Venous Endothelium of Experimental Arteriovenous Fistulas in Rabbits

By J. T. Fallon and W. E. Stehbens

ABSTRACT
Since hemodynamic factors have been implicated in the localization and pathogenesis of atherosclerosis, the effect of hemodynamic stress on endothelium was investigated. The endothelium of the inferior vena cava of 24 rabbits with aortocaval fistulas was stained in situ and examined by the Häutchen technique at postoperative intervals ranging from 1 to 50 weeks. Cell counts revealed that, in the anastomosed vein of the fistula, an initially high frequency of abnormal cells decreased 3-12 weeks postoperatively but rose again within 20-50 weeks to levels significantly higher than those in either normal venous endothelium or sham-operated veins. An originally high mitotic index at 1-2 weeks decreased with time but still remained significantly larger than that observed in sham-operated rabbits. In most rabbits with arteriovenous shunts, a small area of the inferior vena cava near the fistula was devoid of endothelium, covered with a thin deposit of mural thrombi, and considered to be a jet lesion. The findings demonstrate that hemodynamic factors can cause endothelial injury, increased turnover of endothelial cells, and formation of multinucleated endothelial cells characteristic of regenerating endothelium.

KEY WORDS endothelial regeneration endothelial mitosis
atherosclerosis Häutchen preparations jet lesion
hemodynamic endothelial injury multinucleated endothelial cells
inferior vena cava

Bizarre multinucleated cells, so prevalent in the endothelium of atherosclerotic aortas (1-3), are also found during endothelial repair and regeneration (1, 4-6). Recent isotope labeling studies in the aortas of experimental animals have revealed that the endothelial lining of blood vessels is renewed continuously, and an elevated endothelial mitotic index has been observed around the orifices of branches (7-9), which are well-recognized sites of predilection for atherosclerosis in man.

Hemodynamic forces have been incriminated in this enhanced turnover of endothelial cells at branching sites (8, 9) and implicated in the localization and pathogenesis of atherosclerosis (10-13). The inferior vena cava of rabbits with experimental aortocaval fistulas was therefore studied by the Häutchen technique to determine the effect on the endothelium of the severe hemodynamic disturbances accompanying the arteriovenous anastomosis.

Methods
Forty-eight male New Zealand White rabbits were divided into three groups. Group 1, consisting of 7 young (2-3 kg) and 3 aged rabbits (4-5 kg), served as unoperated controls. Group 2 comprised 14 young rabbits subjected to a sham operation. In group 3, there were 24 young rabbits in which an arteriovenous shunt was created between the abdominal aorta and the adjoining inferior vena cava. All rabbits were maintained on a standard pellet diet.

Surgical Techniques.—Rabbits in groups 2 and 3 were anesthetized with sodium pentobarbital given intravenously, and anesthesia was maintained with open ether. Each rabbit was tied on its back with

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its legs extended and tethered to cleats on a small operating table. The fur was closely clipped from the ventral surface of the abdomen, and the skin was cleaned with an antiseptic solution. With full aseptic technique, a ventral midline incision was made in the abdomen, and the inferior vena cava and the abdominal aorta were exposed between the renal and the inferior mesenteric vessels. Using a binocular operating microscope, both vessels were cleaned of fat and loose fascia. In group 2, the inferior vena cava was clamped proximally and distally with small vascular clamps. The vein was opened longitudinally for a distance of approximately 1 cm and then closed with a continuous suture of 8-0 monofilament nylon thread. In group 3, the artery and the vein were clamped proximally and distally and opened longitudinally for a distance of approximately 2 mm. Using 8-0 nylon thread, the margins of the apertures were then sutured together, creating an arteriovenous fistula. The abdomen was then closed in layers, and 300,000 units of penicillin were administered intramuscularly to each rabbit.

**Perfusion Procedures.**—To prevent postmortem clotting, each rabbit was given 1 ml of heparin intravenously approximately 1 minute before a lethal dose of sodium pentobarbital was administered intravenously. A minimum of one rabbit from group 2 and two from group 3 was killed at postoperative intervals of 1, 2, 3, 5, 8, 12, 16, 20, 26, 30, 40, and 50 weeks. Immediately after death, the thorax of each rabbit was opened, and a small cannula was inserted in the supradia-phragmatic inferior vena cava and passed downwards to the level of the renal veins. When perfusing rabbits in group 3, a second cannula was inserted into the thoracic aorta. The femoral vessels were then transected bilaterally, and manual perfusion with Tyrode’s solution was instituted for 5 minutes to wash out the blood. The vessels were then perfused successively with 5% glucose (5 minutes), 0.1% silver nitrate (30 seconds), 5% glucose (5 minutes), 10% buffered formalin (1 hour), Harris’s hematoxylin (10 minutes), and a weak solution of lithium carbonate (10 minutes). Care was taken to maintain continuous perfusion without a drop in perfusion pressure to obviate reentry of blood from branches and tributaries, since plasma interferes with silver staining.

The inferior vena cava from the iliac bifurcation to the renal veins was then carefully dissected from the surrounding tissues, tied to a stainless steel rack to prevent shortening, and removed from the rabbit. Under a dissecting microscope, the vessel was cleaned of excess fat and fascia, opened longitudinally, and pinned out on a sheet of polyethylene with stainless steel entomological pins. The vessel was subsequently dehydrated in ascending grades of ethyl alcohol. Hautchen preparations of the endothelium were made using a modification of the nitrocellulose technique as described by Poole and co-workers.

**Counting Procedures.**—Using a Leitz Orthomat-Ortholux microscope, photographs were taken of random areas of endothelium from the Hautchen preparations. Photographic prints were prepared at a final magnification of 150x. The cells in each photograph were classified as mononuclear, multinuclear, or in mitosis (from late prophase to telophase) and then counted. Due to technical difficulties inherent in the Hautchen preparation, sufficiently large areas of endothelium devoid of artifact were not always available in each rabbit for photography. Nevertheless, all vessels but one were counted in group 2, and at least one vessel from group 3 was counted at each time interval except that of 16 weeks. Statistical analyses of counts were performed using the Mann Whitney U-test. The level of significance was set at $P \leq 0.050$.

**Results**

**MACROSCOPIC FINDINGS**

**Group 1.**—The segments of the inferior vena cava exhibited no abnormal findings when they were examined under a dissecting microscope. They were fairly uniform in size, approximately 7 cm long and 1.5 cm wide (range 1.2 to 1.8 cm).

**Group 2.**—Veins of these rabbits were of similar size to those in group 1, and there was no constriction at the site of phlebotomy. Suture material could be distinguished in their walls at the site of repair, but no lesions were apparent on the luminal surface.

**Group 3.**—Following the release of the vascular clamps in the rabbits of group 3 and the establishment of the arteriovenous shunt, blood flowed readily through the fistula, and swirling blood was clearly observed through the wall of the inferior vena cava. Patchy whitish mottling of the venous wall near to and for a few millimeters distal to the fistula soon appeared, and, at times, small fragments of the mural thrombi washed away.

The rabbits of group 3 at the time of death exhibited cardiomegaly and chronic venous congestion of the liver, as was expected. However, to avoid interference with the perfusion and the preservation of the endothelium, no attempt was made to quantify other
cardiovascular changes. In all but one rabbit, the width of the pinned out vessel in the neighborhood of the fistula approximated 1.5 cm. This zone appeared to be a relative constriction in most vessels, since either the proximal or the distal segments, or both, were dilated, with circumferences varying from 2.0 to 5.5 cm. In all but two of the anastomosed veins examined, an irregularly circumscribed surface lesion (5-8 mm in diameter) was seen opposite the fistula or nearly so, often with a small track extending towards the fistula (Fig. 1). This patch, referred to as a "jet lesion," appeared heavily stained with silver and lacked the sheen of the normally stained endothelium (Fig. 2). Around the jet lesion...
there was a zone 5–10 mm wide in which there were multiple low ridges (Fig. 3). Some of these ridges were crescentic (Fig. 1), and the concavity was generally directed towards the jet lesion. In one vein exhibiting dilatation at the site of the fistula as well as proximally and distally, no jet lesion was observed.

**MICROSCOPIC FINDINGS**

**Group 1.**—The endothelium of the normal rabbit inferior vena cava consisted of a single layer of cells, predominantly spindle-shaped and mononuclear. They were approximately $15 \times 110 \mu m$ and oriented lengthwise in the longitudinal axis of the vessel. Their cell outlines were somewhat irregular and often wavy in appearance (Fig. 4). Binucleated cells, $25 \times 130 \mu m$, were regularly seen between entering tributaries. In the veins of young rabbits, mitotic figures and cells with more than two nuclei were rare. However, in two of the three aged rabbits from group 1, a longitudinal strip 3 mm wide containing a high frequency of cells with two to ten nuclei extended for the length of the vein. These multinucleated cells were roughly elliptical, about $50 \times 150 \mu m$, and contained an average of four nuclei per cell. Stigmas and stomas were seldom seen in control vessels and were usually associated with areas of increased silver precipitation in the cytoplasm.

**Group 2.**—The endothelial lining of veins subjected to phlebotomy showed aberrant patterns associated with the suture line and
the position of the vascular clamps applied during surgery. In the first week, a few small denuded areas were found with the surrounding endothelium exhibiting variability in cell size and nuclear staining, mitotic figures, and multinucleated cells. Along the suture line, the regenerated endothelium consisted mostly of small polygonal cells, $10 \times 30\mu\text{m}$, interspersed with large multinucleated cells (Fig. 5). There was a decrease in the number of mitotic figures and multinucleated cells, although small polygonal cells were still prevalent 2
weeks postoperatively and only one small denuded area was found. By 8 weeks, the endothelial cells were predominantly spindle-shaped, but multinucleated cells were still present along the suture line. A small denuded area associated with a few mitotic figures in the surrounding endothelium was observed in one vein 26 weeks postoperatively. In all other veins, endothelial formation was complete by 12 weeks. These veins rarely contained mitotic figures but still exhibited a few multinucleated cells over the sutures (Fig. 6).

**Group 3.**—The jet lesions were devoid of endothelium and were covered by faintly stained small bodies, generally assumed to be platelets (6, 15), in association with occasional leukocytes, but small islands of endothelial cells were at times observed (Fig. 7). In the
regenerating endothelium surrounding the jet lesions there were many small cells with hyperchromatic nuclei, large irregularly shaped multinucleated cells, and numerous mitotic figures. Away from this actively proliferating edge, the endothelial pattern was generally disordered. The cells were pleomorphic and bizarre. Multinucleated cells were numerous (Fig. 8), and in short-term fistulas mitoses were especially frequent (Fig. 9). Stigmas and stomas were sometimes abundant along the silver outlines, although these changes were irregularly distributed.

No endothelial desquamation was found in the dilated proximal and distal segments, and the endothelial cells were predominantly

FIGURE 9
Three mitoses (arrows) in endothelium near fistula.
spindle-shaped. There were occasional areas of pleomorphic cells, many of which were binuclear, and, overall, the mitotic figures were moderately frequent and randomly distributed (Fig. 10). In isolated areas, silver lines exhibited stigmas and stomas not associated with any staining artifact. Towards the jet lesion, this endothelial pattern merged irregularly with that about the crescentic ridges.

CELL COUNTS

The results of endothelial cell counts are presented in Table 1. In group 1, veins from four young and three aged rabbits were examined. The multinucleated cells in this group were predominantly binuclear with less than 0.1 cells per thousand having three or more nuclei. In two of the aged rabbits, additional counts were made in the high-frequency areas, and, in the 5,483 endothelial cells counted, the average frequency of multinucleated cells was 115.6 per thousand cells with no mitotic figures.

Cell counts from Hautchen preparations for groups 2 and 3 were divided according to areas maximally (near suture line or near fistula) and minimally (away from suture line and away from fistula) traumatized during surgical procedures and according to the time elapsed postoperatively. At 1–2 weeks, multinucleated cells and mitoses near the suture line in sham-operated rabbits were significantly more frequent than they were in normal venous endothelium, with \( P \) values of 0.017 and 0.008, respectively. From 3–50 weeks, the mitotic index dropped rapidly, but the multinucleated cells persisted, although their frequency was not statistically significant from that of group 1.

Except for the frequency of multinucleated cells during the first 12 weeks and of mitoses between 20 and 50 weeks in the dilated segments, the frequency of multinucleated cells and mitoses was significantly greater in the endothelium of group 3 than it was in the veins of group 1. In the areas near the fistula, but excluding the growing edge of endothelium about the jet lesion, the frequency of mitoses at all three periods of time considered in Table 1 was significantly greater than that

<table>
<thead>
<tr>
<th>Area</th>
<th>Weeks postop.</th>
<th>No. animals</th>
<th>No. cells counted</th>
<th>Multinucleated cells/10⁶ cells</th>
<th>Mitoses/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Near suture line</td>
<td>1–2</td>
<td>3</td>
<td>10,926</td>
<td>28.0 ± 10.5*</td>
<td>33.3 ± 10.1*</td>
</tr>
<tr>
<td></td>
<td>3–12</td>
<td>4</td>
<td>15,248</td>
<td>11.0 ± 6.6</td>
<td>2.3 ± 1.0</td>
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<tr>
<td></td>
<td>20–50</td>
<td>4</td>
<td>13,367</td>
<td>10.7 ± 1.8</td>
<td>0.0</td>
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<tr>
<td>Away from suture line</td>
<td>1–2</td>
<td>3</td>
<td>8,844</td>
<td>2.6 ± 0.9</td>
<td>9.8 ± 5.4*</td>
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<tr>
<td></td>
<td>3–12</td>
<td>5</td>
<td>16,731</td>
<td>2.5 ± 0.5</td>
<td>1.9 ± 1.2</td>
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<td></td>
<td>20–50</td>
<td>4</td>
<td>18,488</td>
<td>3.1 ± 1.0</td>
<td>0.6 ± 0.6</td>
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<tr>
<td>Group 2</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Near fistula</td>
<td>1–2</td>
<td>3</td>
<td>12,078</td>
<td>38.7 ± 8.4*</td>
<td>68.0 ± 14.9*</td>
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<tr>
<td></td>
<td>3–12</td>
<td>3</td>
<td>13,095</td>
<td>15.0 ± 1.3*</td>
<td>22.5 ± 2.6*†</td>
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<tr>
<td></td>
<td>20–50</td>
<td>3</td>
<td>13,756</td>
<td>43.9 ± 14.0*†</td>
<td>9.5 ± 2.5*†</td>
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<tr>
<td>Away from fistula</td>
<td>1–2</td>
<td>4</td>
<td>13,725</td>
<td>9.4 ± 2.0</td>
<td>34.7 ± 6.4*</td>
</tr>
<tr>
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<td>3–12</td>
<td>4</td>
<td>19,161</td>
<td>5.4 ± 1.3</td>
<td>11.9 ± 3.7*†</td>
</tr>
<tr>
<td></td>
<td>20–50</td>
<td>5</td>
<td>18,082</td>
<td>12.8 ± 2.4*†</td>
<td>2.8 ± 1.1†</td>
</tr>
</tbody>
</table>

Values are means ± SE.

*Significantly greater than normal frequency seen in group 1, \( P < 0.05 \).
†Significantly greater than corresponding frequency in group 2, \( P < 0.05 \).
near the suture line of sham-operated rabbits with $P$ values of 0.050 for 1-2 weeks, 0.028 for 3-12 weeks, and 0.028 for 20-50 weeks. The frequency of multinucleated cells between 20 and 50 weeks was also significantly increased over that for the sham-operated rabbits ($P = 0.028$). The number of multinucleated cells away from the fistula in group 3 vessels was significantly greater ($P = 0.008$) than that in the veins of group 2 rabbits (away from the suture line) only at 20-50 weeks, whereas mitotic figures were significantly more frequent at both 3-12 weeks ($P = 0.014$) and 20-50 weeks ($P = 0.032$) but not at 1-2 weeks postoperatively.

Discussion

Arteriovenous fistulas were used in this experiment because they provide a means of producing profound hemodynamic disturbances in vivo (16, 17). The chronic changes in the wall of anastomosed veins have the appearance of a nonlipid-containing arteriosclerotic intimal proliferation or phlebosclerosis and seem to be the direct result of the associated hemodynamic stresses (18). Therefore, the experimental fistula provides an excellent model for ascertaining the effects of hemodynamically induced injury of the endothelium.

The vessels were perfused in situ to reduce artifacts to a minimum because venous endothelium is readily damaged by manipulations in the production of en face preparations (15). Because of this susceptibility to injury, the use of sham-operated rabbits was considered essential to establish a baseline for the repair of surgically induced endothelial changes.

This study demonstrated that, in the endothelium of the inferior vena cava of normal rabbits, the mitotic index was low in comparison with epithelial cells of the skin (19) or the intestinal tract (20) but was of the same order of magnitude as that in smooth muscle cells of normal blood vessels (21) and arterial endothelium of guinea pigs (8, 9).

The prevalence of multinucleated cells in the vessels of old animals has been noted previously (1, 3, 5), and, consequently, their occurrence in our aged rabbits was expected. However, their nonrandom distribution in both young and old rabbits has not been previously recognized and is difficult to explain. Hemodynamic factors related to flow patterns associated with the entrance of tributaries or some mechanical disturbance in the venous wall generated by the pulsations of the juxtaposed aorta, or both, might be responsible.

Endothelial regeneration is characteristically accompanied by multinucleated cells (6). Their method of formation, whether due to fusion of cytoplasm (though not of nuclei) or to division of nuclei (though not of cytoplasm) is at present uncertain. The frequency of multinucleated endothelial cells typically increases sharply in the first week, remains elevated until repair is complete, and subsequently decreases with a return of normal endothelial cell patterns (6). Our results demonstrated similar findings in the sham-operated rabbits. Within 3 weeks, the endothelial pattern had almost completely returned to normal except for a small denuded area in two rabbits and a slightly higher frequency of multinucleated cells along the suture line. This persistence of abnormal cells along the suture line in the long-term rabbits might be due to a low-grade, but chronically irritating, action of the suture material itself or to a mechanical disturbance of the tissue secondary to the presence of the rigid nylon thread during the pulsatile movements of the venous wall.

We felt that it was conservative to compare the anastomosed vein near the site of the fistula (excluding the jet lesions with its growing edge and the anastomotic site) with the suture line of the sham-operated veins. The differences in the frequency of multinucleated cells and of mitoses at 20-50 weeks were highly significant.

Considering the influences of both age and operative trauma, the dilated segments of the anastomosed inferior vena cava away from the fistula were most aptly compared to the sham-operated veins away from the suture line. The fact that the frequency of multinucleated cells
and of mitotic figures was high in the first 2 weeks, fell during the next 10 weeks, and rose thereafter suggests that their prevalence is not due to the direct effect of the operation per se but to a long-term effect of the hemodynamic stresses associated with the fistula. The dilatation of the vein must have caused some endothelial proliferation, but this proliferation too might be the consequence of hemodynamic stress (17). However, the prevalence of bizarre endothelial cells in the dilated segments in areas away from the fistula was considerably less than it was closer to the fistula and in the nondilated segments. It is, therefore, likely that their prevalence in the latter two areas is the result of the profound hemodynamic disturbance rather than of other factors (e.g., congestive cardiac failure) which might have a generalized effect on vascular endothelium.

The presence of a jet lesion (an area devoid of endothelium) in most anastomosed veins could be caused by continued and recurrent desquamation of endothelial cells or by some factor impeding formation of endothelium, or by a combination of both. Jet lesions also occur in association with stenotic orifices, including coarctation of the aorta. They are characterized grossly as raised corrugated patches of intima or endocardium (22), and histologically they are composed of nonvascular intimal fibrous tissue, mainly collagenous, although in some instances deposits of platelets and fibrin occur on the surface of the lesion (23). The mechanism underlying the occurrence of the jet lesion is thought to be related to the presence of severe hemodynamic disturbances associated with the fistula, possibly due to a high shearing stress (24) causing trauma at the site of impact of the jet stream of blood. The absence of dilatation of the inferior vena cava in the region of the fistula in most of the rabbits in group 3 might have been due to perivascular fibrosis occurring postoperatively or to fibrosis associated with the jet lesion as described by Edwards and Burchell (23). The present study is the first experimental production of a jet lesion, and the fistulas constitute a useful experimental model of the phenomenon.

The irregularities of the venous wall surrounding the jet lesion might be attributed to formation of endothelium and to organization of the irregular small mural thrombi seen through the wall of the inferior vena cava shortly after the experimental production of the fistula. However, the fate of the mural thrombi is uncertain, since they were not observed 1 week postoperatively. Alternatively, the irregular and unequal intimal proliferation might be related more directly to nonlaminar flow patterns as suggested by the crescentic ridges with their specific orientation towards the jet lesion.

Therefore, the augmentation in mitotic activity, the increased frequency of multinucleated cells, the occurrence of stigmas and stomas, which are believed to represent interendothelial gaps (18, 25), and the finding of a jet lesion devoid of endothelium in the inferior vena cava of rabbits with aorto caval fistulas is consistent with the conclusion that endothelial damage can be the result of local hemodynamic factors. This view, espoused by several investigators, has arisen from indirect evidence from acute studies on canine aortas (24, 26–28) and labeling studies on aortic endothelium (8, 9). It is, therefore, very likely that hemodynamically induced endothelial trauma, with increased permeability to blood constituents, participates in the earliest development of atherosclerotic lesions.

References


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