Progressive Impairment of Regional Myocardial Perfusion After Initial Restoration of Postischemic Blood Flow

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The "no-reflow" phenomenon, the occurrence of areas with very low flow in hearts reperfused after ischemia, is thought to be largely established at the time of reperfusion as a result of microvascular damage induced by ischemia. In the present study we sought to determine whether additional impairment of tissue perfusion might also occur during the course of reperfusion. Open-chest dogs were subjected to 90 minutes of left circumflex coronary artery occlusion and reperfused for 2 minutes (n=7) or 3.5 hours (n=8). Myocardial perfusion was visualized in left ventricular slices following in vivo injection of the fluorescent dye thioflavin-S just before killing. The area of impaired perfusion (absent thioflavin) averaged 9.5±3.0% of the risk region in dogs reperfused for 2 minutes, whereas it was nearly three times as large in dogs reperfused for 3.5 hours (25.9±8.2% of the risk region, p<0.05). Serial measurements of flow by microspheres during reperfusion demonstrated zones within the postischemic myocardium that were hyperemic 2 minutes after reperfusion, with adequate flow still present at 30 minutes, but with a subsequent marked fall in perfusion. After 3.5 hours these areas showed negligible flow (0.13±0.03 ml/min/g) and no thioflavin uptake. Tissue samples showing postischemic impairment in perfusion had received virtually no collateral flow during ischemia (<0.01 ml/min/g), whereas collateral flow was significantly higher in adjacent thioflavin-positive zones (0.04±0.01 ml/min/g in endocardial samples and 0.07±0.02 ml/min/g in samples from the midmyocardium, p<0.001 vs. thioflavin-negative areas). Areas that showed late impairment of flow invariably demonstrated contraction band necrosis, which contrasted with the pattern of coagulation necrosis observed in areas of "true" (i.e., immediate) no-reflow. Intracapillary erythrocyte stasis and marked intravascular neutrophil accumulation (to levels >20-fold that found after 2 minutes reperfusion) were typically observed in areas of delayed impairment to flow. Obstruction to flow at the capillary level was confirmed in additional dogs in which the heart was injected postmortem with silicone rubber to delineate the microvascular filling pattern. Areas of absent capillary filling were much more extensive after 3.5 hours than after 2 minutes reperfusion. Thus, this study shows that the occurrence of areas of markedly impaired perfusion in postischemic myocardium is related only in part to an inability to reperfuse certain areas on reflow. A more important factor is represented by a delayed, progressive fall in flow to areas that initially received adequate reperfusion. This phenomenon develops in regions receiving no collateral flow during ischemia and is associated with neutrophil accumulation and capillary plugging late during the course of reperfusion. (Circulation 1989;80:1846-1861)
Restoration of the blood supply to previously ischemic tissue is not always followed by uniform return of perfusion to the microvasculature. In 1968, Ames et al1 studied cerebral ischemia in the rabbit and reported the occurrence of areas that lacked vascular filling on reperfusion. The authors suggested that microvascular compression from ischemia-induced tissue edema was responsible for an inability of the tissue to reperfuse; hence, the term "no-reflow." The occurrence of the no-reflow phenomenon in the heart was first noted by Krug et al,2 and important pathologic observations were made by Kloner et al3 in a canine model. In Kloner’s study, after coronary artery occlusion for 90 minutes and reperfusion for times ranging from 10 seconds to 20 minutes, portions of the previously ischemic area failed to stain with the fluorescent dye thioflavin-S that was given in vivo as a perfusion marker. In the poorly perfused areas (absent thioflavin), severe capillary damage and coagulation necrosis were observed. Contraction bands were absent, consistent with the notion that these areas had not received significant reflow despite release of the coronary artery ligature. These findings lent further support to the hypothesis that no reflow is the consequence of severe damage to the microvasculature caused by ischemia and that it is largely established at the moment of reperfusion.3–5

However, recent evidence suggests that in the heart as well as other tissues, reperfusion can induce injury over and above the damage caused by ischemia.6 It is possible, therefore, that microvascular damage might also develop during reperfusion, either secondary to myocyte damage or by means of direct vascular injury. Granulocyte7–10 and/or platelet11 adherence to the endothelium, as well as direct free radical attack,12 have been proposed as possible causes of vascular damage in reperfused tissues. If widespread vascular obstruction occurred during the reperfusion phase as a consequence of these or other mechanisms, the mass of myocardium receiving little or no flow should increase progressively over time after reperfusion. Despite the wealth of data on myocardial consequences of reperfusion, little is known about the consequences of posts ischemic reflow on microvascular integrity or about its effects on tissue perfusion.

In the present study, we sought to determine whether progressive damage to the microvasculature occurs in dog hearts during posts ischemic reperfusion. For this purpose we compared the size of the area of myocardium showing low flow immediately after reperfusion ("true" no-reflow) with the size of the low flow zone delineated several hours after reflow (delayed impairment to flow). The ultrastructural features of myocardium within areas of immediate and delayed impairment to flow, as well as of posts ischemic but normally perfused tissue, were also analyzed. Finally, we measured serial changes in regional blood flow in tissue samples from the previously ischemic region to look for evidence of progressive vascular obstruction.

Methods

Surgical Preparation

Mongrel dogs of either sex (20–25 kg) were anesthetized with i.v. sodium thiamylal (18 mg/kg), followed by i.m. α-chloralose (14 mg/kg) and urethane (136 mg/kg), and ventilated with room air. Polyethylene catheters were placed in the right femoral artery and vein, a left thoracotomy was performed, and a catheter was placed in the left atrium. An electromagnetic flowmeter and a pneumatic occluder were placed around the circumflex coronary artery proximal to any major marginal branch. Heart rate, circumflex coronary artery blood flow, and arterial and left atrial pressures were continuously monitored.

Experimental Protocol

After baseline measurements of regional myocardial blood flow (see below), the occluder was inflated. The flowmeter signal was used to verify complete coronary occlusion. Regional myocardial blood flow was measured again 85 minutes after occlusion. After 90 minutes of occlusion, blood flow was restored by deflation of the occluder. Dogs were assigned to two groups. The animals in the first group (n=8) were reperfused for 3.5 hours; regional myocardial blood flow was measured at the time of peak reactive hyperemia (about 2 minutes after the onset of reperfusion) and again after 30 minutes and 3.5 hours of reperfusion. In the second group (n=7), the dogs were reperfused only long enough to obtain the first regional myocardial blood flow measurement at peak reactive hyperemia. All dogs survived until completion of their respective protocols. One animal in each group developed ventricular fibrillation during ischemia and was resuscitated.

Measurement of Area of Impaired Perfusion, Risk Region, and Area of Necrosis

Following the last microsphere injection in both groups, the area of impaired perfusion was delineated by an intra-atrial injection of 1 ml/kg of the fluorescent dye thioflavin-S (Sigma Chemical Co, St. Louis, Missouri). The dye was prepared fresh as a 4% solution in warm (37°C) saline, centrifuged to remove particulate matter, and injected by hand over 15–20 seconds. Approximately 20 seconds later the circumflex coronary artery was reoccluded, and a 1 ml/kg bolus of monoastral blue dye (Heubach Corporation, Newark, New Jersey) was injected into the left atrium to determine the in vivo ischemic area (risk region). The dogs were then killed by an overdose of potassium chloride.

After excision the heart was placed in ice-cold saline containing 20 mM KCl. The right ventricle and the atria were removed, and the left ventricle was cut into six to eight slices parallel to the
TABLE 1. Scoring System of Myocardial and Microvascular Injury

<table>
<thead>
<tr>
<th>Myocyte injury associated with ischemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+ Mild-to-moderate nuclear chromatin clumping, occasional vacuoles, I-bands</td>
</tr>
<tr>
<td>2+ Intracellular edema and vacuoles, more marked nuclear changes</td>
</tr>
<tr>
<td>3+ Further intracellular edema, more vacuoles, extracellular edema, lifting of sarcolemma</td>
</tr>
<tr>
<td>4+ Marked swelling and cellular derangement</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Myocyte injury associated with reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+ Contraction bands confined to a few areas of individual myocytes, minimal intracellular edema</td>
</tr>
<tr>
<td>2+ More extensive hypercontraction and disruption of sarcomeres, intracellular and extracellular edema, lifting and/or tearing of sarcolemma</td>
</tr>
<tr>
<td>3+ Severe disruption of cellular architecture caused by hypercontraction and rupture of sarcomeres, numerous breaks in sarcolemma, dissociation of intercalated discs</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Endothelial injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>1+ Paling of cytoplasm, loss of pinocytotic vesicles, mild nuclear chromatin clumping</td>
</tr>
<tr>
<td>2+ More marked nuclear changes, intracellular swelling, intraluminal blebs, intraluminal fibrin deposition</td>
</tr>
<tr>
<td>3+ Endothelial disruption and hemorrhage</td>
</tr>
</tbody>
</table>

atrioventricular groove. A glass plate was placed over the slices, and the contour of each slice was traced on a clear plastic sheet. The area unstained by monastral blue (risk region) was also traced. With an ultraviolet light source (peak emission wavelength 340 nm), the areas not perfused by thioflavin-S (areas of impaired perfusion) were identified and traced.

Tissue specimens were then taken for regional myocardial blood flow measurements and light and electron microscopic examination. Samples (50–100 mg) were removed under ultraviolet light from thioflavin-negative areas and from immediately adjacent thioflavin-positive tissue within the risk region. Care was taken to avoid samples that showed visible heterogeneity in thioflavin staining. The specimens were cut into 2–3 mm³ pieces, weighed, and placed in cold fixative (4% formaldehyde/1% glutaraldehyde). Microsphere radioactivity was measured, and the samples were processed for light and electron microscopy (see below).

To determine the extent of myocardial necrosis in the 3.5-hour reperfusion group, the left ventricular slices were incubated at 37°C in triphenyltetrazolium chloride (TTC) for 30 minutes. The slices were photographed in color, and the transparencies were projected onto a sheet of paper. The outlines of the slice, risk region, and TTC-negative (necrotic) area were traced, and the size of the risk region and infarct were calculated by planimetry. Infarct size was not measured in one dog.

Measurement of Regional Myocardial Blood Flow

Regional myocardial blood flow was measured with 16 μm diameter radioactive microspheres (NEN-TRAC, New England Nuclear, Boston, Massachusetts). For each flow measurement 4,000,000 microspheres labeled with 141Ce, 113Sn, 103Ru, 85Nb, or 46Sc were injected into the left atrium. Starting before injection and continuing for 2 minutes afterwards, a reference arterial blood sample was withdrawn by a Harvard pump at a constant rate of 2.16 ml/min. For assessment of average regional myocardial blood flow, sampling (1–2 g) was done transmurally in the center of the risk region and in the nonischemic anterior wall for each left ventricular slice. Samples were divided into inner and outer halves, weighed, and counted for radioactivity with the reference blood samples at appropriate energy windows. Myocardial blood flow (ml/min/g) was calculated by standard methods and corrected to account for the combined effects of microsphere loss, local edema, and hemorrhage.

Light and Electron Microscopy

Samples were washed with cold 0.1 M phosphate buffer and postfixed with cold 1% osmium tetroxide, dehydrated in a graded series of alcohol and propylene oxide, and embedded in epoxy resin. Semithin (1 μm) sections were stained with toluidine blue and examined under a light microscope. Ultrathin (75 nm) sections were prepared from tissue blocks with fibers oriented longitudinally and in cross-section. The sections were mounted on copper grids, stained

FIGURE 1. (See facing page.) Top panel: Typical appearance under ultraviolet light of left ventricular slice from a heart undergoing 3.5 hours reperfusion injected with thioflavin-S before death. The yellow-green fluorescence indicates perfused tissue. The brown areas correspond to areas of low flow. In this slice, myocardium in the nonischemic anterior wall shows less intense stain as a result of the partial washout of the dye that occurs in that area during injection of monastral blue to delineate the risk region. Bottom panel: The same slice photographed under normal light after staining with triphenyltetrazolium chloride (TTC). The blue tint of the myocardium in the nonischemic anterior wall is due to tissue uptake of monastral blue. Within the risk region a large pale (TTC-negative) area can be seen, corresponding to necrotic myocardium, surrounded by a rim of brick-red salvaged tissue.
with lead citrate and uranyl acetate, and examined in a JEOL 100S electron microscope.

A semiquantitative assessment was made of the morphologic appearance of myocytes and endothelial cells. For myocyte injury toluidine blue-stained semithin sections (approximately 1 mm²) from each specimen were scanned by light microscopy at a magnification of ×500 in 100-μm² increments using a grid reticle eyepiece. A minimum of 100 grid squares was assessed for each specimen. The presence and severity of tissue injury were graded using the scoring system shown in Table 1 (after electron microscopic confirmation). Moderate-to-severe mitochondrial swelling with intramitochondrial amorphous densities was used as the electron microscopic marker of irreversible myocyte injury. Only 1+ ischemic injury was believed to be potentially reversible; all other categories were believed to be probably or definitely irreversible. Final injury scores in each category for each specimen were expressed as the ratio of the number of grid squares of each grade to the total number of grid squares for each specimen ×100.

Intravascular granulocyte accumulation in each specimen was estimated by counting the number of granulocytes in each 100-μm² grid square and expressing the results as the average number per millimeter squared. A granulocyte was counted if the nucleus and cytoplasm were clearly identifiable within a capillary lumen and distinguishable from the capillary endothelial cell. Intracapillary red blood cell stasis was defined as the occurrence of more than five contiguous erythrocytes per capillary in longitudinal section. The results were expressed as the fraction of grid squares containing capillaries with stasis. Endothelial cell injury was assessed by electron microscopy using the grading criteria in Table 1. The results for each specimen were expressed as the average injury score assigned to capillaries in each specimen.

Microvascular Injection Studies

Visualization of the microvasculature was achieved by postmortem intracoronary injection of microfil silicone rubber (MV-122 Yellow, Canton Biomedical Products Inc, Boulder, Colorado) in four additional hearts. After 90 minutes of circumflex occlusion, two hearts were reperfused for 3.5 hours, while two were excised at the time of peak reactive hyperemia. The hearts were placed on ice after removal, and the left main coronary artery was cannulated with a large bore plastic catheter. Microfil was infused at 100 mm Hg constant pressure until the injection mass flowed freely from the coronary sinus. In one additional heart, also subjected to 90 minutes of ischemia and 3.5 hours of reperfusion, the coronary sinus was cannulated, and microfil was injected retrogradely at a constant pressure of 30 mm Hg. All hearts were left on ice for 2 hours and fixed for 48 hours in cold 4% formaldehyde. The left ventricle was then sectioned into 1 cm thick rings from apex to base, and 1-cm³ blocks were cut from normal and postischemic regions. The blocks were cleared by a process that changes tissue into a semitranslucent amber material through which the microfil-filled microvasculature could be viewed. Samples were sequentially placed for 24 hours in each of the following solutions: tap water, distilled water, 70% ethanol, 80% ethanol, 95% ethanol, 100% ethanol, and a solution of 3 parts benzyl benzoate to 4 parts methyl benzoate. Samples were then cut into 1–2 mm thick slices, stored in the final clearing solution, and viewed stereoscopically and photographed at different magnifications under a dissecting microscope using epi-illumination.

Statistical Analysis

Data are presented as the mean±SEM. Differences in the various groups were tested by Student’s t test for unpaired samples or repeated-measures analysis of variance (ANOVA), as appropriate.

Results

Validation of Thioflavin-S as Flow Marker

After injection of thioflavin-S, the myocardium in the nonischemic anterior wall was characterized by uniformly faint fluorescence under ultraviolet light (thioflavin-positive, Figure 1). In contrast, in the previously ischemic risk region thioflavin-negative (i.e., nonfluorescent) zones were observed in the inner layers (Figure 1). Lack of thioflavin staining was associated with low blood flow. At the time of dye injection, flow in samples taken from thioflavin-negative areas (n=41) averaged 0.13±0.03 ml/min/g tissue in dogs reperfused for 3.5 hours, whereas tissue specimens taken from within the risk region but from the thioflavin-positive areas in the same animals (n=62) showed normal flow (1.18±0.16 ml/min/g). Half of the thioflavin-negative samples had negligible or no flow (<0.05 ml/min/g), and 73% showed flow values lower than 0.2 ml/min/g (Figure 2, upper panel). There was little overlap of flow values between thioflavin-negative and positive areas with a cutoff point around 0.4 ml/min/g (Figure 2, lower panel). Flow in thioflavin-negative areas in dogs sacrificed early after reflow was also low, averaging 0.26±0.04 ml/min/g. Despite the injection of thioflavin-S during hyperemia in these animals, the distribution of flow in thioflavin-negative and positive areas was similar to the 3.5 hour reperfusion group. Flow was lower than 0.4 ml/min/g in 80% of thioflavin-negative samples, whereas in 98% of tissue specimens from thioflavin-positive areas, flow was more than 0.4 ml/min/g, with an average of 2.79±0.25 ml/min/g.

Extent of Area of Impaired Perfusion, Risk Region, and Area of Necrosis

Proximal circumflex occlusion resulted in similar hemodynamic changes in the two groups (Table 2). The size of risk regions was also similar in the two
groups (Figure 3). In dogs sacrificed early after reperfusion, the flow-impaired thioflavin-negative ("true no-reflow") area was confined to the subendocardium and averaged 9.5±3.0% of the risk region (Figure 3). However, in dogs reperfused for 3.5 hours, the thioflavin-negative zone encompassed most of the subendocardium and often extended into the midmyocardium, representing 25.9±8.2% of the risk region (p<0.05, Figure 3).

In the 3.5-hour reperfusion group infarct size averaged 41.4±7.5% of the risk region. In this group, the zone of impaired perfusion was always contained within the area of myocardial necrosis delineated by TTC staining. The sizes of the infarct and low flow areas were linearly correlated (y=0.85x+19.5; r²=0.86), with a minimum infarct size of about 20% of the risk region required before any low flow area was seen.

**Regional Myocardial Blood Flow**

The severity of ischemia, as assessed from collateral blood flow to the center of the risk region, was almost identical in the two groups of dogs (Table 3). On reperfusion average flow in both inner and outer layers increased about twofold over preischemic values. This hyperemic response subsided between 30 and 210 minutes of reperfusion (Table 3).

In addition to these measurements of average flow to the risk region, we analyzed flow changes during 3.5 hours of reperfusion in tissue specimens

![Figure 2. Bar graph of distribution of tissue specimens according to blood flow measured at the time of death (3.5 hours after reperfusion). The results for samples from thioflavin-negative and thioflavin-positive areas are shown in the upper and lower panels, respectively.](image)

![Figure 3. Bar graph of extent of the risk region (RR), expressed as percent of the left ventricle (LV), and of the thioflavin-negative (low flow) area (normalized to that of the risk region) in the dogs killed after 2 minutes or 3.5 hours of reperfusion.](image)

**Table 2. Hemodynamic Measurements**

<table>
<thead>
<tr>
<th></th>
<th>Occlusion (min)</th>
<th>Reperfusion (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline 30 60 90</td>
<td>30 60 90 120 150 180 210</td>
</tr>
<tr>
<td><strong>3.5 hr study</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>132±7 134±11</td>
<td>134±11 123±13 129±18 128±16 107±5 115±15</td>
</tr>
<tr>
<td>SAP (mm Hg)</td>
<td>114±7 114±7</td>
<td>111±9 114±9 115±7 116±7 113±7 118±7 119±8</td>
</tr>
<tr>
<td>DAP (mm Hg)</td>
<td>84±8 81±7</td>
<td>81±8 82±7 86±6 91±6 89±6 99±7 93±7</td>
</tr>
<tr>
<td>MLAP (mm Hg)</td>
<td>3.1±0.8 6.8±1.2</td>
<td>5.6±1.5 4.8±1.1 4.6±1.3 4.9±1.8 4.1±1.5 5.5±1.8 6.7±1.5</td>
</tr>
<tr>
<td><strong>2 min study</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>141±10 143±11</td>
<td>141±11 140±9</td>
</tr>
<tr>
<td>SAP (mm Hg)</td>
<td>132±8 115±10</td>
<td>107±6 107±5</td>
</tr>
<tr>
<td>DAP (mm Hg)</td>
<td>101±6 85±9</td>
<td>85±6 81±6</td>
</tr>
<tr>
<td>MLAP (mm Hg)</td>
<td>5.8±1.0 8.3±1.1</td>
<td>6.3±1.2 5.8±1.5</td>
</tr>
</tbody>
</table>

HR, heart rate; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MLAP, mean left atrial pressure.
TABLE 3. Regional Myocardial Blood Flows

<table>
<thead>
<tr>
<th>Layer</th>
<th>Baseline</th>
<th>Ischemia</th>
<th>Reperfusion (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Center of risk region</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>3.5 hr study</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inner</td>
<td>0.97±0.13</td>
<td>0.05±0.02</td>
<td>2.16±0.41</td>
</tr>
<tr>
<td>Outer</td>
<td>0.90±0.11</td>
<td>0.12±0.05</td>
<td>2.55±0.38</td>
</tr>
<tr>
<td>2 min study</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inner</td>
<td>1.36±0.12</td>
<td>0.05±0.02</td>
<td>1.89±0.62</td>
</tr>
<tr>
<td>Outer</td>
<td>1.28±0.11</td>
<td>0.13±0.03</td>
<td>3.84±0.87</td>
</tr>
</tbody>
</table>

Nonischemic LAD territory

<table>
<thead>
<tr>
<th>Layer</th>
<th>Baseline</th>
<th>Ischemia</th>
<th>Reperfusion (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5 hr study</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inner</td>
<td>0.95±0.09</td>
<td>1.37±0.22</td>
<td>1.18±0.18</td>
</tr>
<tr>
<td>Outer</td>
<td>0.90±0.10</td>
<td>1.32±0.23</td>
<td>1.16±0.20</td>
</tr>
<tr>
<td>2 min study</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inner</td>
<td>1.34±0.16</td>
<td>1.28±0.17</td>
<td>1.41±0.43</td>
</tr>
<tr>
<td>Outer</td>
<td>1.30±0.15</td>
<td>1.34±0.18</td>
<td>1.51±0.44</td>
</tr>
</tbody>
</table>

Flow is expressed in ml/min/g wet tissue wt (mean±SEM). LAD, left anterior descending coronary artery.

selected according to the presence or absence of visible perfusion by thioflavin at the end of the experiment. Samples from thioflavin-negative areas received virtually no collateral flow during ischemia, that is, less than 0.01 ml/min/g in endocardial and midmyocardial samples (Figure 4, upper panel). Flow was significantly higher in samples from thioflavin-positive areas within the risk region in the same animals but was still quite low (0.04±0.01 and 0.07±0.02 ml/min/g in endocardial and midmyocardial samples, respectively; p<0.001 vs. samples from thioflavin-negative areas; Figure 4, lower panel). A similar difference in collateral flow between thioflavin-positive and negative zones was observed in dogs from the short reperfusion group (Table 4).

Tissue specimens from areas showing no thioflavin uptake 3.5 hours after reperfusion exhibited a hyperemic response early after reperfusion (Figure 4, upper panel). Distribution analysis of flow in these samples showed that 2 minutes after reflow, the majority of samples taken from thioflavin-negative zones were being perfused at a flow rate more than 1 ml/min/g, whereas only 10% of the samples had a flow of 0.4 ml/min/g or less (Figure 5). Since lack of thioflavin staining was associated in our study with flows lower than 0.4 ml/min/g (Figure 2), these results suggest that most of the specimens sampled from thioflavin-negative areas 3.5 hours after reperfusion would have actually belonged to the thioflavin-positive zone if delineated early after reflow. Furthermore, flow in these specimens was still relatively high 30 minutes after reperfusion (Figure 4, upper panel). On the other hand, samples from thioflavin-positive areas at 3.5 hours of reperfusion, even though within the infarcted zone, showed higher levels of early reflow, and although flow decreased during the remainder of the study, it did not fall below baseline levels (Figure 4, lower panel).

FIGURE 4. Bar graph of changes in regional myocardial blood flow during the course of the experiment in tissue samples taken from thioflavin-S negative (upper panel) or thioflavin-S positive (lower panel) areas at the end of 3.5 hours of reperfusion. The epicardial layers did not have significant thioflavin-negative areas.
minutes After Reperfusion

4. Thioflavin-Negative Endocardium

These features were characterized by coagulation necrosis with relaxed myofilaments, cellular edema, swollen mitochondria with fragmented cristae, and nuclear changes (Figure 6A and B). These features accounted for more than 95% of the total tissue area analyzed (Table 5). The vascular endothelium demonstrated severe injury with edema, cytoplasmic clearing, loss of pinocytotic vesicles, nuclear changes, and formation of blebs and membrane bound bodies protruding into the vascular lumen (Figure 6B). The mean endothelial cell injury score was 2.5±0.2.

In striking contrast with the pattern observed after 2 minutes of reflow, thioflavin-negative areas from dogs reperfused for 3.5 hours mostly showed contraction band necrosis, involving more than 95% of the total area analyzed (p<0.001 vs. 2 minute dogs). The usual picture was that of severe tissue injury with hypercontracted sarcomeres and swollen mitochondria with disrupted cristae and calcium precipitates (Figure 6C and D, Table 5). Endothelial damage was always prominent (mean injury score, 2.3±0.2).

Ultrastructural Observations

Specimens from thioflavin-negative areas from both groups of dogs showed widespread tissue damage. No zones of normal or only mildly injured myocardium were seen within the thioflavin-negative area in either group. Areas of no-reflow in dogs reperfused for 2 minutes were characterized by coagulation necrosis with relaxed myofilaments, cellular edema, swollen mitochondria with fragmented cristae, and nuclear changes (Figure 6A and B). These features accounted for more than 95% of the total tissue area analyzed (Table 5). The vascular endothelium demonstrated severe injury with edema, cytoplasmic clearing, loss of pinocytotic vesicles, nuclear changes, and formation of blebs and membrane bound bodies protruding into the vascular lumen (Figure 6B). The mean endothelial cell injury score was 2.5±0.2.

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In both groups of dogs, thioflavin-positive areas within the risk region demonstrated moderate tissue injury, with a mixture of coagulation and contraction band necrosis pattern (Table 5). Zones of myocardium showing only mild or no damage represented a substantial portion of the total tissue area analyzed (Table 5, p<0.001 vs. thioflavin-negative areas). The mean endothelial injury scores from the thioflavin-positive areas in both groups of dogs were significantly less than the scores from the thioflavin-negative areas (2 minutes reflow, 0.4±0.1; 3.5 hours reflow, 0.7±0.2; p<0.001 vs. thioflavin-negative areas).

Marked intracapillary red blood cell stasis was found in thioflavin-negative areas from dogs reperfused for 3.5 hours (Figure 6C). Of the tissue area analyzed, 77.3±8.7% exhibited red cell stasis compared with 32.8±7.7% and 33.3±5.9% in thioflavin-negative and positive areas, respectively, from dogs reperfused for 2 minutes and 37.5±3.6% in thioflavin-positive areas from dogs reperfused for 3.5 hours (p<0.001 vs. other groups).

Intravascular neutrophil accumulation was minimal in postischemic myocardium after 2 minutes

<table>
<thead>
<tr>
<th>TABLE 4. Blood Flow in Tissue Specimens Taken From Either Thioflavin-Negative or Thioflavin-Positive Areas in Dogs Killed 2 Minutes After Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocardium</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>T(-), n=9</td>
</tr>
<tr>
<td>T(+), n=17</td>
</tr>
<tr>
<td>Midmyocardium</td>
</tr>
<tr>
<td>T(-), n=6</td>
</tr>
<tr>
<td>T(+), n=22</td>
</tr>
<tr>
<td>Epicardium</td>
</tr>
<tr>
<td>T(+), n=22</td>
</tr>
</tbody>
</table>

T(-), thioflavin-negative; T(+), thioflavin-positive.
*Statistically different from T(-), p<0.001. Flow is in ml/min/g wet tissue wt (mean±SEM).

<table>
<thead>
<tr>
<th>TABLE 5. Morphologic Appearance of Tissue Specimens Taken From Either Thioflavin-Negative or Thioflavin-Positive Areas in Two Groups of Dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>2 min study</td>
</tr>
<tr>
<td>T(-), n=4</td>
</tr>
<tr>
<td>13.0±3.6</td>
</tr>
<tr>
<td>T(+), n=6</td>
</tr>
<tr>
<td>3.5 hr study</td>
</tr>
<tr>
<td>T(-), n=5</td>
</tr>
<tr>
<td>29.4±8.1</td>
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<tr>
<td>T(+), n=10</td>
</tr>
</tbody>
</table>

T(-), thioflavin-negative; T(+), thioflavin-positive. Data are expressed as a percentage of the total tissue area analyzed.
FIGURE 6. (A) Light micrograph of a thioflavin-negative area 2 minutes after reperfusion showing margination and clumping of nuclear chromatin (arrows), various amounts of intracellular edema, and derangement of normal cellular architecture. Swollen mitochondria are seen in the perinuclear region (arrowhead). Open arrow shows abnormal nucleus of vascular endothelial cell (toluidine blue stain of 1 μm plastic-embedded specimen, magnification ×1,275). (B) Electron micrograph of the same region shown in (A). Sarcomeres have prominent I-bands (IB), and mitochondria (M) are swollen and contain amorphous densities (black arrow). A red blood cell (rbc) containing capillary has numerous intraluminal membrane bound bodies (black arrowhead), and the cytoplasm of the endothelial cell is thinned with pinocytic vesicles.
noticeably absent (magnification ×15,000). (C) Light micrograph from a thioflavin-negative area 3.5 hours after reperfusion. Contraction bands (CB) are prominent and some capillaries appear to be blocked by white blood cells (arrowheads) causing stasis of red blood cells (rbc) within the capillary (toluidine blue stain of 1 μm plastic-embedded specimen, magnification ×975). (D) Electron micrograph of the same region shown in (C). Sarcomeres are hypercontracted and overstretched producing contraction bands (CB). A capillary with thinned and disrupted endothelium (black arrowhead) contains a leukocyte (wbc) and red blood cell (rbc). The swollen, disrupted mitochondria (M) contain calcium precipitates (white arrows), another feature typical of reperfusion associated necrosis (magnification ×12,000).
reperfusion whether the area was thioflavin negative or positive (Figures 6A and B). After 3.5 hours reperfusion there was a nonsignificant trend toward more neutrophils in thioflavin-positive areas. However, in thioflavin-negative areas there was a striking, more than 20-fold increase in neutrophils (Figure 7, *p<0.01 vs. all groups). Granulocytes apparently occluding a capillary or clustering in the vascular lumen were often seen (Figure 6C and D).

**Microvascular Injection Studies**

Postmortem injection of the coronary arteries with microfil confirmed that in the hearts made ischemic for 90 minutes and reperfused for only 2 minutes, the microvasculature was largely patent with filling of the majority of capillaries throughout the left ventricular wall. Lack of capillary filling was noted in a small rim of endocardium. Small focal areas of capillary nonfilling were also visualized in the inner layers of the postischemic region (Figure 8B and C). In the two hearts reperfused for 3.5 hours, the picture was dramatically different (Figure 8D and E). Large confluent areas of capillary nonfilling were seen. Filling was seen down to the arteriolar level with frequent, abrupt truncation of the microfil column in the visualized arterioles (Figure 8E). In the one heart in which retrograde microfil injection was performed, a large confluent zone was seen with capillary nonfilling and apparent blockage at the venular level (Figure 8F).

**Discussion**

Restoration of blood flow in a previously occluded coronary artery is not always followed by the return of normal tissue perfusion. The ability to reperfuse certain areas of ischemic myocardium has traditionally been considered to be a consequence of microvascular damage occurring during ischemia and, therefore, to represent a process already established at the beginning of arterial reflow (hence, the term "no-reflow"). In the present study, however, the area of impaired perfusion delineated by thioflavin-S was about three times larger after 3.5 hours than after 2 minutes reperfusion. Furthermore, direct measurements of blood flow showed that myocardium with poor perfusion at 3.5 hours received, for the most part, substantial reperfusion initially, followed by a marked decline in flow over time. These findings demonstrate that in addition to the area of "true" (i.e., immediate) no-reflow found at the time of reperfusion, a substantial portion of postischemic myocardium undergoes progressive vascular obstruction during reperfusion. These areas of delayed impairment to flow were also characterized by distinctive morphologic features.

Previous studies have measured serial changes in blood flow in postischemic myocardium but failed to observe this progressive decrease in flow. However, without a specific attempt to delineate spatially the areas of low flow, specimens from postischemic myocardium generally contain mixtures of low flow and normally perfused or hyperemic tissue. A marked fall in flow to a portion of the sample could go unnoticed because of high flow in the remainder. Cobb et al showed that after 2 hours coronary artery occlusion in the dog, flow remained at near-normal levels in the majority of tissue specimens during 4 hours of reperfusion. However, a marked decrease in flow was found during reperfusion in the lowest flow samples showing extensive necrosis at the end of the study. Because the area of low flow was not spatially delineated in Cobb et al's study, an increase in the amount of poorly perfused myocardium during reperfusion could not be detected.

In a previous study Kloner et al found no differences in the extent of no-reflow after reperfusion periods ranging from 10–12 seconds to 20 minutes. Severe capillary damage with coagulation necrosis was the usual morphologic pattern observed in areas of no-reflow while contraction bands were absent. Our data are in partial agreement with those of Kloner et al because we also found areas of immediate no-reflow early after reperfusion, characterized by myocyte coagulation necrosis with edema but without contraction bands. However, in addition to these findings, we observed progressive impairment to flow in tissue that was initially reperfused. The typical ultrastructural pattern of such tissue was that of endothelial damage in the presence of contraction band necrosis. An explanation for the discrepancy between Kloner et al's data and ours is that 20 minutes of reperfusion were probably not sufficient for delayed microvascular obstruction to develop. Our finding that substantial blood flow was present 30 minutes after reflow in samples showing little or no flow later in the course of the experiment lends support to this hypothesis.
Figure 8. Photomicrographs of cleared sections of left ventricular wall following microfil injection in coronary arteries (A–E) or coronary sinus (F). (A) Nonischemic area (original magnification, ×20). Note extensive uniform capillary filling. (B) Postischemic area after 2 minutes reperfusion, endocardium oriented upwards. Note patchy filling of subendocardial capillaries with Swiss cheese appearance (dots mark endocardial border) (20×). (C) Same as B at higher magnification showing focal area of capillary nonfilling in subendocardium with truncation of several arterioles suggestive of obstruction (arrow) (62×). (D) Postischemic area after 3.5 hours reperfusion; endocardium oriented to left and epicardium to right. Note extensive confluent area of nonfilling involving inner one third to one half of left ventricular wall (×6). (E) Same as (D) at higher magnification showing abrupt truncation of many arterioles (×35). (F) Postischemic area after 3.5 hours reperfusion, endocardium oriented toward lower left corner. There is filling of the small veins and venules but absence of capillary filling over a large confluent area of subendocardium. Normal capillary filling is seen to the right of the section (×35).
Interstitial edema resulting in external compression of the microvasculature has been proposed as a cause of no-reflow. Tranum-Jensen et al.\(^2\) showed that ischemia induced the glycolytic production of osmotically active molecules, potential contributors to tissue edema on reflow. Reperfusion with hypertonic perfusate containing mannitol markedly reduced the extent of no-reflow.\(^2\) Studies by Powell et al.\(^2\) and by Willerson et al.\(^2\) demonstrated that reperfusion after 40–120 minutes of coronary artery occlusion was associated with abnormal myocardial fluid retention and reduced ability to reperfuse, which could be partially prevented by administration of mannitol. Although edema may be the cause of “true” no-reflow, the relation between edema and a delayed flow impairment is less clear because the tissue edema largely develops immediately on reflow.\(^2\) In addition, the results with mannitol do not necessarily support a role for edema because mannitol possesses vasoactive properties\(^2\) and is a scavenger of hydroxyl radicals,\(^2\) as well as being an osmotic agent.

In our study, underperfused areas were not uniformly distributed. Although confined to the subendocardium and midmyocardium, thioflavin-negative areas were often spatially separated by areas of myocardium with much better perfusion. This phenomenon was particularly evident in hearts examined early after reflow. Our data suggest that the basis for this heterogeneity of tissue perfusion was a corresponding heterogeneity of the degree of ischemia. Thioflavin-negative areas received virtually no flow during the period of occlusion, whereas immediately adjacent, thioflavin-positive areas located within the ischemic zone were exposed to significantly higher (although still quite low) collateral flow. Impaired reperfusion in areas exposed to low collateral flow might suggest that a particularly severe ischemic insult may have been necessary to damage the microvasculature. Alternatively, very low levels of flow may have been required to “prime” the myocardium for subsequent reperfusion injury.\(^2\) The setting of severe ischemia, a number of processes may occur that can promote oxygen radical formation during reperfusion, including an increase of hypoxanthine and xanthine,\(^2\) conversion of xanthine dehydrogenase to its oxidase form,\(^2\) loss of scavenging enzymes,\(^2\) and accumulation of granulocytes.\(^2\)

The basis for the focal heterogeneity of ischemia during coronary occlusion is unknown. Flow in certain vessels might cease because of random obstruction by blood elements and/or local increase in extravascular compressive forces. Another possibility relates to the anatomy of the vasculature. Certain branching angles or the number of vascular divisions occurring before a given precapillary vessel is reached may determine the pressure drop along its length and thereby influence blood flow. These factors might also make certain areas of myocardium more susceptible to a progressive increase in coronary resistance, which may occur during ischemia.\(^3\)–\(^5\)

Granulocytes have recently been proposed as a major factor in the no-reflow phenomenon. Studies by Engler et al.\(^6\)–\(^10\) have shown that leukocytes can be seen in individual capillaries from posts ischemic myocardium with red blood cells apparently piled up behind, suggesting mechanical obstruction. Reperfusion greatly enhances accumulation of neutrophils in the posts ischemic myocardium in a time-dependent fashion,\(^7\)–\(^9\),\(^39\) presumably secondary to enhanced adhesion of white cells to the capillary endothelium.\(^40\)–\(^42\) The hypothesis that leukocytes can cause microvascular obstruction is supported by the observation that depletion of leukocytes or administration of agents that interfere with neutrophil function reduces granulocyte and red cell accumulation\(^30,42,43\) and results in less “no-reflow.”\(^8,44\) In our study neutrophil accumulation was much more pronounced in those areas of myocardium undergoing delayed impairment of blood flow. Plugging of the capillaries by neutrophils could have caused vascular erythrocyte packing upstream from the point of obstruction and prevented the penetration of microfil beyond the arteriolar level. Our finding of greater erythrocyte accumulation in areas of delayed flow impairment is also consistent with the possibility of capillary plugging by neutrophils as well as with the notion that the extent of myocardial hemorrhage in reperfused hearts depends on the severity of the ischemic insult.\(^5\)\(^,45\)

Our data suggest that in addition to vascular damage, progressive myocyte damage might have occurred during reperfusion. Of the tissue area analyzed from the thioflavin-positive zone immediately after reflow, 13% was morphologically normal and an additional 30% showed only mild ischemic changes consistent with reversible injury. In addition, endothelial damage was absent or mild. Because the area of impaired perfusion increased threefold over 3.5 hours, we can speculate that some of the tissue that was thioflavin-positive immediately after reflow may have been subsequently converted to thioflavin-negative areas with extensive contraction band necrosis and severe endothelial injury. These observations are consistent with the hypothesis that reperfusion may induce specific damage to nonirreversibly injured myocardium and vascular endothelium as a result of oxygen radical formation and/or granulocyte activation.\(^6,27,35\) While several investigators failed to demonstrate significant tissue preservation from “anti-free radical” interventions,\(^46\)–\(^48\) others have reported substantial reduction of infarct size and/or microvascular damage by administration of oxygen radical scavengers\(^39,49\)–\(^51\) or antineutrophil interventions.\(^30,39,42\)–\(^44,52\)

A major question is whether vascular obstruction during reperfusion may directly contribute to myocyte damage. The available evidence would suggest that at least for “immediate” no-reflow, the die is cast, and the surrounding myocytes are already
dead at the moment of reperfusion. Kloner et al\textsuperscript{53} have emphasized that during myocardial ischemia ultrastructural features of myocyte injury appear to precede microvascular injury and that the area of no-reflow is always contained within the border of myocardial necrosis, suggesting that no-reflow occurs because of myocyte death and not vice versa. However, the situation is less clear for “delayed” no-reflow. Although we observed that the no-reflow area was also contained within the infarcted area after 3.5 hours reperfusion, progressive vascular obstruction in principle could have led to an extension of necrosis, perhaps by converting areas of patchy necrosis to confluent necrosis. Interestingly, in previous studies prevention of reperfusion injury resulted in a patchy distribution of myocardial necrosis, whereas the infarcted region appeared largely confluent in control animals.\textsuperscript{29,49,52}

On the basis of our data, no firm conclusion can be reached as to the existence of a causal relation between progressive damage to the microvasculature and extension of myocyte damage. However, it can be speculated that both phenomena might occur at the same time through a common mechanism. Vascular and myocyte injury could both result at reperfusion from generation of oxygen radicals from several sources, including the xanthine oxidase reaction (occurring mainly in endothelial cells) and granulocytes that have adhered to the endothelial surface. Intraluminally produced free radicals could diffuse into the interstitial space to attack myocytes.\textsuperscript{54,55} After the initial damage, additional granulocyte adhesion might occur, leading to further myocyte and vascular injury and further granulocyte accumulation and microvascular obstruction. In addition, activated neutrophils may release vasoconstricting substances, which could contribute to progressive impairment of flow.\textsuperscript{56} Thus, “delayed no-reflow” might signify a vicious cycle of reperfusion-induced vascular and myocyte injury, initiated by a sufficient degree of ischemic injury to “prime” the reaction and resulting in progressive tissue necrosis.

Preservation of tissue perfusion might have beneficial effects other than prevention of cell necrosis.\textsuperscript{57} The delivery of drugs to the previously ischemic myocardium should be enhanced, as well as the capacity to develop collateral vessels between the postischemic region and adjacent vascular territories. Furthermore, infarct expansion and formation of left ventricular aneurysms might be prevented through an alteration of tissue mechanical properties or promotion of infarct healing. Interestingly, recent studies have demonstrated that late postischemic reperfusion reduces infarct expansion in the rat without altering infarct size.\textsuperscript{58,59} Thus, while it is unclear whether prevention of “delayed no-reflow” will translate into infarct size reduction in reperfused hearts, it is likely that preservation of tissue perfusion will have a number of other favorable consequences.

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**KEY WORDS** • reperfusion • myocardial ischemia • occlusions
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G Ambrosio, H F Weisman, J A Mannisi and L C Becker

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