vivo Responses**

The eggs were placed in a heating mantle and the surface temperature maintained at 38°C. The shell was then removed down to the level of the CAM. Adenosine (27, 270, or 2700 ng in 10 μl Normosol-R) or 10 μl of the vehicle alone was applied locally to the CAM. After 1 minute, the in vivo VDI was estimated as described above, except that counting was done at 45 x. The doses of adenosine and the Normosol-R control were tested in random order and without knowledge of the concentration. Dipyridamole (1 and 5 μg/10 μl) was similarly studied.

**Pellet Release of Adenosine**

Elvax-40 pellets, containing 3 mg of adenosine or 3 mg of adenosine plus 1 mg of dipyridamole, were prepared as described above, except that tritium-labelled adenosine (2,8-3H adenosine) was also included. Each pellet contained approximately 0.012 μCi. The pellets were pre-incubated for 6 hours prior to study. Individual pellets were incubated in 400 μl of Normosol-R at 38°C. Every 24 hours the incubation medium was replaced and the amount of radioactivity released determined by liquid scintillation counting. After the last change, the pellet was dissolved in methylene chloride (200 μl x 2) and the mixture counted to determine the total radioactivity added to each pellet. The release of the tritiated adenosine is presented as the cumulative percent release.

**Materials**

The adenosine, inosine, methyl-isobutyl-xanthine, and dipyridamole were obtained from Sigma. The methylene chloride (99 mol% pure) was purchased from Fisher, and the tritiated adenosine from ICN.

**Results**

During embryonic development of the chick, there is a progressive increase (p<0.001) in the vascular density of the CAM, the site of respiratory exchange for the embryo. This growth parallels the increase in embryo weight (Figure 1). The experiments presented in this study were conducted between Day 10 and 14, that period when vascularization of the CAM is most rapid. Despite this rapid growth, the VDI and the embryo weights exhibited little variation among individuals.
Adenosine released from Elvax polymer pellets stimulated a local dose-related increase \((p<0.01)\) in the vascular density index (VDI) of the CAM (Figure 2). At the 0.2-, 1.0-, and 4.0-mg doses, 7 of 13, 14 of 15, and 14 of 14 CAMs respectively, exhibited increases in vascularity. Inosine, a primary metabolite of adenosine formed by adenosine deaminase, failed to elicit a statistically significant change in the VDI at these same three doses (Figure 2).

The local increase in the VDI stimulated by the 3-mg dose of adenosine was a highly consistent response (Figure 3). Among 44 separate experiments, 43 CAMs responded with an increase in the VDI. The mean increase was 15.1 \(\pm\) 1.5\% \((p<0.001)\). These data are a composite of five separately conducted experiments.

Experiments were conducted to determine whether the increase in the VDI was due to adenosine opening closed, but preexisting, vessels. The effect of topically applied adenosine and of dipyridamole on the \textit{in vivo} VDI was studied. Adenosine, 27-2700 ng applied in 10 \(\mu\)l of Normosol-R, did not significantly affect a local change of the \textit{in vivo} VDI among eggs studied at either 10 or 14 days of incubation (Table 1). Similarly, dipyridamole (1 and 5 \(\mu\)g; \(n=12\)) also failed to alter the \textit{in vivo} VDI of 14 Day eggs \((p<0.650)\) (data not shown).

\textbf{MIX}, at doses to block the adenosine receptors, was injected daily into the albumin on Days 10-14. Regression analysis revealed a significant dose-related inhibition \((p<0.001)\) by MIX on the adenosine-stimulated VDI (Figure 5). MIX at 110 \(\mu\)g/day completely blocked the angiogenic response to the 3-mg dose of adenosine. In the absence of MIX, adenosine stimulated a 15.3\% increase in the VDI. By itself, MIX (110 \(\mu\)g/day) did not significantly alter the VDI of unstimulated CAM vessels (Table 2).
FIGURE 3. Increase in the VDI of the CAM in response to the 3 mg dose of adenosine. The VDI was determined using the 4-, 5-, and 6-mm concentric circles and presented as the actual number of line-vessel intersections counted. Control pellets contained 1 mg of mannitol. Data are mean ± SEM. Number inside bars is group n. *** p < 0.001 vs. control by paired t test.

The vasodilator, dipyridamole, enhanced adenosine's stimulation of CAM vascularity. One milligram of dipyridamole added to the 3-mg adenosine pellet increased the neovascular response an additional 52% (p < 0.005) (Figure 6). A mannitol control pellet was also placed on each of the CAMs. Dipyridamole alone, tested on separate CAMs, had a minimal effect on the VDI.

The effects of the Elvax polymer itself and of mannitol on the CAM vasculature are presented in Table 3. The presence of the Elvax pellet on the CAM had no effect on the VDI. Likewise, no change in vascularity was observed when mannitol was included in the pellet (Table 3, Figure 4). Neither protocol caused a change in the orientation of the vessels surrounding the pellet.

The release of tritiated adenosine from Elvax pellets containing adenosine or a combination of adenosine and dipyridamole is shown in Figure 7. Over 4 days approximately 85% of the labelled nucleoside was released from both pellet preparations into the incubation medium. An initial rapid release occurred during the first 24 hours, after which the rate of release was more constant. The rates of labelled adenosine release were not statistically different (p < 0.382) between the two curves.

Discussion
This study offers direct evidence that adenosine can increase the vascularity of the CAM. The quantitative technique employed in these experiments provides an estimate of the actual vessel density, the endpoint of the angiogenic process. Adenosine's augmentation of vascularization on the CAM appears to be an increase in the number of new vessels. Acute suffusion of adenosine onto the CAM failed to increase the in vivo VDI (Table 1) on either 10-day or 14-day CAM's. The vasodilator, dipyridamole, also failed to affect the in

FIGURE 4. Formalin-fixed CAM photographed after removal from egg illustrating representative responses to a 3-mg adenosine pellet (ADN) and a mannitol control pellet (M). The pellets are 1.2 cm apart.
TABLE 1. Acute Effects of Local Adenosine Suffusion on the in Vivo Vascular Density Index of the CAM at 10 and 14 Days

<table>
<thead>
<tr>
<th>Adenosine dose (ng)*</th>
<th>0</th>
<th>27</th>
<th>270</th>
<th>2700</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-Day (15)</td>
<td>136.3±6.6*</td>
<td>136.4±8.1</td>
<td>139.8±6.5</td>
<td>136.9±8.1</td>
</tr>
<tr>
<td>14-Day (15)</td>
<td>177.0±8.0</td>
<td>175.4±8.4</td>
<td>171.3±8.3</td>
<td>179.5±8.5</td>
</tr>
</tbody>
</table>

*Adenosine applied in 10 µl of Normosol-R, 38°C.

Vascular density index = number of intercepts between vessels and concentric circles over 47.1 mm of circumference.

Data are mean ± SEM; sample size in parentheses.

1-WAY ANOVA (repeated measures): 10-day, p < 0.445; 14-day, p < 0.107.

Adenosine-Stimulated Angiogenesis

Among the experiments summarized in Figures 2 and 3, it is clear that the VDI responses to adenosine are highly consistent, albeit modest. The low variability of this bioassay model (Figure 1) and the absence of an effect on the VDI by the Elvax pellets (Figure 4, Table 3) are obvious advantages for discerning these small changes. Because these experiments were conducted during the time when the normal embryonic in vivo VDI was most rapid (Figure 1), it is not certain whether the effect of the exogenous adenosine was only to accelerate an ongoing mechanism or to superimpose an additional effect of its own. However, the observed VDI increase in the presence of the 3-mg adenosine pellet was not maximal as dipyridamole enhanced adenosine's effectiveness even further (Figure 5).

Recent experiments directly testing the angiogenic activity of adenosine have not, however, documented a clear involvement. Adenosine implanted in the rabbit cornea stimulated an elongation of the surrounding limbus vessels toward the adenosine implant. Inosine, which had no effect in our study (Figure 2), had a more pronounced effect than adenosine.

On the CAM, millipore filters saturated with 10–4M adenosine did not elicit an angiogenic response; nor did a variety of other vasodilators. These investigators, using quantitative techniques similar to ours, only counted vessels that were at least 1 mm in length. A minimum length was not a criterion in our experiment, although with the 1-mm separation of the lines a proliferation of the CAM vessels is most rapid (Figure 1).

Figure 5. Inhibition of the adenosine-stimulated increase in the VDI by methyl-isobutyl-xanthine (MIX). The MIX (55 or 110 µg/day) or Normosol-R was injected into the albumin. Each group had an adenosine (3 mg) and a mannitol (1 mg) pellet on each CAM. The VDI was determined using the 4-, 5-, and 6-mm concentric circles. The mean control VDI was 198.2. Data are mean ± SEM; numbers inside bars are group n. **p<0.001 vs. control; ***p<0.005 vs ADN by paired t tests.

Table 2. Effect of MIX on Vascular Density Index of 14-Day Chick CAM

<table>
<thead>
<tr>
<th>Group</th>
<th>Vascular density index*</th>
<th>%Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (6)</td>
<td>290.0±6.8</td>
<td></td>
</tr>
<tr>
<td>110 µg MIX (5)</td>
<td>280.1±9.6*</td>
<td>−3.4</td>
</tr>
</tbody>
</table>

*Number of intercepts between vessels and concentric circles over 72.2 mm of circumference.

Δp = not significant (unpaired t test) vs. control.

Data are mean ± SEM; sample size in parentheses.

Figure 6. Dipyridamole (DIP) enhancement of adenosine's (ADN) stimulation of the VDI on the CAM. DIP (1 mg) was added directly to the Elvax pellet containing 3 mg of adenosine. DIP alone was tested in a separate group. The VDI was determined using the 4-, 5-, and 6-mm concentric circles. The mean control VDI was 198.2. Data are mean ± SEM; numbers inside bars are group n. **p<0.001 vs. control; ***p<0.005 vs ADN by paired t tests.
be counted. Moreover, their results may have been influenced by the marked reorientation and presence of vascular loops which formed in response to the micropore filter itself, a response observed in our own laboratory (unpublished) and by others. 24

In other studies, a role for adenosine in angiogenesis has been implicated using pharmacological interventions. Dipyridamole, which potentiates the biological activity of adenosine by inhibiting adenosine deaminase and blocking the adenosine re-uptake mechanism, increased capillary density in the heart20 21 and skeletal muscle29 of adult rats. The angiogenic response was evaluated by tritiated thymidine uptake and electron microscope morphometry of the capillary endothelial cells.

The absence of vascular proliferation on the CAM with dipyridamole (Figure 6) as opposed to the response in rats,19 20 21 may be due to the relatively short duration of the experiment compared to the 3- to 4-week studies with rats; or, to the difference in angiogenic criteria employed. It is also quite possible that adenosine does not play an active role in regulating normal embryonic neovascularization, at least on the CAM. Manipulation of the putative endogenous adenosine response mechanism by either MIX or dipyridamole alone failed to alter the VDI (Table 2, Figure 6). The effectiveness of these two compounds in affecting vessel growth was only evident in the presence of exogenously supplied adenosine. Thus, it appears that adenosine is not a primary mediator of normal vascular growth of the CAM.

The data do, however, point to the embryonic presence of functional adenosine receptors. Among the eggs treated with MIX, the adenosine-induced increase in the VDI was suppressed in a dose-dependent manner (Figure 5). At low doses, MIX is an effective adenosine receptor blocker.29 Although this study does not specifically rule out the well-known inhibition of phosphodiesterase by MIX and other xanthines, the Ki for this inhibition is in the millimolar range. In contrast, adenosine receptor blockade by MIX is achieved at significantly lower doses. The Ki for this inhibition is in the micromolar range, the dose level employed in this study.

Under other environmental circumstances, such as long-term hypoxia, adenosine may exert a more active influence. Periods of reduced oxygen availability are often accompanied by vascular proliferation,5 31 as well as the release of adenosine.30 31 Recently, we reported that eggs incubated in a 15% oxygen environment exhibited a 40% increase in the vascular density of the CAM.32 That this response was partially inhibited by MIX at doses comparable to those which blocked adenosine's stimulation of the VDI in this study (Figure 5), supports a vasoproliferative role for adenosine.

The amounts of adenosine incorporated into the pellets obviously do not approximate physiological levels. However, the incubation of pellets containing tritium-labelled adenosine demonstrated a sustained release of the adenosine from the polymer. That approximately 85% of the labelled adenosine was released from the Elvax pellets in vitro (Figure 7) should not be interpreted that 85% of the adenosine incorporated into the experimental pellets was released in vivo. The release kinetics in the incubation medium and on the surface of the CAM undoubtedly are very different. But varying the adenosine content of the experimental pellets does provide a range of concentration gradients to be tested. The efficacy of this protocol is demonstrated by the dose-related response of the CAM vascular density index (Figure 2).

Murray and co-workers33 have shown recently that

![Figure 7.](http://circres.ahajournals.org/)

**Figure 7.** Release of tritium-labelled adenosine from 10% Elvax pellets incubated in 400 μl of Normosol-R at 38°C. The incubation medium was changed every 24 hours. Data are mean ± SEM. Curves not statistically different (p<0.382: 2-WAY ANOVA).
the rate at which substances are released from Elvax can depend on the total amount of material incorporated. This suggests that the dipyridamole-augmented response to adenosine (Figure 6) may be due to an increased release of adenosine from the pellets which contained both compounds. Indeed, the release of tritium-labelled adenosine from these pellets was higher, but only 7–10% (Figure 7). In view of the slope of the adenosine VDI dose-response curve in Figure 2, it is apparent that the magnitude of this increased adenosine release is not sufficient to account for the augmented VDI observed in the presence of dipyridamole.

Qualitatively, a clearly defined response to adenosine is presented in Figure 4. Compared to the control, the vessels surrounding the adenosine pellet exhibit a more radial arrangement with a few larger vessels looping inward toward the adenosine source. This pattern of loops is not as pronounced as that characteristically described for tumor angiogenesis factor, a potent angiogenic stimulus on the chick CAM. A definitive mechanism for adenosine’s stimulation of the increased VDI was not defined in these experiments. During angiogenesis migration and proliferation of endothelial cells are fundamental processes which may be regulated separately. In the rabbit cornea angiogenesis assay, rabbit wound fluid stimulated marked neovascularization. When exposed to an in vitro gradient of this angiogenic factor, capillary endothelial cells exhibited a chemotactic response as well as a morphological orientation to the gradient. The wound fluid did not exert a mitogenic effect. Sepa- rate regulatory mechanisms for endothelial cell proliferation and migration have also been suggested for inflammation-induced angiogenesis in the rat cornea.

In this model, endothelial cell mitosis was suppressed by x-irradiation. However, angiogenic activity, as evidenced by vascular sprouting and cell migration was not abated until after the fourth day. Analogously, adenosine may act on a specific regulatory component. Among cultured aortic endothelial cells, adenosine induced a chemotactic response, but this nucleoside was ineffective in eliciting a mitogenic or proliferative effect. Thus, the elevated VDI with adenosine reported in the present study may be mediated, in part, through a selective enhancement of endothelial cell migration.

With our sampling technique, the increased VDI could be partially explained by the loops and reoriented vessels crossing additional lines of the counting circles. Such reorientation does not necessarily infer the concomitant growth of new vessels, but these loops often form anastomoses between arteries and veins or other sprouts, subsequently giving rise to additional sprouts. This loop formation or radial-like arrangement of vessels is an integral component of the CAM angiogenic mechanism, and therefore its influence on the counted VDI has been retained in our analysis.

Acknowledgments

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**KEY WORDS** • adenosine • angiogenesis • chick chorioallantoic membrane • dipyridamole • vascular density
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