Stimulation of Angiogenesis by Adenosine on the Chick Chorioallantoic Membrane

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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 \(\mu g\) of methyl-isobutyl-xanthine injected daily into the albumin. Partial inhibition was observed with 55 \(\mu g\)/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 decreased oxygen availability associated with chronic exercise,\(^4\) high altitude adaptation,\(^4\) and wound repair.\(^1\) 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 dilatation 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 Duling\(^12\) 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,\(^4\)\(^-\)\(^6\) ADP\(^17\) and lactic acid.\(^18\)\(^-\)\(^19\) 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.\(^20\)\(^-\)\(^21\)

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,\(^8\) 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 x 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.

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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 density was estimated by counting the number of ves- cels which intersected the circles. Because this quanta- tion under a dissecting microscope, and the average number of intercept points on a grid). This allows a more thorough sampling of the experimental area, especially with regard to short newly forming vessels.

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 by counting the number of ves- sels which intersected the circles. Because this quanta- tive technique employs the continuous lines of concentric circles to sample vessel density, the VDI is not limited by a predetermined maximum (e.g., the number of intercept points on a grid). This allows a more thorough sampling of the experimental area, especially with regard to short newly forming vessels.

If a pellet was not present, the intercepts with all four circles (72.2 mm total circumference) were counted; with a pellet present, only the 4-, 5-, and 6-mm circles (47.1 mm total circumference) were counted. Each response was counted 2–3 times at 20 × magnification under a dissecting microscope, and the average of the counts recorded. This technique permits counting the intercepts of vessels 10–12 μ in diameter; it does not separate arterioles from venules. An experimental and a mannitol-containing control pellet were placed on each CAM at least 1 cm apart. The responses on each CAM were evaluated individually as a percent of control. The site of pellet placement relative to underlying vessels was not critical. However, pellets were not placed directly over or immediately next to the largest vessels in order to avoid a counting bias resulting from large diameter vessels in the study area. 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 ×. 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-la- belled adenosine (2,8-3H adenosine) was also includ- ed. 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 × 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-Stimulated Angiogenesis

The dose of adenosine incorporated into individual pellets was confirmed by preparing a sample of pellets (two preparative batches for each dose), weighing the individual pellets, and subtracting the mean weight of similarly prepared pure polymer pellets. The mean weight of 33 Elvax only pellets was 971.7 ± 85.8 μg. The net weight of the 0.2 (n = 25), 1.0 (n = 22), and 3.0 (n = 19) mg pellets was 214.6 ± 22.5, 1287.6 ± 45.8, and 3104.6 ± 79.7 μg, respectively.

Figure 4 illustrates a representative response to a 3-mg adenosine pellet and a mannitol control pellet. The two pellets are 1.2 cm apart. In addition to a greater VDI, the vessels surrounding the adenosine pellets generally exhibited a more radial orientation toward the pellet. This morphological feature was not observed among controls.

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 in vivo VDI was studied. Adenosine, 27-270 ng applied in 10 μl of Normosol-R, did not significantly affect a local change of the in vivo VDI among eggs studied at either 10 or 14 days of incubation (Table 1). Similarly, dipyridamole (1 and 5 μg; n = 12) also failed to alter the in vivo VDI of 14 Day eggs (p < 0.650) (data not shown).

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 μg/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 μg/day) did not significantly alter the VDI of unstimulated CAM vessels (Table 2).

**Figure 1.** Age-related changes of the CAM vascular density index (VDI) and embryo weight during the incubation of fertile chick eggs. The VDI was determined using the 4-, 5-, 6-, and 8-mm concentric circles (72.2 mm total circumference). Data are mean ± SEM. VDI, n = 14-18; embryo weights, n = 19-30; p < 0.001 for each parameter, 1-WAY ANOVA.

Adenosine release 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 ± 1.5% (p < 0.001). These data are a composite of five separately conducted experiments.

**Figure 2.** Changes in the VDI of the CAM in response to various amounts of adenosine or inosine released from 10% Elvax pellets. Matching control pellets placed on the same CAM contained mannitol. The VDI was determined using the 4-, 5-, and 6-mm concentric circles (47.1 mm total circumference) and presented as percent of control changes. The mean control VDI for the adenosine dose-response curve was 217.5 (n = 42); for the inosine dose-response curve, 192.7 (n = 33). Data are mean ± SEM. Number beside each value is group n. Adenosine dose-response curve, p < 0.01; inosine, p < 0.405 by 1-WAY ANOVA.
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.

vivo VDI. It is likely, therefore, that the observed increase in the vascularity of the CAM with adenosine was due to the growth of new vessels and not to a direct vasodilator effect of this nucleoside opening preexisting vessels.

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 proliferation of the CAM vessels is 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 6).

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.26

On the CAM, millipore filters saturated with 10–4M adenosine did not elicit an angiogenic response; nor did a variety of other vasodilators.27 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
Table 3. Effect of 10% Elvax Polymer Pellets and of Mannitol on Vascular Density Index of CAM

<table>
<thead>
<tr>
<th>Group</th>
<th>Vascular density index</th>
<th>%ΔA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pellet (34)</td>
<td>326.2±5.7t</td>
<td></td>
</tr>
<tr>
<td>Elvax only (34)</td>
<td>327.3±5.8t</td>
<td>0.4±0.54</td>
</tr>
<tr>
<td>No pellet (24)</td>
<td>305.0±3.5</td>
<td></td>
</tr>
<tr>
<td>Mannitol - 1 mg (24)</td>
<td>306.8±3.1t</td>
<td>0.67±0.55</td>
</tr>
</tbody>
</table>

*Number of intercepts between vessels and concentric circles over 72.2 mm of circumference.
1p = not significant (paired t test).
Data are mean ± SEM; sample size in parentheses.

The effectiveness of these two compounds in affecting vascular proliferation on the CAM 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 heart and skeletal muscle 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 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. 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, as well as the release of adenosine. Recently, we reported that eggs incubated in a 15% oxygen environment exhibited a 40% increase in the vascular density of the CAM. 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-workers have shown recently that...
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. Separately 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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