Endothelial cell damage and thrombus formation following temporary arterial occlusion

Effects of pretreatment with aspirin or heparin

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The effects of restoration of blood flow on endothelial damage that occurs distal to the site of temporary arterial occlusion with surgical clips, and the effects of heparin and aspirin on thrombus formation at the site of the clip, were studied by scanning electron microscopy (SEM). The right carotid artery of 45 rabbits was occluded with a Heifetz clip for 30 minutes. The clips were then removed and blood flow resumed for periods of 30 minutes, 1 hour, 2 hours, and 24 hours. A second group was pretreated intravenously with heparin (1400 units/kg), and a third group was pretreated orally with aspirin (50 mg/kg). The SEM analysis of arterial segments distal and proximal to the clip indicated that the frequency of occurrence of endothelial crater- and balloon-like vesicular defects decreased to zero within 24 hours of restoration of blood flow. Examination of arterial segments compressed by the clip revealed endothelial desquamation and thrombus formation within 30 minutes of resumption of flow. The maximum degree of thrombus formation occurred within 1 to 2 hours of resumption of flow, with a subsequent decrease in the extent of deposition of platelets, fibrin, erythrocytes, and leukocytes within 24 hours. No change was found in composition or quantity of thrombus formation after pretreatment with heparin although, in the latter specimens, fibrin deposition appeared considerably less. However, pretreatment with aspirin resulted in marked reduction in the overall quantity of thrombus formation at the site of the clip following temporary occlusion.

KEY WORDS - endothelium - carotid artery - vascular occlusion - endothelial damage - thrombosis - scanning electron microscopy - heparin - aspirin - surgical clip

It has been shown that the use of surgical clips, regardless of their precise design, for temporary vascular occlusion, results in damage to the underlying endothelial lining ranging from focal crater- or balloon-like vesicular defects to total desquamation with exposure of subendothelial tissues. It has also been shown that such arterial occlusion results in endothelial cell injury distal and, to a lesser extent, proximal to the site of the occluding clip. This ischemic injury appears as endothelial craters and balloons, as viewed with scanning electron microscopy (SEM). When examined with transmission electron microscopy (TEM), this injury appears as protruding endothelial blebs, intracytoplasmic vacuoles, or pseudopodia from adjacent endothelial cells or underlying smooth-muscle cells.

The present study was designed to determine the effects of resumption of blood flow on endothelial cell damage that occurs distal to the site of occlusion and at the site of the clip, with further observations on the effectiveness of heparin or aspirin as antithrombogenic agents.

Materials and Methods

Forty-five New Zealand white rabbits were lightly anesthetized with sodium pentobarbital (Nembutal, 30 to 40 mg/kg, administered intravenously) and both
common carotid arteries exposed. The right carotid artery of each animal was occluded with a Heifetz clip* at the level of the laryngotracheal junction for 30 minutes. The clips were then removed and blood flow resumed for periods of 30 minutes, 1 hour, 2 hours, and 24 hours (five animals each). A second group of 10 animals was pretreated with heparin (Panheparin 1400 units/kg, administered intravenously) 5 minutes prior to occlusion, and flow was resumed for periods of 2 hours and 24 hours (five animals each). Animals in which flow was resumed for 24 hours received a second dose of heparin 10 hours after the first. A third group was pretreated with aspirin (acetylsalicylic acid, 50 mg/kg, administered orally) 2 hours before occlusion, and flow was resumed for periods of 2 hours and 24 hours (five animals each). In the five remaining animals, the right carotid artery was occluded for 30 minutes without permitting resumption of blood flow. The left carotid artery of each of the 45 animals served as a sham-operated control. The carotid arteries were fixed in situ by intracardiac perfusion of 1.6% glutaraldehyde in 0.8 M Sørensen’s phosphate buffer (pH 7.4, 310 mOsm/liter, 120 mm Hg), at room temperature. Arterial segments 1 cm in length were excised from four areas: the site of the clip, 5 mm distal to the clip, 5 mm proximal to the clip, and from the sham-operated control (left) carotid artery. All tissues were then immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for at least 24 hours, and prepared for scanning electron microscopy by the critical-point drying technique as described previously. Specimens were coated with gold palladium and examined in Cambridge S4-10 or Joel JSM-35 scanning electron microscopes.

The frequency of occurrence of endothelial cell alterations (craters and balloons) in distal, proximal, and sham-operated control segments was determined from a series of 20 random SEM fields at ×1000 magnification from each of the three arterial segments of all animals. P-values were calculated by application of Student’s t-test.

Results

The SEM examination of the sham-operated, unoccluded left common carotid arteries revealed normal, well organized endothelium, which is consistent with previous descriptions of normal endothelial cell morphology (Fig. 1). The SEM examination of arterial segments subjected to ischemia for 30 minutes (distal to the site of the clip) without subsequent reflow revealed numerous crater- and balloon-like vesicular defects (1 to 10 μ in diameter) in the endothelial lining (Fig. 2). When examined with TEM, these defects appeared to repre-

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sent protruding blebs, intracytoplasmic vacuoles or pseudopodia of adjacent endothelial cells or underlying smooth-muscle cells. The frequency of occurrence of endothelial alterations (craters and balloons) was significantly greater in the distal arterial segment as compared with either proximal (p < 0.05) or sham-operated control segments (p < 0.025) (Table 1). However, after removal of the clip, the numbers of craters and balloons in the distal segments decreased markedly within 30 minutes of resumption of blood flow (p < 0.05), to the level of sham-operated control within 1 hour (p < 0.025), and to zero within 24 hours (p < 0.01) (Table 1). A reduction was also found in the frequency of endothelial alterations in proximal (0 versus 30 minutes, p = not significant; 0 versus 1 hour, p < 0.05; 0 versus 24 hours, p < 0.025) and sham-operated control segments (0 versus 30 minutes, p = not significant; 0 versus 1 hour, p = not significant; 0 versus 24 hours, p < 0.05).

In a previous study the marked endothelial damage that is detectable in arterial segments compressed by the surgical clip for as little as 5 minutes. Further examination of these specimens indicated that the severity of this damage varied directly with its location beneath the V-shaped clip blades. In areas beneath the periphery of the clip blades, the site of minimal arterial compression, the endothelium appeared relatively normal, except for numerous craters and balloons such as those seen in segments distal to the site of the clip (Fig. 2). Beneath the center of the clip blades, the site of maximal arterial compression, marked endothelial damage was seen ranging from flattening of the cells, particularly over the area of the nucleus, to extensive fragmentation and disruption of endothelial cell continuity (Fig. 3). Desquamation of large areas of the endothelial lining was also found with exposure of subendothelial connective tissue.

After removal of the clip and resumption of blood flow, marked attachment of platelets to damaged endothelial cells and to exposed subendothelial tissues was seen with subsequent secondary aggregation of

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**TABLE 1**

Average number of craters and balloons in distal, proximal, and sham-operated control segments of arteries subjected to occlusion for 30 minutes

<table>
<thead>
<tr>
<th>Segment</th>
<th>Duration of Reflow</th>
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<tr>
<td></td>
<td>0 mins</td>
<td>30 mins</td>
<td>1 hr</td>
<td>2 hrs</td>
<td>24 hrs</td>
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<tr>
<td>distal segment</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>craters</td>
<td>19.8*</td>
<td>3.2</td>
<td>1.2</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>balloons</td>
<td>12.6</td>
<td>4.0</td>
<td>3.0</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>craters and balloons†</td>
<td>32.4 ± 11.3</td>
<td>7.2 ± 2.0</td>
<td>4.2 ± 1.6</td>
<td>1.6 ± 1.2</td>
<td>0</td>
</tr>
<tr>
<td>proximal segment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>craters</td>
<td>7.0</td>
<td>5.4</td>
<td>0.6</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>balloons</td>
<td>1.8</td>
<td>1.2</td>
<td>0.4</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>craters &amp; balloons</td>
<td>8.8 ± 3.8</td>
<td>6.6 ± 2.0</td>
<td>1.0 ± 0.8</td>
<td>1.2 ± 1.2</td>
<td>0</td>
</tr>
<tr>
<td>control segment</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>craters</td>
<td>3.0</td>
<td>2.2</td>
<td>0.8</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>balloons</td>
<td>1.8</td>
<td>1.0</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>craters and balloons†</td>
<td>4.8 ± 2.4</td>
<td>3.2 ± 1.0</td>
<td>1.0 ± 0.6</td>
<td>0.6 ± 0.4</td>
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</tr>
</tbody>
</table>

*Average number of lesions for the five animals calculated from 20 random scanning electron micrograph fields in each arterial segment of each animal.
†Mean ± standard error.
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platelets, deposition of fibrin and leukocytes, and entrapment of erythrocytes (Figs. 4 and 5). Table 2 summarizes the changes in relative quantity of platelets, erythrocytes, and leukocytes at the site of endothelial desquamation following resumption of blood flow. The maximum degree of thrombus formation occurred within 1 to 2 hours of reflow. The luminal surface area occupied by endothelial desquamation remained constant from 0 to 24 hours reflow. The extent of platelet deposition and aggregation appeared to reach a maximum at 1 hour with a slight decrease by 24 hours. The quantity of entrapped erythrocytes increased to a maximum 2 hours after resumption of flow, followed by a marked reduction by 24 hours. However, leukocytes appeared only after 1 hour of reflow, then declined within 2 hours, but increased again by 24 hours following resumption of flow.

Examination of arterial segments of animals pretreated with heparin before clipping and resumption of blood flow for periods of 2 hours and 24 hours revealed no apparent change in nature or extent of endothelial damage, platelet attachment, or deposition of erythrocytes and leukocytes from the nontreated group (Table 3, Figs. 4 and 5). However, the extent of fibrin deposition appeared to be considerably less than in the nontreated group.

As with the nontreated and heparin-pretreated groups, the animals pretreated with aspirin before occlusion and resumption of blood flow for periods of 2 hours and 24 hours showed large areas of endothelial desquamation at the site of the clip. However, the animals pretreated with aspirin showed a marked

<table>
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<tr>
<th>Duration of Reflow</th>
<th>0</th>
<th>30 mins</th>
<th>1 hr</th>
<th>2 hrs</th>
<th>24 hrs</th>
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<tr>
<td>platelets</td>
<td>0</td>
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<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>erythrocytes</td>
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<td>++</td>
<td>++++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>leukocytes</td>
<td>0</td>
<td>0</td>
<td>++++</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

*0 to ++++= none seen to maximum seen. Each notation represents the average of five animals.
reduction in the extent of platelet attachment to exposed subendothelial tissues (Table 3, Figs. 5 and 6). The quantity of entrapped or adherent erythrocytes was also considerably less in the aspirin-pretreated group. The number of leukocytes adherent to altered endothelial cells or entrapped in the platelet-fibrin meshwork appeared to be the same in animals pretreated with aspirin before occlusion and resumption of flow for 2 hours as in the nontreated animals; but, in the group in which blood flow was resumed for 24 hours, the number of leukocytes was noticeably less following aspirin pretreatment.

This difference in overall quantity of thrombus formation between animals that received no treatment or heparin before occlusion and those animals that were pretreated with aspirin is clearly seen by viewing the clip site from the edge of a longitudinal section parallel to the long axis of the blood vessel (Fig. 7). In the animals that received no treatment or heparin, the thrombus protruded from 50 to 150 μ into the lumen (5% to 15% occlusion). By contrast, in the animals pretreated with aspirin, thrombus formation was always limited to the adherence of one or two discontinuous layers of platelets, fibrin, erythrocytes, and...
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leukocytes to damaged endothelial cells or exposed subendothelial tissues.

Discussion

We have demonstrated that arterial occlusion with surgical clips results in the formation of numerous endothelial craters and balloons distal and, less frequently, proximal to the site of the clip. In previous reports we have presented evidence that craters actually represent ruptured or collapsed balloons.\(^{15,16}\) It has also been shown that injury appearing as craters and balloons on scanning electron microscopy, represents blebs and vacuoles as seen with transmission electron microscopy.\(^{10,11}\) These alterations have been reported following a wide variety of injurious stimuli as well as “spontaneously” at branch orifices\(^{20}\) and it appears that these alterations represent a nonspecific reaction of endothelial cells to injury.

We have shown, by SEM analysis, that the number of craters and balloons in the endothelium of carotid arteries subjected to ischemia by arterial clipping for 30 minutes decreased markedly within 30 minutes of removal of the clip and resumption of blood, and fell to zero within 24 hours. These observations indicate that craters and balloons may resolve upon removal of the injurious stimulus. However, additional SEM and TEM studies are needed in order to determine whether these lesions may become irreversible or may be followed by more extensive endothelial damage if the insult were allowed to persist, such as following longer periods of occlusion or as a result of persistent rheological trauma at points of arterial branching.

It has recently been shown, with the aid of the scanning electron microscope, that the use of the currently available surgical clips, frequently referred to as "atraumatic," including the cerebral aneurysm clips\(^{11}\) and the small artery microclips,\(^{6}\) results, immediately upon application, in marked endothelial cell damage at the site of the clipping. This damage ranges from focal craters and balloons to total desquamation of the endothelial lining with exposure of the subendothelial layers of the vascular wall. In the present study we have shown that following removal of the surgical clip and resumption of flow, thrombus formation ensues as evidenced by marked attachment of platelets to exposed subendothelial tissues with further deposition of fibrin, erythrocytes, and leukocytes. The maximum

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration of Reflow</th>
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<tbody>
<tr>
<td></td>
<td>2 hrs</td>
</tr>
<tr>
<td>platelets</td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>+</td>
</tr>
<tr>
<td>heparin</td>
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<tr>
<td>aspirin</td>
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<tr>
<td>erythrocytes</td>
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<tr>
<td>none</td>
<td>++++</td>
</tr>
<tr>
<td>heparin</td>
<td>+</td>
</tr>
<tr>
<td>aspirin</td>
<td>+</td>
</tr>
<tr>
<td>leukocytes</td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>+</td>
</tr>
<tr>
<td>heparin</td>
<td>+</td>
</tr>
<tr>
<td>aspirin</td>
<td>+</td>
</tr>
</tbody>
</table>

*0 to ++++ = none to maximum seen. Each notation represents the average of five animals.

FIG. 7. Cut edge of longitudinal sections parallel to the long axis of the carotid artery at the site of occlusion by surgical clip for 30 minutes followed by restoration of flow for 2 hours. Note the greater quantity of thrombus formation at the site of the clip in the nontreated (left) as compared with the aspirin pretreated animals (right). Arterial wall (W), erythrocyte (E), leukocyte (L), vessel lumen (Lu). Direction of flow is from right to left. Left: \(\times 1400\). Right: \(\times 2000\).
degree of thrombus formation occurred within 1 to 2 hours, with protrusion of 50 to 150 μ into the lumen (5% to 15% occlusion) and a subsequent reduction in overall quantity of thrombus within 24 hours. Rosenbaum and Sundt reported a similar pattern of intraluminal thrombus formation and subsequent resolution at the suture lines of micro-arterial end-to-side anastomoses between the two common carotid arteries of the rat. The time course of thrombus formation and subsequent dissolution was somewhat shorter in the latter study (maximum, 15 minutes; dissolution, 30 minutes), and the thrombi were frequently larger with respect to luminal diameter. However, these differences might be accounted for by differences in extent of endothelial damage, possible dissimilarities in nature or time course of the mechanisms of hemostasis between the two species, or by differences in hemodynamic patterns between the two systems.

Dujovny, et al., reported the long-term vascular effects of temporary arterial occlusion with microsurgical clips. In the latter study, SEM evaluation of the middle cerebral artery of the dog following occlusion with six currently available microclips, followed by resumption of blood flow for 1 month, revealed persistent disorganization of the endothelial lining at the site of the clip with disruption of interendothelial junctions. In a study of the long-term effects of large artery clamping, DePalma, et al., reported marked endothelial damage following occlusion of the carotid and femoral arteries of the dog with DeBakey or Potts clamps, trauma that persisted 10 days and 13 months after resumption of flow. Light microscopic examination of the arterial wall 10 days after clamping revealed disruption of the internal elastic lamina, smooth-muscle proliferation, and focal thickening of the intima. Moreover, animals subjected to arterial clamping in association with a hypercholesterolemic diet developed grossly visible atheromatous lesions at the site of application of the clamp. Thus, the present studies, when considered together with those of DePalma, et al., and Dujovny, et al., indicate that the danger exists for partial or total arterial occlusion at the site of the clip as compared with control or heparin-pretreated animals. The precise mechanism of action of aspirin on platelet aggregation has not been clarified. It should be emphasized that there are a number of uncertainties concerning the effects of heparin on platelet aggregation and release, but its effects on aggregation induced by other agents such as adenosine diphosphate (ADP) and collagen are uncertain. In fact, the exact stimulus for platelet attachment to exposed subendothelial tissues, be it collagen itself or ADP that is simultaneously released locally upon endothelial desquamation, is also uncertain. It is known that one component of the subsequent platelet release reaction is the release of platelet factor 4 (heparin neutralizing factor). However, the exact nature and possible local effects of this factor on platelet aggregation have not been clarified. It should be emphasized that there are a number of uncertainties concerning the effects of heparin on platelet aggregation, and it is therefore not unreasonable to expect a negative or unpredictable effect of heparin administration on thrombus formation following surgical procedures similar to that reported in the present study.

By contrast, pretreatment with aspirin resulted in a marked reduction in overall quantity of thrombus formation at the site of the clip as compared with control or heparin-pretreated animals. The precise mechanism of action of aspirin on platelet aggregation is the subject of considerable controversy, but there is agreement that aspirin inhibits the platelet-aggregation reaction and subsequent secondary phases of platelet aggregation leading to thrombus formation. Although the extent of deposition of platelets, erythrocytes, and leukocytes appeared to be markedly reduced, the adhesion of platelets to altered endothelial cells and exposed subendothelial connective tissue was not totally prevented. This is consistent with the observations of Baumgartner and Muggli and Weiss, et al., that, although aspirin inhibits the
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secondary phase of platelet aggregation leading to thrombus formation, it does not prevent the attachment of platelets, in flowing blood, to exposed subendothelial tissues (the first phase of platelet aggregation).

Finally, there is considerable evidence that suggests that atherosclerosis is preceded by damage to the endothelial lining. This damage, in addition to permitting the infiltration of large lipoproteins and lipid-laden macrophages, serves as a stimulus for smooth-muscle cell proliferation following the adherence of platelets to subendothelium and release of mitogenic substances. Thus, additional long-term SEM studies on this model of endothelial damage, with correlative light and transmission electron microscopy, should be conducted to determine whether aspirin (or other antiplatelet-aggregating agents that might be more effective in preventing the adherence of platelets to subendothelium as well as the release reaction) might also be beneficial for the prevention of parenchymal ischemia, and for subsequent functional deficits as a consequence of critical arterial obstruction by either thrombus formation or atherosclerosis following surgical procedures which require temporary vascular occlusion.

Acknowledgments

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References

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