Time Course of Increased Collateral Arterial and Venous Endothelial Cell Turnover after Renal Artery Stenosis in the Rat

NENAD Ilich, NORMAN K. HOLLENBERG, DEBORAH H. WILLIAMS, AND HERBERT L. ABRAMS

SUMMARY We induced left renal artery stenosis in rats and studied collateral arterial formation by angiography, histology, and radioautography with tritiated thymidine. Endothelial cell turnover was estimated by radioautography with tritiated thymidine in the periureteric blood supply of 10 normal and 38 collateral-forming kidneys 1 to 100 days after stenosis. Periureteric arterial endothelial cell labeling showed a highly significant \( P < 0.005 \) increase, apparent within 1 day and gradually falling as the vessels grew, until a baseline was reached in 35 days. A smaller but statistically significant increase in the labeling index also was found in endothelial cells of the renal vein during the first week \( (P < 0.01) \), and had a similar time course. A marked increase in epithelial cell labeling in the ureters draining the stenotic kidneys also was evident \( (P < 0.005) \). Thus, collateral vessel development is characterized by active DNA synthesis in the cellular elements which is maximal during the first week. A humoral factor is implicated in the vascular response by the parallel proliferation of venous and uretic cellular elements that are unlikely to experience the biophysical forces, such as increased blood flow or tangential wall force, which might stimulate proliferation in the arterial vessels.

WHEN a major arterial vessel is occluded, the rapidity with which collateral channels are recruited and the degree to which the blood supply is restored are major determinants of tissue survival. Despite their importance, however, the time course of the response and the mechanisms responsible for collateral development remain obscure. The present study was initiated to define the time course of arterial endothelial cellular proliferation during collateral arterial expansion following renal artery stenosis. It also confirmed a significant increase in endothelial cell labeling of renal veins and epithelium of the ureter draining the kidney, previously demonstrated in this laboratory (Cowan et al., 1978). This observation provides further support for the presence of a humoral substance participating in vascular neogenesis (Cuttino et al., 1975; Schaper et al., 1971).

Methods

Sprague-Dawley white rats weighing about 350 g were anesthetized with intraperitoneal pentobarbital (30 mg/kg). A left flank incision was made with sterile technique, and the main renal artery was exposed along a short segment. A silver clip was placed around the exposed artery and gradually tightened until the kidney became soft and blue, leaving an incomplete but severe stenosis. The incision was closed, and the rat was returned to its cage and assigned randomly to one of six follow-up time periods. In 10 rats an identical sham procedure was performed but the renal artery was not stenosed permanently. All data were reviewed on a coded basis and the code was not broken until the data analysis was complete.

The abdominal aorta was catheterized from the femoral artery with PE 90 polyethylene tubing, and blood pressure was monitored continuously with a Statham P23DC transducer and Grass recorder. To define and localize the collateral arterial supply, an aortogram at five times magnification was performed 1 to 100 days after surgery. The catheter in the abdominal aorta also was used to infuse tritiated methyl thymidine (5 \( \mu Ci \)) into the aorta at the level of the diaphragm over a 3-hour period; the rats...
were killed with intravenous potassium chloride 4 hours after the initiation of infusion. The renal fascia, capsular vessels, perihilar area, and periureteric tissues were inspected for unusual vascularity and then were removed along with a piece of small bowel for radioautography. The degree of increased vascularity was graded 3+ if it was striking, 2+ if it was unequivocal but less marked, and 1+ if it was equivocal. A similar approach was used to assess the aortograms. All assessments were made on a coded basis, without reference to either the date of surgery or, in the case of the direct inspection, whether a stenosis had been created.

The tissues were fixed for a minimum of 24 hours in 10% buffered formalin and processed automatically on the Auto Technicon tissue processor (Technicon Co.) for dehydration in serially increasing ethanol concentrations. They then were cleared with xylene, impregnated with Paraplast, and embedded in Paraplast blocks which were cooled on ice and sectioned at 5μm (AO 820 microtome). 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 placed in light-tight black boxes with a desiccating agent. A 12- to 13-week exposure period was found optimal for endothelial labeling. The slides were developed with Kodak Dektol 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% (Cowan et al., 1978; Schaper et al., 1971; Bevan, 1976).

In each rat we examined four 5-μm sections, including one from the renal hilum and three from the periureteric tissues. Labeled nuclei were assessed on a duplicate, coded basis from transverse serial sections of the ureter and periureteric arterial and venous vessels under oil immersion light microscopy. A nucleus was considered to be 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 to 3+. Zero and 1+ indicated, respectively, either no or an occasional tritiated thymidine-labeled 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 labeling indices were defined by counting all positive and negative endothelial cells (Cowan et al., 1978), 1+ reflected a turnover rate of about 0.1%, and 3+ a 50-fold increase to 5−6%. For presentation, 0 and 1+ have been considered “negative” and 2+ and 3+ “positive”; little was gained from the finer ordinal gradations, and these terms are used in the following description.

In the case of ratio data, mean values have been presented with the standard error of the mean as the index of dispersion and statistical significance was assessed by Student’s t-test. For the nonparametric data, primarily involving the ordinal assessment of gross or microscopic evidence of collateral formation, median values have been presented and statistical significance was assessed by nonparametric tests including the χ² or Fischer exact test. The null hypothesis was rejected when P was less than 0.05.

Results

A brisk increase in endothelial cell turnover of the periureteric arterial vessels was apparent on the first day, with every rat showing a striking increase in the number of positive endothelial cells (Figs. 1 and 2). On the 4th day, every rat studied also showed increased endothelial cell turnover in the arteries; the percentage of rats showing a positive response gradually fell to the low basal level by the 35th day (Fig. 2). The time course of the venous and ureteric proliferative response was similar, but a smaller fraction of the rats showed a positive response (Fig. 2).

Among the 10 control, sham-operated rats that were distributed randomly throughout the sections analyzed, only one arterial system was read as positive (and that was 2+, not 3+), and none had a positive venous or ureteric system.

Among the seven rats assessed on day 1, mean arterial pressure had increased significantly (P < 0.05) from the normal 107 ± 2 to 120 ± 6 mm Hg. Hypertension, defined as an arterial pressure more than two standard deviations above the mean, i.e., over 130 mm Hg, was present in two rats. Arterial pressure in all rats rose progressively to the 100th day when pressure had reached 205 ± 7 mm Hg (Table 1). Only the occasional rat did not have hypertension following the 4th day.

During the surgical exploration in each case an ordinal gross assessment of the collateral vessels was made. On the first and 4th day, the median response was 1+ (Table 1), which rose to a consistent 2+ to 3+ by the 21st day and thereafter. Only 40% of the rats showed gross collaterals during the first week but thereafter only the occasional rat did not. Despite the surgical procedure from 1 to 35 days before follow-up, none of the normal rats showed evidence of gross collateral formation (Table 1).

Arteriographically demonstrable collaterals became evident on the 10th day; thereafter no indication of increasing density or magnitude of the collaterals was evident.
Discussion

Stenosis or occlusion of the main renal artery reduced the distal intravascular pressure and resulted in an increase in the pressure gradient from the aorta through the residual renal artery lumen and through preexisting unoccluded small arterial vessels. Increased flow velocity through these small arteries could stimulate dilation and growth through a "biophysical" mechanism, such as shear rate at the plasma-endothelial cell interphase (Rodbard, 1971) or increased arterial pressure (Bevan, 1976). Also, cell hypoxia invokes release of both the products of anaerobic metabolism and intracellular constituents such as potassium from the injured cells. These are potent vasodilators which can dilate the preformed collaterals at their junction with the ischemic tissue and thus augment blood flow (Schaper et al., 1971). In an elegant study, May (1978) defined the response of vascular elements to hypoxia in vitro. Hypoxia reduced the turnover rate of vascular cells in tissue culture, rather than increasing turnover. Although the results of an in vitro experiment may not be applicable to the in vivo situation, May's study weighs against the direct cellular effects of hypoxia or the catabolic products of hypoxic metabolism serving as the stimulus to cell replication. It seems unlikely, moreover, that the latter mechanism is solely responsible, since with a modest reduction in renal blood flow and decreased glomerular filtration rate, renal oxygen utilization is diminished in parallel, and the arteriovenous oxygen difference across the kidney tends to remain constant (Thurau, 1964). The kidney is ischemic in the sense that it does not receive its normal blood supply, but tissue hypoxia may not be present (Hollenberg and Adams, 1976).

Whereas increased endothelial cell labeling of the aorta through the residual renal artery lumen and through preexisting unoccluded small arterial vessels, increased flow velocity through these small arteries could stimulate dilation and growth through a "biophysical" mechanism, such as shear rate at the plasma-endothelial cell interphase (Rodbard, 1971) or increased arterial pressure (Bevan, 1976). Also, cell hypoxia invokes release of both the products of anaerobic metabolism and intracellular constituents such as potassium from the injured cells. These are potent vasodilators which can dilate the preformed collaterals at their junction with the ischemic tissue and thus augment blood flow (Schaper et al., 1971). In an elegant study, May (1978) defined the response of vascular elements to hypoxia in vitro. Hypoxia reduced the turnover rate of vascular cells in tissue culture, rather than increasing turnover. Although the results of an in vitro experiment may not be applicable to the in vivo situation, May's study weighs against the direct cellular effects of hypoxia or the catabolic products of hypoxic metabolism serving as the stimulus to cell replication. It seems unlikely, moreover, that the latter mechanism is solely responsible, since with a modest reduction in renal blood flow and decreased glomerular filtration rate, renal oxygen utilization is diminished in parallel, and the arteriovenous oxygen difference across the kidney tends to remain constant (Thurau, 1964). The kidney is ischemic in the sense that it does not receive its normal blood supply, but tissue hypoxia may not be present (Hollenberg and Adams, 1976).


days post-stenosis

<table>
<thead>
<tr>
<th>CONTROL</th>
<th>1</th>
<th>4</th>
<th>7-10</th>
<th>21-28</th>
<th>35-42</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARTERIES</td>
<td>100</td>
<td>100</td>
<td>75</td>
<td>56</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RENAL VEIN</td>
<td>0</td>
<td>29</td>
<td>60</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>URETER</td>
<td>0</td>
<td>40</td>
<td>13</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 2. Time course of the proliferative response after renal artery stenosis. The ordinate represents the percentage of rats in which a coded assessment revealed an increase in turnover of endothelial cells in arteries or the renal vein or epithelial cells in the ureter.
TABLE 1  Time Course of Hypertension and of Grossly Evident Collaterals following Renal Artery Stenosis

<table>
<thead>
<tr>
<th>Days after stenosis</th>
<th>Normal</th>
<th>1</th>
<th>4</th>
<th>7-10</th>
<th>21-28</th>
<th>35-42</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>7</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>BP (mm Hg ± SEM)</td>
<td>107 ± 2.3</td>
<td>120 ± 5.9</td>
<td>158 ± 5.1</td>
<td>168 ± 13.6</td>
<td>166 ± 10.6</td>
<td>188 ± 11.3</td>
<td>205 ± 7.3</td>
</tr>
<tr>
<td>% with hypertension</td>
<td>0</td>
<td>(29)</td>
<td>(100)</td>
<td>(89)</td>
<td>(86)</td>
<td>(100)</td>
<td>(100)</td>
</tr>
<tr>
<td>Gross collateral</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++-+++</td>
<td>++-+++</td>
<td>++-+++</td>
</tr>
<tr>
<td>(median)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gross collateral</td>
<td>0</td>
<td>(43)</td>
<td>(40)</td>
<td>(71)</td>
<td>(86)</td>
<td>(100)</td>
<td>(83)</td>
</tr>
<tr>
<td>(% positive)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

perirenal vessels could represent a response to local injury, no such explanation can be applied to the perirenal vessels, to the veins, or to the ureter, which has an independent blood supply. Indeed, flow and pressure are reduced in the renal vein after arterial stenosis, and venous oxygen concentration tends to remain constant. Ureteral epithelial cell labeling cannot be attributed to hypoxia or to unintended damage by the surgical procedure, since the vascular supply of the ureter is independent of that to the kidney throughout most of its length and because the sham operation failed to induce a similar response.

The most likely explanation for both the increased renal vein endothelial and ureteral epithelial cell labeling is the release of a transferable chemical factor into the renal venous blood and into the urine. Others have demonstrated a similar increase in renal pelvic and bladder epithelial cell mitosis following acute renal ischemia in the rat (Dunn et al., 1976), and we have documented it in the dog (Cowan et al., 1978).

Earlier attempts to demonstrate growth-promoting factors in the drainage from ischemia tissues generally were performed late following arterial stenosis, in the 2nd to 4th week (Cuttino et al., 1975), primarily because the experimental protocol demanded arteriographic documentation of collateral formation. This study has confirmed that these vessels are generally difficult to visualize by angiography or gross inspection early in the course, when the mitogenic activity is at its height. As a consequence, the concentration of mitogen extracted in earlier studies might have been underestimated and the capacity to document its presence uniformly reduced because the experiments were performed so late after renal artery stenosis. This study has made it clear that the optimal time to collect tissue and fluids for mitogen assay is during the first week. The results are also provocative in suggesting that the mitogen responsible for enhanced vascular turnover also was responsible for increased ureteric turnover via its presence in urine. If so, urine could prove a fruitful source of this interesting agent.

Acknowledgments

It is a pleasure to acknowledge the able assistance provided in various aspects of this study by Robert John, Patricia Dunne, Carolyn Connelly, and Elaine Goncal

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Time course of increased collateral arterial and venous endothelial cell turnover after renal artery stenosis in the rat.
N Ilich, N K Hollenberg, D H Williams and H L Abrams

Circ Res. 1979;45:579-582
doi: 10.1161/01.RES.45.5.579
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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