Myocardial Myoglobin Following Coronary Artery Occlusion

An Immunohistochemical Study

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To evaluate morphologic changes and myoglobin content in normal, ischemic and necrotic myocardium, the authors studied human (n = 13) and dog (n = 28) myocardium by triphenyltetrazolium chloride staining, light and electron microscopy, periodic acid–Schiff stain for glycogen loss, and by an immunoperoxidase technique. Myocardium from autopsied patients with infarction 10–24 hours old showed loss of myoglobin from necrotic fibers. Dogs with infarcts after 3 hours or more of coronary occlusion showed myoglobin loss in fibers shown to be necrotic. In 4 dogs with 50% reduction in left main coronary artery flow for 3 hours, which demonstrated ischemia without necrosis (glycogen loss with no triphenyl tetrazolium chloride evidence of necrosis), myoglobin staining in myocardial sections was similar to nonischemic and positive control tissues. By comparison of immunoperoxidase staining with concomitant study by light and electron microscopy and histochemistry, loss of myoglobin from necrotic myocardium was demonstrated, while ischemic but not necrotic fibers stained normally. These findings indicate that necrosis is necessary for myoglobin loss from myocardium to be detected by this immunoperoxidase technique. (Am J Pathol 1983, 111:374–379)

WITH THE ADVENT of sensitive immunoassays for serum myoglobin, the potential clinical utility of serum myoglobin measurement for the diagnosis of myocardial infarction has increased.[1-11] Recent clinical studies indicate that increased serum myoglobin can be detected as early as 1 hour after the onset of chest pain and that peak levels can occur within 5–6 hours. Thus, measurement of serum myoglobin may supplement more frequently used serum enzyme measurements (creatine kinase (CK) and its isoenzymes) for the early detection of myocardial infarction. Myoglobin, an oxygen-carrying protein found only in skeletal and cardiac muscle cells, is relatively small (mol wt ~17,800 daltons) and is thought to be released through the deteriorating cell membrane in necrotic myocardium. In order to better understand the relationship between myoglobin release, appearance in the serum, and myocardial damage, changes within the actual tissue as well as in the serum must be examined.

The present study was directed toward answering two unresolved questions about myoglobin loss from myocardium. First, is myoglobin lost from tissues that are ischemic but not necrotic? Second, how early during the progression of an infarct can a loss of myoglobin from the affected myocardium be detected? To answer these questions, we utilized an immunoperoxidase technique to evaluate myoglobin content in human myocardium from patients with ischemic heart disease and from canine myocardium rendered ischemic or infarcted in experimental studies of myocardial ischemia. By using experimental animals, in which the degree and duration of ischemic injury were known we could study the time course of myoglobin loss and the relationship between myoglobin loss and other markers of ischemic injury.

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Materials and Methods

Human Myocardial Samples

After pilot studies had shown that normal myocardium obtained at autopsy (6–24 hours after death), as well as at surgery, consistently showed positive immunostaining for myoglobin, 13 human cases of coronary artery disease were selected for study. Ten specimens were obtained from autopsies of patients who had died with myocardial infarction and 3 from excised cardiac muscle from patients undergoing surgery for complications of myocardial infarction (acquired ventricular septal defect, papillary muscle dysfunction). As determined clinically and histologically, infarcts ranged in age from less than 24 hours to several weeks, some with significant scar formation. All tissues were formalin-fixed and processed by routine laboratory procedures: dehydration in increasing concentrations of alcohol, clearing in xylene, and embedding in paraffin. Sections 6 μ thick were cut.

Dog Myocardial Samples

Cardiac tissue from 28 dogs were used for this study. To study the status of myocardial myglobin in dogs with ischemia alone, tissue from 4 dogs in which coronary artery blood flow was reduced 50% by controlled closed chest perfusion of the left main coronary artery (LMCA) was used. These dogs underwent closed-chest cannulation of the LMCA. This model utilized a controlled roller pump and allowed control of LMCA perfusion by application of a screw-clamp on the perfusion tubing. LMCA flow was reduced from 98 ± 8 to 47 ± 3 ml/min/100 g.[12] LMCA pressure and flow, as well as left ventricular function, were monitored. Dogs were sacrificed after 3 hours. To study the time course of myoglobin loss in evolving myocardial infarction, tissues from 24 dogs with ischemia produced by balloon occlusion of the left anterior descending coronary artery (LAD) were used.[13] The dogs were sacrificed after the following time periods: 45 minutes (2 dogs), 3 hours (8 dogs), 5–6 hours (8 dogs), 15–24 hours (6 dogs). Each excised heart was rinsed in sterile saline, cut into 1-cm-thick transverse slices, and incubated in triphenyltetrazolium chloride (TTC). TTC stains the tissues with intact dehydrogenase enzyme systems a dark red; the necrotic tissues are not stained. This technique has been shown to reliably delineate areas of necrosis as early as 2–3 hours after coronary artery occlusion.[14] After staining in TTC, the sections were photographed and fixed in formalin. Tissue for H&E staining and for myoglobin immunoperoxidase studies was chosen from those areas that contained both normal and damaged myocardium. Processing was as described for the human tissues.

To more clearly define the degree of myocardial damage, we conducted other studies on the same tissues. Periodic acid–Schiff (PAS) staining for glycogen was utilized on Carnoy's-fixed tissue as a marker for ischemia.[15] Glycogen loss can be observed within 20 minutes of coronary artery occlusion and thus can delineate regions that are ischemic but not necrotic.[16] In 3 dogs with only 3 hours of occlusion, glutaraldehyde-fixed tissue was studied by electron microscopy to confirm TTC evidence of necrosis. By examining tissue with no TTC or histologic evidence of necrosis but the glycogen loss, we could study the myoglobin content of tissue that was ischemic but not necrotic.

Antiserum

The immunoperoxidase staining technique used in this study utilized rabbit anti-human myoglobin IgG (DAKO Laboratories, Santa Barbara, Calif). The antiserum was prepared according to the method of Kagen and Christian[17] with two modifications: first, human cardiac muscle myoglobin was used instead of human pectoral muscle myoglobin, and second, a DEAE Sephadex column was used instead of DEAE cellulose. Precipitin tests showed that a neutralized solution does not stain control tissues (personal communication, DAKO Laboratories).

The cross-reactivity of this antiserum to rat myoglobin has been shown to be very high.[1] No attempt to quantify the cross-reactivity to dog myoglobin was made, although considerable cross-reactivity was shown to exist, because the dog tissues stained similarly to the human tissues.

Immunoperoxidase Staining Procedure

Myoglobin in the cardiac tissue was visualized by a modification of the three-step immunoperoxidase technique of Sternberger.[18] Formalin-fixed paraffin-embedded sections were first deparaffinized and rehydrated through a graded series of ethanol. Slides were then incubated with a mixture of 50 ml 100% methanol and 10 ml 3% hydrogen peroxide for 15 minutes to remove endogenous peroxidase activity. After washing in phosphate-buffered saline (PBS), a 1:20 dilution of normal swine serum in PBS with 10% albumin was applied to the slides to reduce background staining.[19] Following incubation for 15 minutes in a humidity chamber (PBS-saturated atmosphere), the serum dilution was tipped off and rabbit anti-human myoglobin antiserum was applied to the slides in a

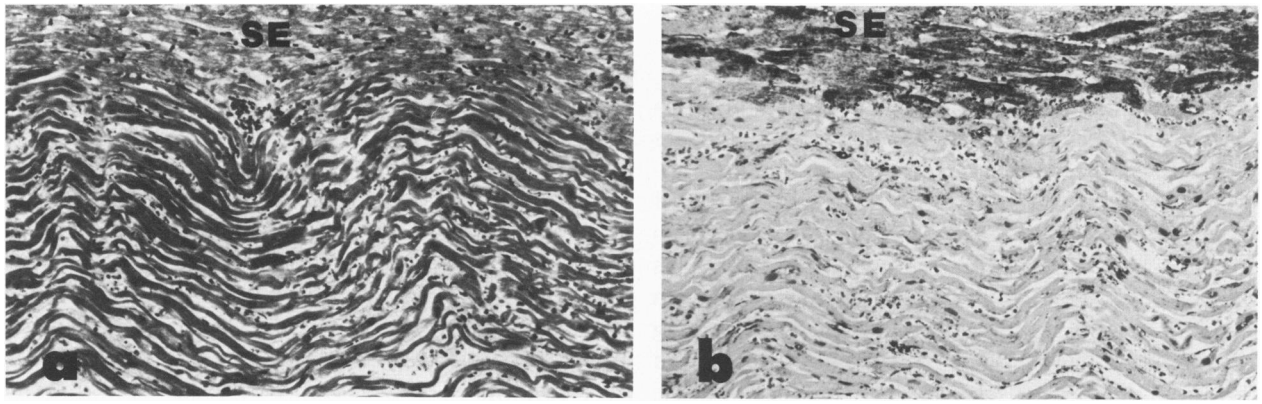

Figure 1 — Sections of myocardium from a patient who died of infarction 12 hours after the onset of chest pain. **a** — H&E-stained section showing hypereosinophilia (darker staining) and waviness of necrotic fibers with interstitial edema and acute inflammatory cell infiltration; subendocardial fibers (*SE*) are spared. **b** — Section stained for myoglobin showing loss in necrotic fibers and positive staining in non-necrotic subendocardial fibers. (×40)

1:50 dilution for 45 minutes. The slides were then rinsed in PBS and incubated for 30 minutes with a 1:20 dilution of swine anti-rabbit IgG. After a rinse with PBS, a 1:100 dilution of horseradish-peroxidase–rabbit anti-peroxidase–soluble complexes was applied for 30 minutes, followed by a rinse in PBS. A fresh solution of 4 mg 3,3′-diaminobenzidine (DAB), 0.2 ml dimethylsulfoxide (DMSO), and 0.05 ml 30% hydrogen peroxide in 10 ml PBS was then applied to the slides for 1–5 minutes for visualization of the peroxidase activity. The reaction was stopped when positive control tissue showed dark brown staining. After counterstaining in dilute hematoxylin, the slides were dehydrated and coverslipped.

Specificity of staining was validated by the use of both negative and built-in positive controls. Negative control slides were included for all cases by substituting the primary rabbit anti-human myoglobin anti-

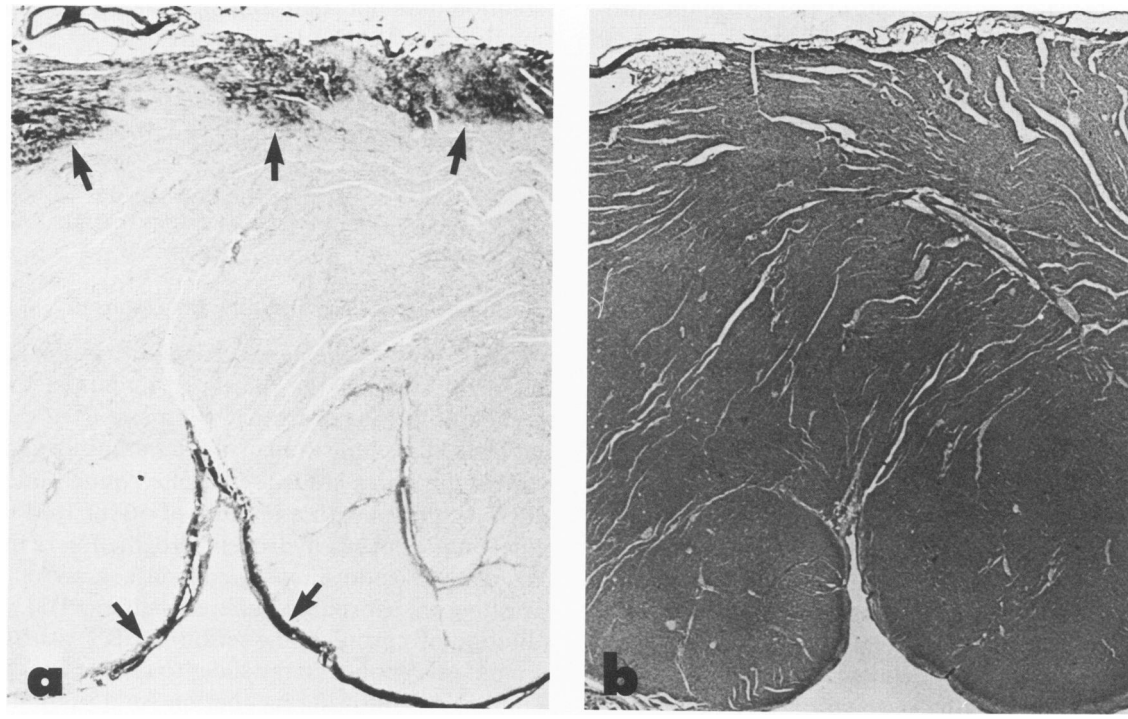

Figure 2 — Sections from the left ventricle of a dog with 3 hours of 50% coronary flow reduction. TTC stain was normal, and electron-microscopic study showed ischemic changes without necrosis. **a** — A Carnoy's-fixed, PAS-stained section with diffuse glycogen loss, indicating ischemia. Only subendocardial (*lower arrows*) and subepicardial (*upper arrows*) fibers contain glycogen. **b** — A section stained for myoglobin. Note that there is diffuse staining with no loss of myoglobin detectable. (×4)

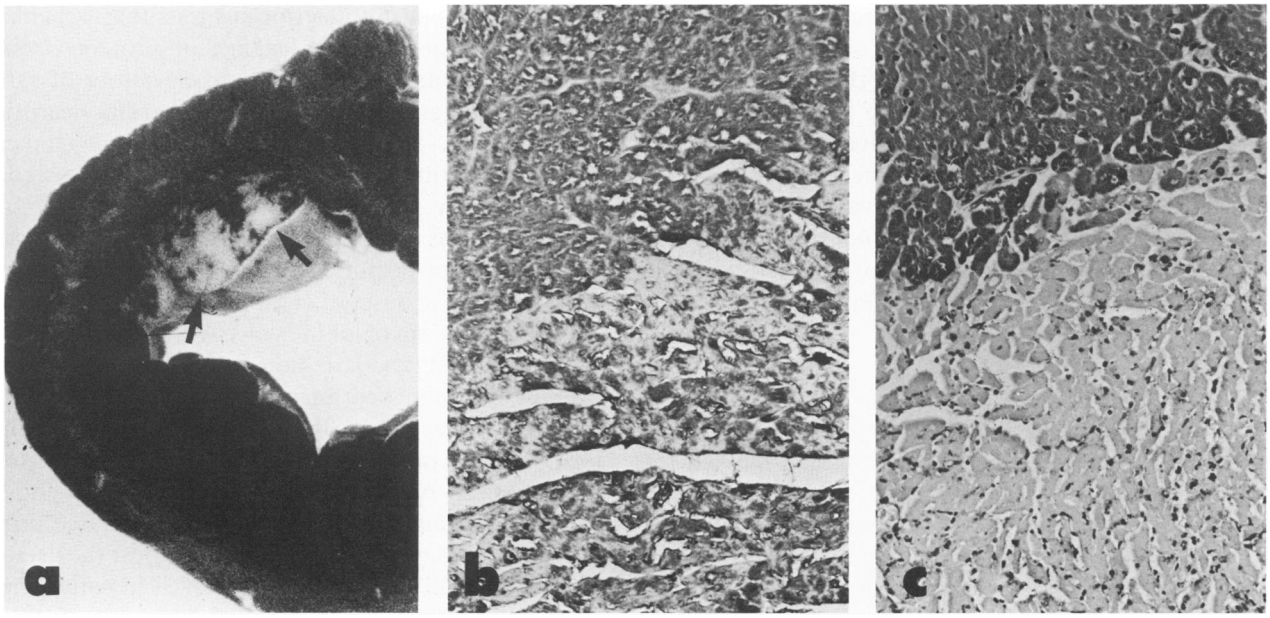

Figure 3a – Gross photograph of a transverse slice of a dog left ventricle stained with TTC, showing a 3-hour-old subendocardial infarction (*arrows*). **b** – Histologic section of tissue shown in **a**, taken from the junction of viable and necrotic tissue, stained for myoglobin. Note the patchy but definite loss of staining in the lower half of the section. **c** – Similar section from a dog with a 6-hour-old infarct. Myoglobin loss is now more severe and diffuse. (×40)

serum (and in selected cases the bridging antiserum) with PBS or normal rabbit serum. These controls revealed no staining, except for a few cases in which the necrotic tissue, often a site of nonspecific immunoperoxidase staining,[19] showed mild nonspecific uptake. This staining was usually faint. When it was more than faint, the staining of that case was repeated with freshly prepared reagents, which gave acceptable results. Each slide contained areas of normal as well as injured myocardium, providing a built-in positive control. This demonstrated two important characteristics of the stain: 1) specific positive staining by normal myocardium, and 2) lack of staining of tissues known not to contain myoglobin (collagen, vessel walls etc.), indicating that no significant nonspecific staining was occurring.

Results

Human Tissue

In all cases the areas of necrosis could be defined by use of the hematoxylin and eosin (H&E) and TTC stains. In the areas of necrosis the immunoperoxidase technique showed loss of myoglobin, indicated by the loss of immunostaining. Because of the varying ages of the human myocardial infarcts, granulation and scar tissue were also present. These tissues did not stain, indicating that they contained no myoglobin. Normal myocardial cells adjacent to necrotic areas stained darkly, indicating that the viable cells retained myoglobin. Subendocardial sparing, a characteristic of myocardial infarcts, was demonstrated by the dark staining of a narrow band of cells adjacent to the endocardium (Figure 1).

Dog Tissue

As with the human tissue, viable cells stained darkly for myoglobin, while necrotic fibers showed absent or markedly reduced staining. The myocardium from the 4 dogs with 50% flow reduction for 3 hours and 2 with total occlusion for 45 minutes had no detectable reduction of immunostaining for myoglobin. TTC and H&E stains showed no evidence of necrosis, but PAS staining of these tissues demonstrated significant glycogen loss, indicating ischemia was present (Figure 2). In 3 of 8 dogs with infarcts 3 hours old decreased immunostaining for myoglobin was detected, first manifested as a patchy decrease in staining in subendocardial areas (Figure 3). Longer periods of coronary occlusion resulted in an increasingly distinct depletion. In the other 5 dogs with infarcts 3 hours old, no definite myoglobin loss was observed. Necrosis was confirmed in 3-hour infarcts by TTC staining of all cases and electron microscopy in 3 (amorphous matrix densities in mitochondria along with other ischemic changes).[20] Five to 6 hours of occlusion resulted in clear and definite loss of myo-

globin in the region shown to be necrotic by TTC and H&E (Figure 3). Longer coronary occlusions resulted in myocardium that had much the same staining characteristics as that subjected to 5- and 6-hour occlusion. Necrotic zones, as defined by TTC and H&E, showed markedly diminished staining, while normal myocardial fibers stained very darkly, with a sharp border between the two. A normal myocardial cell would stain very darkly, and an adjacent necrotic cell would show absence of staining.

Discussion

The recent development of rapid radioimmunoassays for serum myoglobin determination has led to renewed interest in the diagnostic value of this test for detecting acute myocardial infarction. Clinical data show that elevated myoglobin levels can be detected as early as, and perhaps even earlier than, both lactate dehydrogenase and CK elevation,[1-11] and thus suggest that accurate and rapid assays for myoglobin could provide valuable early information on myocardial damage. In addition, in dogs, preliminary studies indicate a good correlation between infarct size and peak serum myoglobin levels ($r = 0.83$).[21] Kent, who has recently demonstrated myoglobin in formalin-fixed paraffin-embedded myocardium by both immunofluorescent and immunoperoxidase techniques, showed that loss did occur in necrotic tissue.[22] It has been suggested, however, that serum myoglobin elevations (myocardial myoglobin loss) may occur with myocardial ischemia alone not associated with actual necrosis.[2-4] Our study attempted to further define myoglobin content of myocardium and study its changes in evolving myocardial infarction with the use of an immunoperoxidase staining technique. With human myocardium obtained at autopsy, and surgically resected, the myoglobin depletion could be clearly demonstrated. However, to study the specificity of loss for necrosis and its time course, it was necessary to study tissues from experimental animals with well-characterized ischemic injury.

Ischemic tissues could be identified by PAS staining, revealing glycogen loss, but normal TTC staining and lack of ultrastructural criteria of necrosis. Ischemia without necrosis was produced in three circumstances. The first was controlled closed-chest perfusion, in which blood flow through the LMCA was reduced 50%.[12] The second was complete occlusion for 45 minutes, and the third was one case in which in spite of the LAD being completely occluded for 3 hours, no necrosis resulted. Immunoperoxidase staining of these tissues showed no detectable myoglobin loss. This investigation of myocardium that was ischemic but not necrotic suggests that ischemia alone does not result in leakage of proteins from myocardial cells and that if serum elevations of cardiac proteins are present, some myocardial necrosis has occurred. Since immunoperoxidase techniques are not quantitative, we cannot completely exclude the possibility that some undetectable leakage of myoglobin has occurred from fibers that were ischemic but not necrotic.

Following the onset of chest pain, elevations of the serum levels of myoglobin evolve with time, reaching peak values at about 6 hours. Immunoperoxidase staining of the myocardium revealed that in some of the dogs subjected to 3-hour occlusions, myoglobin loss was detectable as patchy loss in focal subendocardial areas. At 5 and 6 hours of occlusion the loss was more dramatic and extensive, and increased progressively with time. Some observers report the detection of elevated serum myoglobin levels in patients as early as 1 hour after the onset of chest pain. In this group, however, the exact duration of ischemia or necrosis cannot be determined with certainty. In a closed-chest canine model of myocardial infarction, Willerson et al reported elevated serum myoglobin in some dogs within 2 hours of coronary occlusion.[21] Because we were able to detect myoglobin loss in 3 of 8 dogs with infarcts 3 hours old, it is suggested that the immunoperoxidase technique can demonstrate myoglobin loss from myocardium at a very early stage of the necrotic process. The significant loss demonstrated in all dogs with infarcts 5–6 hours old is concordant with the observed peak serum levels occurring within 4–6 hours reported by Willerson et al.[21]

In conclusion, this study shows that immunoperoxidase techniques can be utilized to demonstrate loss of myoglobin from necrotic myocardium. Ischemic myocardium that is not clearly necrotic does not demonstrate diminished immunostaining for myoglobin; actual necrosis is required before myoglobin loss from myocardium can be detected.

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