Injury and Repair of Endothelium at Sites of Flow Disturbances Near Abdominal Aortic Coarctations in Rabbits

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The acute and chronic effects of flow disturbances on arterial endothelium were studied by locally constricting the diameter of the rabbit abdominal aorta by 62% ± 2.4% (mean ± se). This procedure produced a region of elevated shear stress immediately upstream from the coarctation. A region of small irregular vortices was formed in the first 5 to 7 mm downstream, whereas an annular vortex was observed in the region from 2.0 to 2.5 cm further downstream. Morphologic changes to the endothelium near these coarctations were assessed by scanning electron microscopy; endothelial cell replication rates as a function of time after coarctation were monitored with 3H-thymidine. These studies established that profound alterations in endothelial cell shape caused by changes in local flow conditions immediately downstream from coarctations are primarily the result of alterations in morphology of pre-existing cells, rather than the proliferation of new cells with altered morphology. Definitive morphologic evidence of injury to endothelium was not seen at any sites after coarctation.

Indeed, any early injury to endothelium caused by the flow disturbances was not sufficiently severe to cause a significant increase in endothelial cell turnover rate during the first week of coarctation. On the other hand, we observed a major increase in cell turnover (over 100-fold) by 30 days after coarctation at sites immediately upstream and immediately downstream of the coarctation. This finding suggests that flow-induced trauma can ultimately injure the cell sufficiently to cause cell death if the source of injury is persistent. Finally, we demonstrated that the high shear stress immediately upstream from the coarctation and the secondary flow disturbances immediately downstream can retard migration of endothelial cells into sites of injury, whereas repair was enhanced in the region of the primary annular vortex. These findings suggest that specific flow conditions, possibly characterized by relatively low and unidirectional shear forces, optimize migratory repair of endothelium. In conclusion, this study indicates that hemodynamic factors may influence the distribution of atherosclerotic lesions both by contributing to endothelial injury and by influencing endothelial repair. (Arteriosclerosis 6:146–154, March/April 1986)
to lesion formation by influencing endothelial repair, regardless of the mechanism of injury. To assess this hypothesis, repair of a well-defined aortic endothelial injury was monitored near sites of flow disturbances in the vicinity of coarctations.

**Methods**

**Experimental Design**

Experiments were performed on male New Zealand White rabbits weighing between 2.5 and 3.5 kg. Before surgery, rabbits were premedicated with 0.7 to 1.0 ml Innovar-Vet (0.4 mg/ml fentanyl, 20 mg/ml droperidol) then anesthetized with 3.0 to 6.0 mg/kg sodium pentobarbital (Somnotol). The midabdominal aorta was exposed by a midline incision. A length of 000 silk suture was passed under the aorta 5 cm caudal to the origin of the left renal artery and tied so as to constrict the aorta to slightly over 50% of its original diameter. Then the incision was closed.

**Assessment of Flow Conditions near Coarctations**

**Measurement of Flow Velocities by Pulsed Doppler Flowmetry**

Flow conditions near coarctations were assessed in three acute and two chronic (2-week) preparations with a pulsed Doppler ultrasonic flowmeter (Bioengineering, University of Iowa, model 545C-4, Iowa City, Iowa). Pulsed Doppler devices use crystals that are mounted on the blood vessel wall and that emit a brief ultrasonic signal, and that then switch to a receiving mode to pick up the reflections of the outgoing wave from cellular components of flowing blood. The velocity of the blood cells causes a Doppler shift in the frequency of the reflected signal which is related to the velocity of the cells by the equation:

\[ v = \frac{1}{2} c f_0 \cos \theta \]

where \( v \) is the velocity of sound in the fluid, \( f_0 \) is the transmitted frequency, \( \Delta f \) is the Doppler difference frequency, and \( \theta \) is the angle between the acoustic axis of the signal and the direction of flow. By varying the time between emission and reception at the crystal, the flow at different sites across the vessel wall can be monitored. Our device has a "range" control that adjusts the distance from the crystal (hence the depth within the vessel) from which flow velocity is being sampled.

We placed a blood vessel cuff containing a crystal oriented at 45° to the vessel axis around the aorta so that the crystal was 0.5 cm upstream from the coarctation. A second cuff positioned three crystals at 0.5, 1.5, and 2.5 cm downstream from the coarctation (Figure 1). All crystals were directed upstream. Each crystal was used to repeatedly sample velocities across the aorta before and after coarctations were applied. Abdominal incisions in rabbits used in chronic studies then were closed, and the wires attached to the crystals were routed to the nape of the neck. Two weeks later the rabbits were reanesthetized and these wires were brought to the exterior through a small neck incision. The incision was closed and blood velocity recordings were made before and after recovery from anesthesia. Findings from acute and chronic preparations were similar, although the results were most consistent and reliable in the chronic, awake animals.

Blood velocity determinations are ultimately limited by the resolution and accuracy of the probes. Our flowmeters sampled and averaged velocities from a volume of blood of about 1 mm³ and this ultimately limited the spatial resolution of our velocity determinations. Accuracy of velocity measurements is primarily limited by how well the angle of probe orientation is defined with respect to flow direction. Simultaneous, quantitative determinations of velocity (Equation 1) for the region near the vessel axis yielded very similar results from all four crystals before coarctation, a finding that suggests that the 45° orientation of the probe consistently was achieved.

**Predicted Flow Patterns and Rationale for Probe Placement**

Theoretical analyses and studies of hydraulic models or in vivo experiments have yielded results pertaining to the nature of flow near arterial coarctation. We have integrated the results of these studies to predict flow patterns near our coarctations. These predictions are illustrated schematically in Figure 1. The placement of velocity probes described above and shown in Figure 1 was chosen to most rigorously assess the predicted flows.

Three distinct zones of abnormal flow about the coarctation site were predicted. Zone I included approximately 5 mm immediately upstream from the coarctation where flow was funneling into the site of constriction. Stable, laminar flows were predicted here because the mean Reynolds number in the normal rabbit aorta is about 250, well below the value that elicits turbulence in blood vessels (800–2000) and because converging channels are known to stabilize flows. However, the increased velocities in the converging flow will increase wall shear in this region. Quantitative estimates of these shears for pulsatile flow are not available, but shear in such regions increases as the inverse cube of the vessel radius for steady, laminar flow.

Blood leaving the coarctation has too much momentum to follow the rapidly expanding geometry of the aorta at this site. Previous model studies indicate that a slowly expanding funnel of high velocity fluid traps an annulus of fluid downstream from the coarctation. The shear forces transmitted to this region from the center stream flow initiate rotational flow so that an annular vortex is formed (Zone III, Figure 1). For a mean Reynolds number of about 250 prevailing in the rabbit abdominal aorta, this vortex should extend 6 to 7 aortic diameters downstream (vortex...
length extrapolated from the data of Macagno and Hung\textsuperscript{8}). Karino and Goldsmith\textsuperscript{h} have shown that pulsatile flow simply causes the vortex length to oscillate around the length predicted for mean flow.

In idealized systems, the annular vortex should extend upstream to the coarctation site.\textsuperscript{6,8} However, modest geometric irregularities at and around the coarctation site may produce small flow disturbances in the region immediately downstream from the coarctation. The existence of these disturbances appears to be confirmed in this and previous studies by the organization of endothelial cells, which are always aligned in the direction of time-averaged flow, into swirling vortex-like patterns in the 5 to 7 mm immediately downstream from the coarctations (Zone II, Figure 1). We will refer to such disturbances as secondary vortex formation.

The flow patterns illustrated in Figure 1 permit the following specific predictions (these predictions were testable by probes positioned as described above):

1. The mean centerline velocities at all sites above, through, and below the coarctation should be directed downstream. The velocities should be maximal at and near the coarctation site.
2. The velocities at all sites above the coarctation and below the site of re-attachment of flow beyond the primary annular vortex should be directed downstream.
3. In the region of the primary vortex, the velocities near the wall should be directed upstream, whereas those near the centerline should be directed downstream.
4. In the region immediately downstream from the coarctation, significant velocities should be detected only near the axis of the vessel since secondary vortices are probably characterized by low velocities.

**Endothelial Responses to Coarctation**

Rabbits were killed 0.5, 1, 3, 7, 14, or 30 days after coarctation by a barbiturate overdose. The thoracic aorta was cannulated immediately after sacrifice and the abdominal aorta was perfused with 1% glutaraldehyde, 1% paraformaldehyde in phosphate buffer under a pressure of 100 mm Hg. The right atrium was opened to facilitate perfusion. After at least 1 hour, the abdominal aorta was excised and immersed in fixative for 24 hours. Very narrow, longitudinal injuries were made along the complete length of the aorta by a barbiturate overdose.

Endothelial cell replication rates near the coarctations were assessed in four groups of rabbits (three per group) killed at 0, 2, 8, and 30 days after surgery. \(^{3}H\)-Thymidine (1.67 mCi) was injected intravenously at 17, 8, and 1 hour before the animals were sacrificed, and the tissue was fixed as described above. Standard Hautchen preparations for counting labeled endothelial cells cannot be prepared from surfaces with uneven topology like coarctations; therefore, the "whole mount" method of Reidy and Schwartz\textsuperscript{13} was employed. Briefly, the fixed abdomin aorta was opened longitudinally, was pinned flat on Teflon and was dehydrated and dried as described above. The specimens were then dipped in radiographic emulsion (Kodak NTB-2), were allowed to dry, and were stored in darkness for 2 weeks. Tissue was then developed, dried, and mounted on SEM stubs, and coated with gold for viewing by SEM. Labeled endothelial cell nuclei are clearly visible by SEM using this technique.

The percentage of cells labeled with \(^{3}H\)-thymidine was determined in fields measuring 1100 \(\mu\text{m} \times 1500 \mu\text{m}\). All fields around the circumference of the vessel were counted starting from immediately below the coarctation and continuing downstream until scans of two consecutive circumferences yielded no labeled cells. The procedure was then repeated moving upstream from the coarctation.

**Repair of Pre-Injured Endothelium at Aortic Coarctations**

Very narrow, longitudinal injuries were made along the complete length of the aorta by using a modification of the technique of Reidy and Schwartz.\textsuperscript{13} The endothelium was injured with the device illustrated in Figure 2. This consisted of two strands of 000 monofilament nylon bonded together with cyanoacrylate glue 6 mm from their tip and advanced through a length of PE 50 tubing. Prior to insertion in the PE tubing, each nylon strand was bent at the point of bonding so that their tips splayed apart when advanced past the end of the catheter.

Rabbits were anesthetized, and the abdominal aorta was exposed as described above. The aorta was clamped just upstream from the iliac bifurcation. The catheter described above was inserted into the aorta via a small puncture wound distal to the clamp, with the nylon strands withdrawn so that their tips were flush with the catheter tip. The clamp was removed, and the catheter was advanced to the level of the diaphragm. The nylon strands were advanced...
about 8 mm, thus their tips separated outside the catheter and came into contact with the aortic wall. The catheter was withdrawn at a steady rate so that the nylon strands made fine longitudinal wounds along the aorta. The puncture wound at the point of insertion was closed with a No. 5-0 surgical suture. Finally, after wounding of the aorta, a coarctation was placed at the midabdominal aorta as described above.

Some preliminary studies used a single length of nylon to injure the endothelium. The above technique generally produced more consistent injury, presumably because each strand prevented the other from leaving the endothelial surface.

Assessment of Wound Repair

Wound healing rates were assessed by measuring the width of the wound that persisted at various times after injury. Initially, this was done by measuring the wound width from SEM images of fixed tissue. However, fixation, dehydration, and drying causes marked tissue shrinkage, and it is possible that differential shrinkage at coarctations will distort the tissue and introduce systematic errors in wound measurement. To avoid such errors, we employed the vascular casting technique of Reidy and Levesque for examining negative impressions of the intimal surface of the arteries. This technique preserves the in vivo geometry of unfixed vascular tissues.

Rabbits were killed by anesthetic overdose at various times after endothelial injury and aortic coarctation. Immediately after sacrifice, the thoracic cavity was opened and the aorta was cannulated with a short (7 cm) wide-bore (4 mm) Tygon tube. A femoral artery was cut, and the aorta was flushed with physiological saline. Silver nitrate (50 ml, 0.25%) was then flushed through the aorta for 30 seconds before a second saline flushing. This staining procedure distinguishes from normal midabdominal aortic flows in animals without coarctation. This insensitivity to coarctation of flows upstream from the region where the vessel wall were low when compared with the centerline velocities at all levels above and below the coarctation, because of the drag exerted by the wall. The comparisons among velocities near the wall were limited to the direction of flow (forward vs reverse) since large velocity gradients at these sites make quantitative comparisons of velocity too dependent on radial position. In contrast, quantitative comparisons of well-defined centerline velocities were possible because very blunt velocity profiles are observed in vivo.

Blood velocities measured just 0.5 cm upstream from the coarctation (Figure 3 A and B, top trace) were indistinguishable from normal midabdominal aortic flows in animals without coarctation. This insensitivity to coarctation of flows upstream from the region where the vessel wall were low was confirmed in acute experiments. These flows were little affected by repeatedly removing and repositioning the coarctation.

The probe positioned 0.5 cm below the coarctation consistently recorded a higher mean centerline velocity (Figure 3 B) than the upstream probe. The blood velocities near the vessel wall were most often positive at this level, although flow reversal throughout systole was occasionally observed.

The mean centerline velocities decreased between 0.5 and 1.5 cm downstream from the coarctations presumably because the jet of blood exiting from the coarctation (Figure 1) had started to expand by this point. The flow reversal near the wall was always observed at this point (systolic blood flow was below zero, i.e., directed upstream, in Figure 3 A, third trace), a finding that strongly implies that the annular vortex shown in Figure 1 was consistently produced by the coarctation. At 2.5 cm downstream from the coarctation, the mean centerline velocities had returned to upstream levels. This velocity shown in Figure 3 is actually below the upstream levels, probably because of a slightly
Figure 4. A. Low-power scanning electron micrograph of the coarctation. The vessel has been opened longitudinally so that the luminal surface is visible. B. Micrograph of plastic cast of aortic coarctation. Same magnification as A. Direction of blood flow is from left to right in this and succeeding figures, unless otherwise stated. Bar = 100 μm.

Figure 5. A. Scanning electron micrograph of endothelial cell morphology typical of sites distant from the coarctation. Cells resemble endothelium from aortas of control animals. B. Elongated cells seen 0.5 mm upstream of the middle of the coarctation 24 hours after surgery. Same magnification as A. C. Typical endothelial cell morphology seen 0 to 5 mm downstream of the coarctation from 3 days after surgery. Markers are 10 μm.

wider vessel cross section at this point. Flow reversal near the vessel wall was observed in most, but not all cases. On several occasions, the flow at the wall changed from reverse to forward and, at the same time, the probe located 0.5 cm downstream from the coarctation recorded a change from forward to reverse flow. This observation suggests an annular vortex that may vary in length and position according to vascular regulatory adjustments.

Taken together, these recordings indicate that coarctation had a major effect on downstream blood velocities. Specifically, the findings are consistent with the formation of an expanding jet of blood leaving the coarctation and the establishment of an annular vortex outside of the jet that extends from about 0.5 to about 2.5 cm downstream from the coarctation. The vortex caused reversed flow near the wall. On most occasions, this reversal of flow did not penetrate the 0.5 cm immediately downstream of the coarctation, where endothelial cell morphology implies irregular secondary-vortex formation.

En Face Endothelial Morphology and Cell Proliferation

Figure 4 A is a low-power SEM view of a coarctation site that has been opened, pinned flat, and dried by the critical-
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point method. Figure 4 B is a similar view of a plastic cast of the coarctation region. The measurements from vascular casts prepared at physiological pressures indicated that our coarctations reduced aortic diameter by 62.0% ± 2.4% (mean ± se, n = 9). The influences of coarctation on endothelial cell shape and orientation were usually restricted to sites that were within 0.5 cm of the constriction. Thus, cells at the upstream (left) and downstream (right) margin of this region exhibited surface morphology that resembled normal arterial endothelium, i.e., the cells were elongate and oriented along the long axis of the blood vessel (Figure 5 A). The morphology of cells closer to the coarctation was altered as early as 12 to 24 hours after surgery. Cells immediately upstream from the constriction remained oriented along the long axis of the vessel but were narrower and almost twice as long as cells far from the coarctation (Figure 5 B). At this time, no changes were noted in cells overlying, or downstream, of the coarctation. By 3 days after surgery, cells in the area 2 to 3 mm downstream from the coarctation exhibited the marked changes in cell orientation that have been described previously. Cells throughout this region were generally less elongate than in control vessels and they were organized into swirling patterns suggestive of the vortex formation anticipated in this region (Figure 5 C). By 7 days after surgery, this region usually extended 5 mm downstream from the coarctation, with occasional involvement of sites up to 7 mm downstream. Surface morphology stabilized after this time. Marked changes in the shape or orientation of cells immediately over the coarctation were not observed by these methods. Furthermore, no morphological evidence of injury or denudation of the endothelium at or near the coarctation was observed by SEM. Cells more than 7 mm downstream from the coarctation were not distinguishable from normal abdominal aortic endothelium.

Cell Replication near Coarctations

Figure 6 A shows the typical appearance of a replicating endothelial cell when processed for whole-mount autoradiography. Exposed silver granules are clearly visible over the cell nucleus. Labeled endothelial cells were very rarely seen at sites distant from coarctations, and endothelial cell replication rates were less than 0.01% per day, a finding consistent with previous measurements of turnover of endothelium under normal conditions. Replication rates remained low immediately downstream from the constrictions for 1 week after surgery (Figure 7). However, focal areas of high cell turnover rates were seen in this downstream region 30 days after coarctation (Figure 6 B), and the mean replication rate over this area rose by over 100-fold to 1.5% per day in this time (Figure 7). This zone of high turnover rate extended to approximately 5 mm downstream from the site of coarctation. A chronic elevation of cell turnover rates was also observed immediately upstream from the coarctation (Figure 7), with cell replication reaching 2.8% per day by 30 days after surgery. The zone of elevated replication extended approximately 5 mm upstream from the site of coarctation.

Endothelial Cell Migration Into Wounds at Coarctation

Figure 8 A shows the typical appearance of a longitudinal wound to endothelium. The injuries had well-defined
boundaries and were covered with platelets and an occasional adherent white cell. Although the initial wound width varied from animal to animal, presumably because of variability in the tip of the nylon filament used to scratch the vessel, the initial wound width varied very little along the aorta under control conditions.

Examination at various times following injury revealed that wounds were covered through migration of adjacent cells over a period of about 24 hours at sites distant from flow disturbances (Figure 8 B). Typically, partial covering of wound area was seen at 12 hours (Figure 8 C); consequently, this time period was used to assess the influence of flow disturbances on this aspect of endothelial repair.

Figure 9 shows the mean width of the wound that persisted 12 hours after injury at sites upstream, through, and downstream from coarctation applied immediately after the injury was made. The wound was widest (repair slowest) in the high shear region just upstream from the coarctation and in the region of disturbed flow just downstream from the coarctation. At both sites, wound width significantly (p < 0.05) exceeded that observed in control regions (sites more than 10 mm upstream of the coarctation). This inhibition of repair was largely, but not totally, restricted to the region where the aorta had been narrowed by the coarctation (see broken line, Figure 9). Repair inhibition is probably underestimated by the current technique since constriction of the aorta will reduce wound width near the coarct site.

The narrowest wounds (most rapid repair) were observed at sites 10 to 25 mm downstream of the coarctation,
where the annular vortex imposes a slow reverse flow near the vessel wall. Hence, wound repair significantly exceeded that seen in control regions in this zone.

Discussion

The blood flow measurements and observations of the flow-induced alignment of endothelial cells were consistent with flow patterns predicted from previous theoretical and model studies. We conclude that our coarctations induced a region of high shear stress immediately upstream from the coarctation (Zone I), a region of secondary vortex formation immediately downstream, (Zone II), and an annular vortex that extended to 2 to 3 cm downstream from the coarctation (Zone III). Further studies indicated that the flow conditions peculiar to each of these zones had a marked effect on endothelial function and/or morphology.

Morphologic changes in Zone I occurred by 12 hours after coarctation. These changes involved an exaggeration of the elongate shape of the cells. This elongate shape depends on local flow conditions; it is not characteristic of cells grown in static culture or under conditions of zero flow in vivo. Therefore, it is likely that elevation of shear stresses, due to funneling of blood flow into the constricted area, enhances this elongation.

We previously showed that endothelial cells immediately downstream from abdominal aortic coarctations in rabbits (Zone II) lose their well-defined alignment with the long axis of the vessel and become organized into patterns suggestive of the flow disturbances expected in this region. The current study confirms this finding (Figure 5C). Since it is the long-term, time-averaged blood flow that orients endothelial cells, it is likely that cell morphology in this region reflects secondary vortex formation produced by the coarctation geometry. Previous studies did not establish whether the altered cells just downstream from coarctations were pre-existing cells that were modified by changes in the local hemodynamic environment or whether the hemodynamic changes caused cell death and then proliferation of new cells with altered morphology. The current study shows that major realignment of these cells occurred within 3 days, whereas endothelial cell turnover rates remained very low in this region for the first week after coarctation. By this time, fewer than 2% of the cells had gone through cell division. These data indicate that cell reorientation in vivo is due predominantly to reorientation of pre-existing cells. This finding is in agreement with data on flow-induced reorientation of endothelial cells in culture except that our study indicates that endothelial cells may reorient more slowly in vivo.

No marked morphologic changes to the endothelium were observed in Zone III. Here the annular vortex imposes shear forces that consistently are aligned axially, albeit in the upstream direction. Therefore, it is not surprising that endothelial cells exhibit normal orientation in this region.

Chronic exposure to flow anomalies in Zones I and II induced cell injury, as indicated by the 100-fold increases in cell replication rates after 30 days, although cell turnover occurred without detectable denudation. This is consistent with our previous finding that even very high hemodynamic stresses do not denude endothelium. Non-denuding injury in Zone I is probably attributable to chronic shear elevation, but this explanation is unlikely in Zone II. The high velocity, mainstream flow separated from the vessel wall at the coarctation and did not reattach for more than 2 cm; thus, a volume of blood with low velocity and exerting low shear was trapped near the wall in Zones II and III. Zone II is unique in that shear direction varies from instant to instant during the cardiac cycle and also with regulatory adjustments in flow. Thus cells are only transiently exposed to shears that are aligned with the long axis of the cell. If this feature of flow underlies the cell injury seen in this region, then it suggests that the consistent alignment of cells with the flow seen at most arterial sites has an adaptive significance. It is noteworthy that large arterial branch sites also exhibit only transient alignment of instantaneous flow along endothelial cell axes. Endothelial cell replication rates are elevated at these sites which are also prone to atherosclerotic lesion development.

If endothelial trauma is a primary initiator of atherogenesis, then the local distribution of lesions may be linked to mechanisms of endothelial repair as well as injury. In this study, flow conditions in Zones I and II inhibited the repair of injury to endothelium. These regions also exhibited evidence of cell injury, as indicated by elevated cell turnover rates when coarctations were chronic. Therefore, it is possible that inhibition of repair is caused by cell dysfunction secondary to hemodynamic injury. Alternatively, flow influences cell cytoskeleton, and the critical role of cytoskeletal rearrangement on cell migration may account for our observations.

Repair of endothelial injury was enhanced in the region of the annular vortex downstream from the coarctation. It is not yet clear why flow conditions in this region favor cell migration into wounds. Shear stresses are modest and remain aligned with the cells when blood flow rate varies. Again, an influence of flow on endothelial cell cytoskeleton may account for our observations. There is also evidence from the model studies of Karino and Goldsmith that blood cells and platelets migrate from such annular vortices, leaving a region of reduced hematocrit, which could influence endothelial cell function. We did not test for such localized hemodilution in our in vivo studies.

In summary, the morphologic changes in the endothelium near sites of coarctation are not contingent upon cell turnover, but chronic coarctation does cause elevated cell...
turnover in the high-shear region immediately upstream from the coarctation and in the region of irregular secondary vortex formation immediately downstream. We infer that these flow conditions, when acting chronically, cause endothelial injury. Finally, these same flow conditions inhibit migratory repair of endothelium, whereas low shears of consistent orientation that characterize the annular vortices observed in our experiments may enhance repair. If endothelial injury is important in early atherogenesis, then these observations may explain why sites of unusual hemodynamic stresses show a predilection for lesion development.

References

Index Terms: hemodynamic stress • blood flow • atherosclerosis • endothelial injury
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