Despite the importance of the collateral arterial supply for tissue survival following arterial occlusion, surprisingly little is known about the factors responsible for collateral arterial growth. One broad category of hypotheses is based on the possibility that biophysical factors related to the increase in flow they carry play a role. Several lines of evidence have also suggested that humoral factors participate in collateral vessel growth. We have shown that collateral arterial endothelial cell tritiated thymidine uptake occurs remarkably quickly, being evident with 24-48 hours after renal artery occlusion. Our working premise was that an increase in endothelial cell labelling would occur simultaneously throughout the length of the collateral arteries if biophysical factors related to blood flow were the responsible mechanism, because blood flow must be increased simultaneously throughout the length of the small, preformed collateral arterial vessels. On the other hand, if the information spread from the ischemic zone, one would anticipate centripetal spread of the endothelial cell hyperplasia in a retrograde direction from the ischemic zone. With the periureteric collateral arterial supply as the model, we performed serial studies of tritiated thymidine labelling following renal artery stenosis in the rat. As anticipated, endothelial cell labelling rose sharply within 24 to 48 hours, first evident in the area immediately adjacent to the renal hilum. Thereafter, a progressive, time-related centripetal gradient in endothelial cell tritiated thymidine labelling occurred (p < 0.01). These findings indicate that the factors responsible for endothelial cell hyperplasia are less related to blood flow in the lumen than to downstream, ischemic events. Although the mechanism responsible for the centripetal spread remains speculative, the communication system is likely to involve cell-to-cell contact in the vessel wall. (Circulation Research 1987;60:398-401)

Centripetal Spread of Arterial Collateral Endothelial Cell Hyperplasia After Renal Artery Stenosis in the Rat

Norman K. Hollenberg and Teruo Odori

The factors responsible for collateral arterial growth after major artery occlusion remain obscure, despite their importance for tissue survival. An increase in endothelial cell labelling with tritiated thymidine, as an index of collateral arterial growth, occurs early after renal artery occlusion. Our observation provides an approach to distinguishing between local biophysical factors in the arterial lumen or in the wall.

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clips, and the rats were returned to their cages. In the 20 rats described as "sham operated," the silver clip was removed prior to wound closure. In the remainder, the silver clip was left in place.

If evidence of renal infarction was found at the follow-up study, the rat was removed from the investigation.

A random number series was employed both to select the sham-operated and stenosis groups and to define the interval of follow-up prior to repeated study. The rats were randomly assigned to follow-up study at time zero or at 24-hour intervals up to 96 hours, on the basis of our earlier studies, with 12 rats in each group. The approach to radioautography with tritiated (H3) thymidine employed in this laboratory has been described in detail.34 In brief, two tritiated methyl thymidine injections (100 μCi/100 g body wt) were administered intraperitoneally, one hour apart. Two hours after the second injection, rats were killed, and blocks including the kidney, the ureters with periureteric tissue, and the bladder at the point of entry of the ureter were excised on each side. The blocks were fixed in 10% buffered formalin for at least 24 hours prior to processing automatically on the Auto Technicon tissue processor (Technicon Co., Tarrytown, N.Y.) for dehydration in serially increasing ethanol concentrations. Longitudinal blocks embedded in paraplast were made of each periureteric area from the kidney to the bladder, and cross sections were cut at 16 equally spaced intervals along the length of the ureter. Duplicate 5-μm sections cut at each level (A0820 microtome) resulted in 32 samples of each periureteric area for examination.

The sections were floated onto albumin-coated slides, deparaffinized in xylene, hydrated in ethanol and water, and air-dried in preparation for dipping. Slides were dipped individually in Kodak NTB-2 nuclear track emulsion, and the dry slides were placed in lightproof black boxes with a desiccating agent. A 12-13-week exposure period was optimal for endothelial labelling. The slides were developed with Kodak Dektop and Rapid-Fix at 21°C and then stained with hematoxylin and eosin. After dehydration and clearing with xylene, the slides were mounted from xylene with Paragon "Supermount." Attention to detail was critical because of the very low spontaneous turnover of vascular endothelial cells, normally less than 0.1%.33,34

Labelled nuclei were assessed under oil immersion light microscopy. A nucleus was considered endothelial when it was unequivocally at the surface of the lumen with no evidence of cytoplasm medially. Because of the very low spontaneous turnover of endothelial cells and the large number of rats and sections examined, an ordinal assessment system was adopted, ranging from 0-3+. Zero and 1+ indicated, respectively, either no or an occasional tritiated thymidine-labelled endothelial cell. Conversely, 3+ indicated a truly striking increase in endothelial cell turnover and 2+ a less striking but unequivocal increase over the low, spontaneous normal turnover rate. In an earlier assessment of vessel turnover in which formal labelling indices were defined by counting all positive and negative endothelial cells,1+ reflected a positive rate of about 0.1-1.0% and 3+ a fiftyfold increase to 5-6%. For presentation, 0 and 1+ were considered "negative" and 2+ and 3+ "positive"; little was gained from the finer ordinal gradations.

In a subset, 5 rats in each group at day 0, 1, and 2 were selected at random, and the percent of thymidine-labelled endothelial cells was estimated by counting positive and negative cells. At least 400 cells were counted in each ureteric quarter.

To assess the presence of a gradient in endothelial tritiated thymidine uptake, data from the histological sections were pooled to represent the quarter of the ureter nearest the renal hilum, the quarter of the ureter nearest the bladder, and the two intervening quarters. A code number from a random number series was assigned to each histologic section, and the code was not broken until all the sections had been assessed following radioautography.

Mean values have been presented with the mean ± SEM as the index of dispersion. Where appropriate, because multiple comparisons were made, statistical probability was assessed by one way analysis of variance. Chi-square and the Fischer exact test were employed for nonparametric analysis of categorical information. The null hypothesis was rejected for p < 0.05.

**Results**

The sham-operated rats showed no increase in tritiated thymidine in endothelial cell uptake on any day after the procedure (Table 1). The rats with renal artery stenosis, on the other hand, showed the anticipated brisk increase in endothelial cell tritiated thymidine labelling (p < 0.01), which was evident 48 hours after renal artery stenosis and thereafter (Figure 1 and Table 1). Because the normal endothelial cell labelling index ranges from 0.1-1.0%, a histologic section containing more than one labelled endothelial cell was extremely uncommon in normal animals and in the sham-operated group.

The periureteric vessels from rats with renal artery stenosis showed clear evidence of centripetal spread of increased tritiated thymidine endothelial cell labelling from the portion nearest the kidney and extending with time toward the region nearest the bladder (Figure 1). Within 24 hours, the portion of the periureteric vessels nearest the kidney showed a significant increase (p < 0.05), whereas there was no increase more distally. By 48 hours, the endothelial cells nearest the renal hilum were routinely positive for tritiated thymidine labelling, and labelling was also evident, but less consistent and less striking, in the mid-quarters (Table 1). The portion nearest the bladder remained negative at 48 hours but became positive thereafter (Table 1).

**Discussion**

The working premise in this study was that the pattern of collateral arterial endothelial cell hyperplasia following renal artery occlusion could provide insight
Table 1. Ordinal Assessment of Collateral Arterial Endothelial Cell Tritiated Thymidine Labelling

<table>
<thead>
<tr>
<th>Day</th>
<th>Near kidney</th>
<th>Proximal mid-quarter</th>
<th>Distal mid-quarter</th>
<th>Near bladder</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%+</td>
<td>Median</td>
<td>%+</td>
</tr>
<tr>
<td>A. Stenosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>33</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>100</td>
<td>+ + +</td>
<td>58</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>91</td>
<td>+ + +</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>100</td>
<td>+ + +</td>
<td>83</td>
</tr>
<tr>
<td>B. Sham</td>
<td>0-6</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

0 = No evidence of increased labelling; + + = unequivocal increase in labelling but not immediate in degree; + + + = striking increase in labelling.

into the responsible mechanisms. Renal artery stenosis induces an immediate, sharp drop in distal arterial blood pressure and, thus, an immediate increase in the pressure gradient along small, preformed arteries between the aorta and the intrarenal arterial tree. These presumably serve as the major potential collateral arterial pathway. One hypothesis to account for endothelial cell hyperplasia in this setting assumes that an increase in blood flow through such channels, perhaps acting via viscous drag at the plasma–endothelial cell interface, acts as a growth stimulus by analogy with the growth of vascular elements in an arteriovenous fistula. Indeed, the remarkable rapidity with which collateral arterial endothelial cell hyperplasia occurs following renal artery stenosis, a brisk response routinely being evident within 24–48 hours, made factors related to local biophysical forces an attractive possibility.

However, an unequivocal centripetal spread of the tritiated thymidine endothelial cell labelling was found. While no attempt was made to quantitate endothelial cell turnover, it was quite clear that the collateral vessels were growing very rapidly at that time and endothelial cell number must have been increasing. The response reflects true hyperplasia.

Local trauma related to the surgical procedure is an unlikely source of artifact because sham-operated rats did not show increased arterial thymidine labelling. Indeed, to the extent that removing the clip was traumatic, the region of the renal pedicle was subjected to more local trauma in sham-operated rats than in the rats in which the clip was left in place.

Two potential sources of bias were dealt with by the experimental design. First, rats were assigned to sham or stenosis groups and to days of follow-up before the surgical procedure on the basis of a coded random number. Thus, any bias created by events during the surgical procedure could not have contributed to the result. Second, assessment of radioautographs involves a subjective element, although the response is truly striking, but all assessments were made on a coded basis. The reader did not know whether the sections were made from the sham or stenosis group, the day of follow-up, or whether proximal or distal portions of the ureter were being evaluated by the assessment. Bias, therefore, could not have contributed to the finding of a coherent, progressive, time-related centripetal gradient in endothelial cell tritiated thymidine labelling.

Perhaps the most interesting question involves how the information spreads along the artery. Retrograde movement along the arterial lumen against blood flow...
seems unlikely. It is difficult to imagine that forces involved in diffusion can overcome the vector created by inflowing arterial blood to create a concentration gradient up the arterial tree over 3–4 cm, as was involved in this model. Diffusion of some product of ischemia through the tissue planes is possible but also seems unlikely. We have shown that a vascular mitogen is found in high concentration in lymph draining an ischemic kidney, raising the possibility that major lymphatic channels draining an ischemic zone serve as the conduit for the stimulus. Periureteric lymphatic drainage of a mitogen could account for our surprising finding that basal layers of the ureteric epithelium become hyperplastic after ipsilateral renal artery stenosis despite the fact that the urine does not contain a mitogen. The mitogen, however, appears to be a large molecule, and major lymphatics are normally not permeable to large molecules, a characteristic central to their primary role in draining proteins from the interstitium.

The most likely possibility involves cell-to-cell contact and communication. This phenomenon, clearly documented in a number of systems, operates in vascular elements as well. Ryan et al. described a similar gradient of endothelial cell division after the creation of an injury in an in vitro tissue culture of endothelial cells. Clearly, no ischemia was involved in that model. Although a number of chemical mediators responsible for communication via cell-to-cell contact have been identified, in general they have been smaller molecules than the vascular mitogen appears to be.

We have recently demonstrated centripetal spread of endothelial cell hyperplasia along the “feeder artery” to a malignant tumor in the rat, as the feeder vessel grows. It seems possible that the growth of a feeder vessel to a tumor and growth of a collateral arterial blood supply include a hyperplastic component that shares a common communication system and mediator. Arterial vessels also grow through unexplained mechanisms during embryogenesis and fetal growth. All three situations share a common feature: The metabolic demands of fetal growth, the metabolic demands of tumor growth, and the requirements of tissue rendered ischemic by arterial occlusion all induce hyperplasia of vascular elements of the local arterial supply. Whether or not identical factors are responsible in each case, insight into the responsible mechanisms for one is likely to provide insight into the responsible mechanisms for all. The control of vascular wall growth is likely to be complex. Endothelial cells synthesize a mitogen, the endothelial cell-derived growth factor. This factor or one like it may contribute to cell-to-cell communication in the hyperplastic response that characterizes collateral arterial growth.

References

KEY WORDS • tritiated thymidine • ischemia
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