To investigate the morphologic basis of blood flow-dependent adaptive vascular enlargement, we quantitated endothelial cell density, dimensions, and structure in canine carotid arteries that were flow-loaded for 4 weeks, i.e., just before the development of significant adaptive enlargement. Increased flow was produced in the right common carotid artery of seven adult beagle dogs by an arteriovenous shunt to the right external jugular vein. The left common carotid artery was used to produce sham-operated controls. Five additional animals were used to produce sham-shunted controls, and two dogs were used as nonoperated controls. The blood flow rate (BFR) and wall shear rate (WSR) were markedly increased immediately after anastomosis in the proximal segment of the shunted artery (BFR=719±142 ml/min, WSR>4127±1002/sec) and after 4 weeks (BFR=628±157 ml/min, WSR>2919±388/sec) compared to the same artery before anastomosis (BFR=154±50 ml/min, WSR=904±314/sec, p<0.01x10^-3 for both comparisons) and to the contralateral control artery after 4 weeks (BFR=365±110 ml/min, WSR=2136±876/sec, p<0.01 and p<0.05, respectively, compared to the shunted side). In the shunted artery, endothelial cell density was markedly increased (6.15±0.68x10^3 cells/mm^2 compared to 3.33±0.70x10^3 cells/mm^2 for the controls, p<0.001). Endothelial cells on the high flow side were markedly narrowed in both axial and circumferential directions, but were radially thickened; nuclei became prolate-spheroid in shape. On the control side, cells were relatively flat and thin. We conclude that elevated wall shear stress induces an early increase in endothelial cell number and that this increase precedes the development of significant blood flow-dependent vascular enlargement.


A continuous lining of intact endothelial cells is maintained in arteries under conditions of normal flow. It has become increasingly evident that endothelial cells undergo configurational modifications in response to flow in vitro and in vivo. Both the shape and orientation of endothelial cells have been related to local hemodynamic conditions. Blood flow-dependent adaptive vascular growth and dilation, first pointed out by Thoma in 1893, has recently been demonstrated to be closely associated with wall shear stress in experimental animal models.14,15 Although the response is thought to be mediated by endothelial cell reactions to shear, studies have thus far dealt with the flow-related changes in artery radius, which appear to stabilize when wall shear stress values are equal to about 15 dynes/cm^2. To further characterize the participation of endothelial cells in the process, we studied endothelial cell density and structure in canine carotid arteries subjected to an increased flow load by an arteriovenous (AV) shunt. The present report deals with the changes evident at 4 weeks after anastomosis, just before significant shear stress-dependent adaptive dilation became evident.14

Methods

Animals

Fourteen female beagle dogs, 12 to 18 months old, each weighing 7.0 to 12.5 kg, were used for these experiments. Anastomosis between the right carotid artery and the right external jugular vein was performed on seven animals (ages, 15.6±2.4 months; weights, 10.7±1.6 kg). All the operative procedures except for the shunting were performed on the left side in the same animals to control for operative manipulation without anastomosis (sham-operated controls). To examine the effects of the complete shunting procedure, five dogs were used as a second set of controls. In these animals, the anastomosis was performed, but the shunt was closed after the operation and before the hemostatic clamps were released. These animals were kept for 1 week (three animals) or for 4 weeks (two animals). Two dogs served as nonoperated controls (ages, 15 and 18 months; weights, 9.5 and 12 kg). The same hemodynamic measurements, fixation techniques, sampling, and morphologic studies were performed on all the animals.

Although canine right and left carotid arteries are of different lengths (the left branches from the brachiocephalic artery at about 2 cm from the aortic orifice, while the
right branches at about 1 cm from the left), we elected to perform all of the anastomoses on the right side and to use the vessels on the left as the sham-operated controls. Preliminary studies of 20 adult female beagle dogs in our laboratory revealed that the outer diameter was the same for the right and left carotid artery (3.5 mm) and that blood flow was identical for both sides: 146±34 ml/min on the right and 147±29 ml/min on the left (r=0.97, p<0.001). The flow patterns in the two vessels were also indistinguishable. In addition, detailed ultrastructural observations on four adult female beagle dogs showed no differences between the right and left arteries.

**Operative Procedures**

The seven dogs subjected to shunting were anesthetized with sodium pentobarbital (30 mg/kg). The right common carotid artery and the right external jugular vein were exposed, and an AV shunt was constructed under sterile conditions as described by Kamiya and Togawa.14 The AV anastomoses were performed at a distance of 10 cm from the origin of the artery. On the left, the common carotid artery was sutured to the left external jugular vein along the adventitia after having been subjected to the same manipulation and clamping as on the right. These vessels served as controls for operative manipulations without anastomosis (sham-operated controls). Measurements of blood flow rate (BFR) were performed before operation and immediately after operation proximal to the anastomotic site on the shunted side and at the same level on the control side by using an electromagnetic flowmeter (Nihon Kohden Company, MFB 2100, Japan). These animals were kept for 4 weeks, and the flow patterns in the two vessels were also indistinguishable. In addition, detailed ultrastructural observations on four adult female beagle dogs showed no differences between the right and left arteries.

For transmission electron microscopy (TEM), the remaining one-fourth of the resected segment was postfixed for 1 hour in 3% glutaraldehyde in phosphate buffer (pH 7.4) at 4°C and then for 1 hour in 1% osmic acid in phosphate buffer (pH 7.4) at 4°C. Specimens were transected in both axial and transverse directions to obtain precisely oriented longitudinal and transverse planes of section. After dehydration through alcohols and embedding in Epon, ultrathin sections were stained with lead citrate and uranyl acetate and were observed in an LEM 2000 (Akashi Company, Japan) transmission electron microscope. Samples from the seventh dog with an AV fistula were used to obtain silver deposit preparations to mark intercellular junctions. For this preparation, the carotid arteries were flushed in situ with 5% dextrose, were infused with 20 ml of 0.25% silver nitrate in 5% dextrose for 30 seconds, were flushed with the same volume of 5% dextrose, and then were perfused with normal saline solution. Fixation under pressure was subsequently performed, and the tissues were sampled for light microscopic and SEM in the manner described above.

**Tissue Preparation**

For light microscopic and ultrastructural studies, the common carotid arteries were fixed in situ while dis tended. In six of the shunted and in the two nonoperated animals, controlled pressure perfusion-fixation was carried out as described previously20 by using 3% glutaraldehyde in phosphate buffer (pH 7.4). The height of an inflow reservoir and the outflow resistance were adjusted to maintain a constant perfusion pressure of 100 mm Hg. After 30 minutes, a segment of the common carotid artery, 10 mm in length, was resected just proximal to the site of the blood flow measurement on the shunted side at 7 cm distal to the origin of the artery and at the corresponding level on the control side (i.e., 8 mm distal to the origin). One-half of the length of the resected segment was used for light microscopy. Precise transverse sections were embedded in paraffin, sectioned at 1.5 μm, and stained with hematoxylin and eosin.

For scanning electron microscopy (SEM), one-fourth of the resected segment, 2.5 mm in length, was postfixed for 1 hour in 3% glutaraldehyde in phosphate buffer (pH 7.4) at 4°C. This was dehydrated through alcohols and was critical-point dried. The samples were coated by evaporating gold-platinum in a high vacuum. The endothelial surface was observed in a JSM-T200 (JEOL Company, Japan) scanning electron microscope.

Hemodynamic Computations

To calculate the hemodynamic parameters, we obtained the internal radius (r) using the expression:

\[ r = \frac{1.25L}{2\pi} \]

(1)

where L is the luminal circumference on transverse section and 1.25 is a correction factor for shrinkage occurring during fixation, dehydration, and paraffin embedding.23 The circumference was determined by projecting the transverse section of the artery onto a sheet of paper at an enlargement of 100× (Nikon Company, V-16A, Japan), then tracing the lumen contour and measuring the circumference by means of an image analyzer system (Nikon Company, Cosmoszone-1, Japan). For purposes of computation, the internal radii of the shunted (right) arteries and of the control (left) arteries before operation and immediately after operation were assumed to be the same as those of the control (left) arteries at 4 weeks after operation.

**Density of Endothelial Cell Population**

Three complimentary methods were used. We determined the mean of the total number of endothelial cell nuclei visible along the entire circumference on 10 successive transverse light microscopic sections. In addition, the number of endothelial cells and nuclei per unit of circumferential length was obtained from counts on a composite of 20 to 30 TEM photographs encompassing a 0.4 mm segment of the circumference of a transverse section. The photographs were taken at a magnification of 10 000×. The abluminal width of each endothelial cell:

\[ w_{C_1}, w_{C_2}, \ldots, w_{C_{n-1}}, w_{C_n} \]

(2)
was measured, and the total abluminal length of the composite segment (WC) was then obtained:

\[ WC = wC_1 + wC_2 + ... + wC_n + wC_n (\mu m) \]  

(3)

where \( n \) = the total number of endothelial cells in one composite transverse TEM photograph. The number of endothelial cells per millimeter of length (1000 \( \times \) n/WC) was then calculated. Similarly, the number of endothelial nuclei per millimeter of length (1000 \( \times \) m/WC, where m= the total number of endothelial nuclei in one composite, transverse TEM photograph) was determined from the ultrathin transverse sections. In each composite, transverse TEM photograph, 100 to 150 endothelial cells and 15 to 20 endothelial nuclei in the control common carotid artery and 150 to 200 endothelial cells and 20 to 30 endothelial nuclei in the shunted common carotid artery were measured. Finally, the number of endothelial cells per unit of lumen surface area was assessed from SEM photographs taken at a magnification of 1000 \( \times \). The number of endothelial cells on a photograph divided by the area of the photograph was determined for each photograph. The cell density (cells/mm\(^2\)) for each artery was expressed as the average of the determinations from 10 SEM photographs for each artery.

Size and Shape of Endothelial Cells

Several assumptions were made in obtaining estimates of the shape and size of endothelial cells and nuclei: 1) All the endothelial cells and nuclei in a given common carotid artery specimen were the same shape and size. 2) The long axes of the cells were oriented parallel to the longitudinal axes of the blood vessels. 3) Cell body and nuclear shapes were symmetrical in both transverse and longitudinal dimensions. 4) The distribution of these structures was random. When the endothelial layer is precisely transected, transversely or longitudinally, each nuclear and cellular profile represents part of a nucleus and a cell at a given level. TEM ultrathin sections are very thin (approximately 70 nm) compared to the size of an endothelial cell nucleus (10 to 15 \( \mu m \) in length). Therefore, endothelial cell and nuclear profiles appearing on a composite, transverse TEM photograph enlarged 10 000 \( \times \) were used to estimate the dimensions of a single endothelial cell and nucleus within a constant thickness of section \( d (\mu m) \), where \( d = LE/n, \) and \( LE = \)length of an endothelial cell (\( \mu m \)). For the same composite photograph, \( d \) is the same for both cell and nucleus:

\[ d = LE/n = LN/m \]  

(4)

where LN = length of endothelial nucleus (\( \mu m \)). The same considerations are applicable to the longitudinal sections. In practice, however, it was difficult to identify endothelial cell junctions and, due to the elongated spindle shape of the endothelial cells, to obtain a sufficient number of cells and nuclei in a composite longitudinal TEM photograph covering 0.4 mm of actual length. Therefore, we used the longitudinal sections to determine the length of endothelial nuclei (LN in microns) by measuring the longest nuclear profile on a composite longitudinal TEM photograph. From these data, we obtained the \( d \) value for \( d = LN/m \).

To measure endothelial cell and nuclear profile areas on transverse TEM photographs, we resorted to point counting with 5 \( \times \) 5 mm grids. \(^{21}\) The endothelial profile areas of successive endothelial cells in the composite, transverse TEM photographs were taken as: \( aC_1, aC_2, ..., aC_n, \) and \( aC_n (\mu m^2) \) and the successive nuclear profile areas as: \( nN_1, nN_2, ..., nN_m, \) and \( nN_m (\mu m^2) \). Reconstructions of endothelial cell and nuclear profiles were obtained by superimposing all of the transverse profiles appearing in a composite, transverse TEM photograph according to the incremental size of endothelial cells and nuclear widths within a distance of 0.5 \( \times \) d along a lengthwise central index line. The endothelial abluminal area, CA (\( \mu m^2 \)), was obtained from:

\[ d (wC_1 + wC_2 + ... + wC_n + wC_n) = d \times WC = LN \times WC/m \]  

(5)

The endothelial volume CV (\( \mu m^3 \)) was obtained from:

\[ d (aC_1 + aC_2 + ... + aC_n + aC_n) = LN \times AC/m \]  

(6)

where AC was the sum of endothelial cell section profile areas \( (AC = aC_1 + aC_2 + ... + aC_n + aC_n) \) appearing in a transverse composite TEM photograph. The endothelial nuclear area \( (NA, \mu m^2) \) was obtained from:

\[ d \times WN = LN \times WN/m, \]  

(7)

where WN was the sum of widths of endothelial nuclei appearing in a composite transverse TEM photograph \( (WN = nN_1 + nN_2 + ... + nN_m + nN_m) \).

Although these assumptions were justified by the findings in canine carotid arteries given in a previous report\(^{19}\) and by the SEM findings in the present experiments, endothelial cells actually showed some variation in angle of orientation, size, and shape. According to the quantitative data on endothelial orientation, size, and shape furnished by Cornhill et al.\(^{1}\) in the casts of rabbit aortas, nearly 95% of endothelial cells were oriented in their long dimension at an inclination within 25° of the axial direction of the vessel, and 100% were oriented within 45°. In the same study, 60% of endothelial cells were found to be 600 to 900 \( \mu m^2 \) in area, and 80% had a width/length ratio of 0.2 to 0.4. If we assume that the data of Cornhill et al. are similar for all large arteries and, therefore, for the canine carotid artery as well, the area obtained from our expression, \( d \times WC \), is no more than 1.12 larger than the actual value, because 0.95/cos 25° and 0.05/cos 45° = 1.12, and \( d \times WC \times 1.12 \) gives the maximum value for the abluminal area. Referring to the histogram (in steps of 100 \( \mu m^2 \)) of endothelial surface areas provided by Cornhill et al., we note that we had more than 100 cut profiles in each of our composite photographs and a sufficient number (7 to 20) of sections to match each histogram step from Cornhill et al. Because the number of cells in each of the histogram steps is expected to correspond to the frequency of occurrence of each of the cut section profiles, our equation for area, \( d \times WC \), will give the mean area of endothelial cells. Similarly, the volume, length, and width of cells obtained from our equations will be mean values. There are no comparable published data for nuclei, but the nuclear deviation angles should be almost the same as for cells. From the literature,\(^ {3,4} \) however, nuclei appear to be uniform in size and shape, and the number we
CONTROL SIDE AT 4 WEEKS AFTER ANASTOMOSIS ARE SIGNIFICANTLY LARGER THAN JUST AFTER ANASTOMOSIS (P<0.005, TPP<0.005). VALUES ON THE SHUNTED SIDE ARE SIGNIFICANTLY LARGER THAN THE CONTROL SIDE (§P<0.001, §§P<0.05). VALUES JUST AFTER ANASTOMOSIS ARE SIGNIFICANTLY LARGER THAN AT 4 WEEKS AFTER ANASTOMOSIS (***P<0.005, §§§P<0.005). VALUES ON THE CONTROL SIDE AT 4 WEEKS AFTER ANASTOMOSIS ARE SIGNIFICIANTLY LARGER THAN JUST AFTER ANASTOMOSIS (###P<0.005, ####P<0.005) OR BEFORE ANASTOMOSIS (#P<0.001, §§P<0.01), OR IN NON-OPERATED CONTROLS (##P<0.01, ###P<0.05).

### Table 1. Hemodynamic Changes in Canine Carotid Arteries after Arteriovenous Anastomosis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Before anastomosis</th>
<th>Just after anastomosis</th>
<th>4 weeks after anastomosis</th>
<th>Nonoperated controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shunted (right)</td>
<td>Control (left)</td>
<td>Shunted (right)</td>
<td>Control (left)</td>
</tr>
<tr>
<td>Internal radius* (cm)</td>
<td>0.157</td>
<td>0.157</td>
<td>0.157</td>
<td>0.157</td>
</tr>
<tr>
<td>Flow rate (ml/min)</td>
<td>154±50</td>
<td>154±48</td>
<td>719±142††††††</td>
<td>187±59</td>
</tr>
<tr>
<td>Flow velocity (cm/sec)</td>
<td>34±12</td>
<td>35±11</td>
<td>156±31††††††††</td>
<td>42±14</td>
</tr>
<tr>
<td>Reynolds number</td>
<td>368±118</td>
<td>371±118</td>
<td>1695±312†††††</td>
<td>449±139</td>
</tr>
<tr>
<td>Shear rate (1/sec)</td>
<td>904±314</td>
<td>921±381</td>
<td>4127±1002†††††††</td>
<td>1096±432</td>
</tr>
<tr>
<td>Shear stress (dynes/cm²)</td>
<td>27±9</td>
<td>28±11</td>
<td>124±30††††††</td>
<td>33±13</td>
</tr>
</tbody>
</table>

All values are means±standard deviation. The Reynolds number is calculated using 0.03 poise for viscosity and 1.054 g/cm³ for specific gravity. Shear rate and shear stress are calculated assuming Poiseuille flow. *The value for internal radius used for computations for arteries before and immediately after anastomosis is shown in parentheses. The value was assumed to be the same as the value measured for the controls at 4 weeks. †Values on the shunted side are significantly larger than before anastomosis (P<0.05 x 10⁻³). ‡Values on the shunted side are significantly larger than the control side (§P<0.001, §§P<0.05). Values just after anastomosis are significantly larger than at 4 weeks after anastomosis (**P<0.005, §§§P<0.005). Values on the control side at 4 weeks after anastomosis are significantly larger than just after anastomosis (###P<0.005, ####P<0.005) or before anastomosis (##P<0.001, §§P<0.01), or in non-operated controls (##P<0.01, ###P<0.05).

### Statistical Analysis

Our results are expressed as the means±the standard deviations of the mean. Statistical analysis was performed by Student's t test. Differences were considered significant if p values were less than 0.05.

### Results

#### Blood Flow

The thrill at the AV anastomoses in the shunted animals persisted in daily examinations throughout the experimental period. The mean blood pressure was 134±12 mm Hg in the seven shunted animals and 130 and 150 mm Hg in the two nonoperated controls. BFR and mean blood flow velocities (U) (U=BFR/60πr²), with the value for lumen radius (r) obtained on the control side at 4 weeks are given in Table 1. Immediately after anastomosis, BFR and U in the shunted arteries were more than four times greater than before anastomosis (719±142 ml/min compared to 187±59 for BFR, p<0.01, and 156±31 cm/sec compared to 42±14 for U, p<0.05). BFR and U in the control arteries at 4 weeks were, however, less markedly, but significantly, elevated compared to values in controls before operation or just after operation (365±110 ml/min compared to 187±59 for BFR and 81±27 compared to 42±14 for U, p<0.005 for both).

#### Shear Rate and Shear Stress

When the shear rate is about 1000/sec, as is normal for many large blood vessels, non-Newtonian behavior becomes insignificant and the apparent viscosity of blood approaches a value asymptotically in the range of 0.03 to 0.04 poise.28,26 By using 0.03 poise for blood viscosity24,25 and 1.054 g/cm³ for blood specific gravity,27 Reynolds numbers (Re) were calculated on the basis of the expression:

\[ Re = 1.054 \times 2 \times U/r^0.03 \]  

(8)

Re was nearly identical in the right and left common carotid arteries before operation (368±118, right and 371±118, left). These values are well below the critical value of 2000 at which turbulent flow occurs in a smooth straight tube. In these arteries, the entrance length for full development of the steady laminar flow profile was calculated to be 3.5 cm, using the expression:

\[ r = 0.03 \times 2 \times Re \]  

(9)

where r is the radius and 0.03 is an experimentally determined constant.28 This computation is valid for Re values between 10 and 2500. The entrance length value of 3.5 cm is much shorter than the location of the segments that form the basis of this report (7 cm on the right and 8 cm on the left). Wall shear rate (WSR) and wall...
shear stress (WSS) for right and left were apparently identical to the levels calculated assuming Poiseuille flow:

$$W_S R = \frac{4(BFR)}{60\pi r^2}$$  \hspace{1cm} (10)

and

$$WSS = 0.03 \, (WSR) \text{ dynes/cm}^2$$  \hspace{1cm} (11)

After anastomosis, Re was high in the shunted arteries, reaching $1695\pm312$ just after anastomosis and $1403\pm289$ at 4 weeks. In one shunted artery, Re was nearly 2300 immediately after anastomosis. The entrance lengths just after anastomosis and at 4 weeks proved to be about 16 cm and 14 cm, respectively. The actual values of WSR and WSS would be expected to be larger than values calculated assuming Poiseuille flow, because the flow profiles were flatter than would be present with a fully established Poiseuille flow. Turbulent flow was likely in the one artery with Re equal to 2300. In control arteries after operation, Re and entrance length were $449\pm139$ and 4.2 cm, respectively, just after operation and $873\pm256$ and 8.2 cm at 4 weeks. In these arteries, WSR and WSS appeared to be identical to the levels calculated, assuming Poiseuille flow. The values for WSR and WSS shown in Table 1 were calculated with an assumption of Poiseuille flow. In the shunted arteries, WSR and WSS were high ($4127\pm1002/$sec and $124\pm30$ dynes/cm²) immediately after anastomosis and were still at a high level ($2919\pm386$/sec and $88\pm12$ dynes/cm²) at 4 weeks. In the control arteries, WSR and WSS were not significantly changed immediately after operation, but were moderately elevated at 4 weeks ($2136\pm876$/sec compared to $1096\pm432$ for WSR, $64\pm26$ dynes/cm² compared to $33\pm13$ for WSS; $p<0.005$ for each).

Morphologic Features

The endothelial layer was relatively thick, with nuclei protruding prominently from the lumen surface in the shunted arteries (Figure 1A), while the endothelium was flat and thin on the control side (Figure 1B). The intercellular junctions were much more numerous in the shunted arteries (Figure 2A) than in controls (Figure 2B). Endothelial cells on transverse sections of shunted arteries were much narrower than on the control side. Examination by TEM revealed the cells to be radially thickened, closely packed, and circumferentially narrowed in the shunted arteries (Figure 3A and Figure 4). The nuclear contours were round and protruded markedly into the lumen on the transverse sections, and the cell surfaces showed occasional microvilli. The endothelial cells and nuclei on the control side were flat (Figure 3B and Figure 4). The intercellular junctions of shunted arteries were long and straight on the transverse section (Figure 5A), while those of control arteries were short and convoluted (Figure 5B). Viewed by SEM, endothelial cells in the shunted artery appeared slender with rounded, protruding surfaces in their midportions (Figure 6A). Microvilli were observed at the tips of these protrusions. Control arteries had relatively flat surface contours and few microvilli (Figure 6B). In shunted arteries, endothelial cells occasionally showed a constricted or cleaved nucleus, and some cells were binucleate (Figures 7A and 7B). Pairs of endothelial cells of nearly identical shape were occasionally arranged symmetrically in relation to sharp crevices (Figures 7C and 7D). In the nonoperated controls, the endothelial cells were flat and did not differ from the controls in light microscopic or ultrastructural features. In the right common carotid artery of the control sham-shunted animals, the endothelial cells were flat and did not differ in light microscopic or ultrastructural appearances from nonoper-
Figure 3. Transmission electron microscopic views of the endothelial cell layer of canine carotid arteries. A. Shunted artery. B. Control artery. Endothelial cells are thickened, and nuclei protrude markedly on the shunted side, while the cells are thin and flat in the control artery. i=intercellular junction, n=nucleus. ×3000

Figure 4. Diagrammatic representation of the endothelial layer of canine carotid arteries on transverse section. wC=abluminal endothelial cell width, aC=endothelial cross-sectional area, wN=endothelial nuclear width, aN=endothelial nucleus cross-sectional area.

Morphometry

The histometric data on endothelial cell density are given in Table 2. All five quantitative determinations revealed significant increases in the shunted arteries compared to the contralateral control arteries and to the nonoperated controls. The total number of endothelial cell nuclei on the transverse light microscopic sections of the shunted arteries was about 1.5 times greater than that on the control side (488±69 nuclei compared to 317±40, p<0.001). Similarly, endothelial cell density of shunted arteries determined from the light microscopic and TEM material was 1.5 times higher than in the controls (p<0.001 and 0.0001, respectively). On SEM, the endothelial cell density of shunted arteries was 6.15±0.68×10⁵ cells/mm², which was 1.64 times greater than that of control arteries (3.33±0.20×10⁵ cells/mm², p<0.001).

Endothelial cells lining the shunted arteries were narrow and thick, in marked contrast to the wide and thin configuration in the controls. The dimensions of the endothelial cells are presented in Table 3. The endothelial cell density (4.1×10⁵ cells/mm²) as computed from the abluminal endothelial cell area in the shunted arteries (241±45 μm²) was slightly lower than that obtained from SEM determinations (Table 2). This discrepancy is likely to be due to differences in tissue shrinkage during processing, because the ratios of endothelial cell density in shunted arteries to that of controls obtained by the two different methods were almost identical. The endothelial cell volume in shunted arteries was 0.77 times that of the controls (p<0.05), while the volume relative to nonoperated controls was somewhat smaller. The average thickness of endothelial cells was 1.37 μm in shunted arteries, 1.00 μm in the controls, and 0.82 μm in the nonoperated controls. Although the average nuclear volume was the same for shunted and control vessels, the shape of endothelial cell nuclei in shunted arteries was prolate-spheroid and that in control arteries, oblate-spheroid (Figure 8). The average nuclear volume of the nonoperated control vessels was somewhat smaller than that for operated animals. The average dimensions of endothelial cells on the silver-stained preparations as studied by SEM were 49±6 μm for length, 4.9±0.6 μm for width, and 197±26 μm² for surface area in the shunted arteries; 79±7 μm for length,
Figure 5. Endothelial intercellular junctions (i) in the canine carotid arteries. A. Shunted artery. B. Control artery. Intercellular junctions in the shunted artery are long and simple, while those of control arteries are short and irregular. X16000

8.6±1.4 μm for width, and 418±72 μm² for surface area in the controls.

Discussion

In 1893, Thoma proposed that blood flow velocity was intimately related to the growth and dilation of blood vessels. More recently, Kamiya and Togawa showed that the increased blood flow in a canine AV shunt model resulted in an increase in radius of the carotid artery corresponding to a wall shear stress level of about dynes/cm². Zarins et al. demonstrated that an increase in radius resulted in the reestablishment of baseline wall shear stress of about 15 dynes/cm² in the monkey iliac artery 6 months after anastomosis to the iliac vein. Conversely, Guyton and Hartley showed that flow restriction inhibited the normal increase in carotid artery diameter during growth in the rat. It has, therefore, been suggested that the internal radius of arteries is altered in response to changes in flow so as to maintain a constant wall shear stress. The mechanisms underlying wall shear stress-dependent arterial growth and enlargement are not fully understood, nor is the sequence of morphologic changes that correspond to this apparent adaptive process well defined. In their experiments, Kamiya and Togawa found that more than 6 months were required for the canine carotid artery lumen diameter to increase sufficiently to reestablish the baseline shear stress, but that lumen diameter had not changed by 1 week after AV shunting. Early diameter changes could not be followed as easily in the dog as in the monkey, because the canine carotid artery diameter changed much less than the monkey iliac artery.
The observations of Kamiya and Togawa and those of Masuda et al. suggest that immediately before a significant increase in carotid internal diameter (i.e., at 4 weeks) endothelial cells become very active. The present experiments were, therefore, designed to characterize and quantify the morphologic changes of endothelial cells in the flow-loaded canine carotid artery at 4 weeks, the onset of arterial dilation. Since the interaction between wall shear stress and the artery wall occurs mainly at the blood-endothelial interface, our objective was to further define the role of the endothelial cell in shear stress-dependent arterial growth and dilation.

In our experiments, WSS increased sharply immediately after anastomosis and remained elevated during the 4-week experimental period on the shunted side. On the control side, WSS was unchanged immediately after shunting and was only moderately elevated at 4 weeks. This elevation was probably due to the collateral flow through the circle of Willis from left to right because blood pressure in the right common carotid artery distal to the AV anastomosis was presumably decreased. WSS on the shunted side was, however, always much higher than on the control side. Blood flow profiles on the shunted side were apparently flatter and flow was possibly turbulent, while on the control side, the Poiseuille flow was maintained.

We found that the endothelial cells in the shunted arteries were closely packed and radially thickened, protruding markedly into the lumen at 4 weeks. Endothelial cell density was elevated, and the abluminal transverse width of the cells was markedly narrowed. The nuclei on the shunted side were prolate-spheroid but no greater in volume than those on the control side. Endothelial cell volume per unit of lumen surface area was about 1.4 times greater on the shunted side than on the control side, and the volume of the endothelial cell nuclei per unit lumen surface area was 1.8 times larger in
Figure 7. Higher magnification of scanning electron microscopic views of endothelial cells of shunted arteries show a series of changes that suggest mitotic activity. A. A nuclear constriction. B. A dumbbell-shaped nucleus. C and D. Endothelial cells arranged symmetrically with an intervening slit or crevice. Bar=5 μm. ×4800
Table 2. Endothelial Cell Density

<table>
<thead>
<tr>
<th></th>
<th>Light microscopy</th>
<th>Electron microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lumen circumference (mm)</td>
<td>Total nuclei per section</td>
</tr>
<tr>
<td></td>
<td>8.26±0.64</td>
<td>488±69‡</td>
</tr>
<tr>
<td>Shunted (n=7)</td>
<td>7.91±0.91</td>
<td>317±40</td>
</tr>
<tr>
<td>Control (n=7)</td>
<td>8.67±1.37</td>
<td>277±38</td>
</tr>
<tr>
<td>Nonoperated control (n=4)</td>
<td>8.07±1.37</td>
<td>263±28</td>
</tr>
</tbody>
</table>

All values are means±standard deviation. Values on the shunted side are significantly larger than on the controls: $p<0.01$, ‡$p<0.0001$, and §$p<0.0001$ compared to operated controls; **$p<0.01$, ††$p<0.005$ compared to nonoperated controls.

Table 3. Dimensions of Endothelial Cells and Nuclei

<table>
<thead>
<tr>
<th></th>
<th>Cells</th>
<th>Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (µm)</td>
<td>Width (µm)</td>
</tr>
<tr>
<td>Shunted (n=6)</td>
<td>77±13‡</td>
<td>7.9±1.1*§</td>
</tr>
<tr>
<td>Control</td>
<td>97±17**</td>
<td>11.2±0.9</td>
</tr>
<tr>
<td>Nonoperated control (n=4)</td>
<td>66±10</td>
<td>12.8±1.0</td>
</tr>
</tbody>
</table>

All values are means±standard deviation. Values in the shunted arteries are smaller than in controls: *$p<0.01$, ‡$p<0.001$, and §$p<0.001$ compared to operated controls, ||$p<0.01$, ††$p<0.05$ compared to nonoperated controls. Values in operated controls are larger than in nonoperated controls, **$p<0.01$, †††$p<0.005$ compared to nonoperated controls.

Figure 8. Representations of endothelial cells of shunted and control arteries. The upper pair are shunted on the high wall shear stress side. The lower pair are the control side with only a slight increase in wall shear stress. The drawings are derived from computer-assisted histograms of the morphometric studies obtained from the composite transmission electron micrographic (TEM) photographs. The lower drawings of each pair were obtained by simple smoothing by eye of the block-like drawings after superimposing scanning electron microscopic and TEM findings.
shunts. After intraperitoneal injection of BrdU (50 mg/kg), we found that 5% to 10% of the endothelial cells on the shunted side had incorporated BrdU at 2 weeks after the operation, while less than 1% of the cells on the control side had incorporated BrdU. At 4 weeks, we found that 20% of the endothelial cells on the shunted side had incorporated BrdU compared to less than 1% on the control side. A complete 6-month time-course study is underway and will be published in detail. On the basis of the findings reported in this communication, we conclude that endothelial cells proliferated on the shunted side but were crowded along the arterial circumference, which had not yet become sufficiently enlarged to accommodate the cells in a normal configuration. The preliminary data of the BrdU studies provide additional support for a proliferative effect.

Although there have as yet been no other reports in which endothelial cell density or replication have been related to wall shear stress in vivo, several reports that suggest that blood flow affects endothelial cell activity have appeared. These include local differences in aortic endothelial cell turnover, increased mitotic activity of aortic endothelial cells about branchings in normal guinea pigs, and more rapid regeneration of endothelial cells in the axial direction than about the circumference in the rat after denudation. Increased endothelial cell density after 40 days of experimental hypertension and after a sharp rise of thymidine index at 7 to 10 days has been observed in the rat aorta. Increase of endothelial cell density also occurred after experimentally induced stenosis of the rabbit aorta, as well as increased mitotic activity proximal to experimental aortic coarctation in guinea pigs. Low shear stress, turbulence, altered flow velocity, and pressure were considered to be the underlying hemodynamic stimuli in these reports.

In vitro studies have also indicated that endothelial cells are activated by hemodynamically related stresses, including elevated wall shear stress. Ando et al. suggested that endothelial migration and proliferation could be elicited by increased shear stress with no relationship to pressure, pulsation, or chemical growth factors. Davies et al. demonstrated that turbulent stress induced vascular endothelial turnover, while Levesque and Nerem found that, compared to steady-flow shear stress, pulsatile shear stress produced an enhanced proliferative response in subconfluent endothelial cell monolayers. These findings tend to indicate that wall shear stress elicits endothelial cell responses under a variety of differences in pattern of shear development (i.e., steady, turbulent, or pulsatile).

Kamiya and Togawa suggested that wall shear-stress-dependent enlargement is induced by a humoral factor entering the media from the circulation and that the entry of this factor into the artery wall is modulated by wall shear stress. Others have proposed that proliferating endothelial cells have leaky junctions. Our demonstration of straight simple junctions between endothelial cells just before the onset of artery enlargement would tend to support the suggestion of Kamiya and Togawa, as well as the possibility that endothelial cell proliferation results in increased permeability due to leaky junctions.

Vasoactive agents, such as endothelial-derived relaxation factor and endothelial-derived constriction factor, may be released in response to wall shear stress. These agents may also mediate the more chronic type of wall shear-stress-dependent arterial adaptive enlargement that forms the basis of the present study. An appropriate time-course study of these effects, as well as the corresponding changes in the media at both the subacute and chronic phases of the response, should help to illuminate the mechanisms by which wall shear stress modulates or mediates flow-dependent arterial growth and dilation.

Fluid dynamic factors, and wall shear stress in particular, have also been shown to be determinants of plaque localization in athrogenesis. Endothelial damage was induced by markedly elevated experimental wall shear stress, and shear stress-related injury has been proposed as the basis for plaque localization. Zarins et al. found, however, that intimal thickening and atherosclerosis were localized in low shear regions of the human carotid bifurcation and that increased flow velocity inhibited experimental atherogenesis. Ross et al. suggested that endothelial injury, including possible focal desquamations related to altered wall shear stress, may eventuate in smooth muscle cell proliferation and that this effect may be mediated by growth factors derived from platelets, endothelial cells, and macrophages. The observations reported in the present report indicate that endothelial cells proliferate and change size and configuration in response to increased wall shear stress before the onset of adaptive enlargement. The mechanisms by which fluid dynamics influence the induction and evolution of atherosclerosis may be closely related to those that determine vessel enlargement, including the adaptive enlargement and modelling that accompanies early human athrogenesis. Further investigations of both phenomena should eventually provide clinically useful insights into the adaptive potential of arteries in health and disease.

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References

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