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The Role of the Neutrophil and Free Radicals in Ischemic Myocardial Injury

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Introduction

The association of coronary artery thrombosis with the onset of acute myocardial infarction has provided the rationale for the development of pharmacologic and/or physical methods for the restoration of coronary artery blood flow. The institution of pharmacologic methods for myocardial reperfusion has resulted in a reduction in mortality leading to acceptance of thrombolytic therapy as the standard approach to the management of patients with an evolving acute myocardial infarction [*1*]. It is a well established fact that prolonged myocardial ischemia leads to a time-dependent loss in the viability of myocardial cells in the jeopardized region of the heart and that the restoration of coronary artery blood flow is fundamental in order to arrest the progression of cell death and for the restoration of myocardial function. The restoration of blood flow is essential for repair of the reversible changes induced by ischemia and for the continued survival of the myocardial cells at risk of permanent damage. The reintroduction of oxygen at the time of reperfusion, however, may be detrimental to the reoxygenated myocyte as well as being beneficial. A number of recent reviews have been devoted to an examination of the question of whether reactive species of oxygen or oxygen radicals can contribute to the development of irreversible myocardial cell injury during the period of reperfusion [*2*, *3*, *4*, *5*]. The purpose of this editorial is to focus attention on the potential role of the polymorphonuclear leukocyte (PMN) as a determinant of the ultimate extent of irreversible myocardial injury after ischemia and reperfusion, and to call attention to those factors which modulate the inflammatory response to myocardial cell injury.

Neutrophil-dependent mediation of vessel wall and myocyte injury

The PMN possesses unique functions designed expressly for the defense of the host organism. Under certain conditions of altered physiology, the PMN may react in a manner that leads to tissue injury. The tissue damage resulting from myocardial ischemia activates a cascade of events which represents an inflammatory response that occurs independently of any improvement in myocardial reoxygenation. The concept we wish to propose is that the inflammatory response and the invading leukocytes contribute to the ultimate extent of myocardial injury. Therefore, interventions directed against the PMN or against the cytotoxic products produced by the invading cells, should result in a reduction in the extent of tissue damage associated with myocardial ischemia and/or reperfusion.

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Neutrophils invade a region of ischemic myocardium long before the transmural progression of irreversible injury reaches the subepicardial myocardial region [*6*]. Histologic studies by Sommers and Jennings [*7*] demonstrated that PMN were visible in the ischemic myocardium within 50 mins of reperfusion after coronary artery occlusion for 40 mins. Biochemical studies indicate that the infiltration of ischemic myocardium by PMN begins before reperfusion, and increases progressively after reperfusion. The myocardial activity of myeloperoxidase, which correlates with the histologic extent of neutrophil infiltration, exhibited a 6-fold increase after 90 mins of coronary artery occlusion without reperfusion, and a 23-fold increase after 90 mins of ischemia followed by 5 h of reperfusion [*8*]. PMN labeled with indium-111 have been employed to confirm that there is substantial accumulation of neutrophils within ischemic myocardium as early as 1 h after transient coronary artery occlusion for 40 or 90 mins [*9*]. Thus, reperfusion is essential in order to prevent further extension of ischemia-induced necrosis, but neutrophil-mediated injury would progress despite the restoration of blood flow to the myocardial region at risk. The factors involved in the initiation and the termination of the myocardial inflammatory response are not understood in detail, but would serve as an interesting target for the development of new therapeutic interventions aimed at preventing an extension of tissue injury either during the ischemic period or shortly after reperfusion. A number of studies (reviewed in [*4*]) have demonstrated that interventions which limit neutrophil function or which interfere with the cytotoxic factors produced by the infiltrating phagocytic cells can reduce ultimate infarct size resulting from a period of regional myocardial ischemia followed by reperfusion in the experimental animal. The role of the neutrophil as a contributor to myocardial injury in response to ischemia was suggested initially in 1971 [*10*]. More direct studies demonstrated that ultimate infarct size was reduced by 43% in the canine heart when neutropenic as compared to control animals were subjected to 90 mins of regional myocardial ischemia followed by reperfusion for 6 h [*11*]; an observation which subsequently was confirmed by other investigators using alternative means of inducing neutropenia [*12*].

Additional support for the concept of neutrophil mediated myocardial injury derives from the pharmacologic modulation of neutrophil function during myocardial ischemia and reperfusion. Recognition of the mechanism of the protection afforded by ibuprofen on experimental myocardial injury was an important step in addressing the role of the neutrophil in promoting reperfusion injury. In contrast to other inhibitors of cyclo-oxygenase such as indomethacin and aspirin [*13*, *14*], ibuprofen was noted to possess myocardial protective properties without altering regional myocardial blood flow or the relationship between myocardial oxygen supply and demand [*15*, *16*]. An analysis of the results from the several studies mentioned, indicates that of the non-steroidal anti-inflammatory agents examined, only ibuprofen reduced neutrophil infiltration to the reperfused myocardium suggesting that the PMN contributes to the extension of the tissue injury [*11*, *12*]. Aspirin did not reduce infarct size or the *in vitro* neutrophil respiratory burst in response to stimulation of the PMN with C5a. This was in contrast to the response of the PMN in the presence of ibuprofen which prevented the complement induced activation of PMN and the production of oxygen radicals [*14*]. These studies indicate that ibuprofen, a non-steroidal anti-inflammatory agent, does not exert its protective effect upon the myocardium by inhibition of the cyclo-oxygenase pathway of arachidonic acid metabolism or by altering those factors which restore the balance between myocardial oxygen supply and demand. Further evidence for the role of the neutrophil as a mediator of reperfusion injury was derived from studies in which prostacyclin or a related analog, iloprost, as well as prostaglandin E_1 limited ultimate infarct size in a canine model of regional ischemia and reperfusion. The mechanism of action was attributed to the ability of the prostanoids to inhibit the formation and release of neutrophil derived cytotoxic metabolites of oxygen [*17*, *18*, *19*]. The evidence derived from a number of studies is convincing and suggests that the pharmacologic inhibition of neutrophil function correlates with the observed beneficial effects on ischemic myocardial injury.

The role of the Chemotactic factors in the activation and recruitment of neutrophils

One of the most important sources of inflammatory mediators involved in the acute inflammatory response is the complement system. Activation of the complement system has been shown to generate a potent neutrophil chemotactic factor, C_{5a}, that plays an important role in the recruitment and activation of neutrophils, to sites of tissue injury. Proteolytic enzymes from both plasma and cell sources may also react with individual complement components resulting in the formation of inflammatory mediators, without causing generalized activation of the complement cascade. The presence of a tissue protease in ischemic myocardium was initially suggested as being involved in cleaving the third component of complement into chemotactically active fragments that may stimulate the attraction of neutrophils to the myocardium [*20*]. Recent evidence indicates that the cardiac lymphatic drainage from the ischemic myocardium contains molecules of mitochondrial and subcellular origin that are bound to C_{1q} which may activate the complement cascade, promote the release of leukotactic anaphylatoxins, and stimulate the migration of PMN's to sites of tissue injury [*21*]. The central role of the complement system in the recruitment and activation of the PMN within the ischemic myocardium is illustrated by the finding that depletion of complement with cobra venom factor significantly reduced PMN infiltration into ischemic myocardium and resulted in a decrease in tissue injury associated with regional ischemia and reperfusion [*10*, *22*]. Complement derived products can exacerbate ischemic tissue injury and cardiac dysfunction by mechanisms which are independent of the presence of neutrophils. Neutrophil independent injury by complement may be associated with the vascular actions of the C3a, C4a, and C5a, known to enhance vascular permeability and alter vessel tone as well as through the formation of the cytolytic membrane attack complex (C5b–9) which can result in the production of transmembrane channels and facilitate the influx of extracellular calcium and disruption of myocyte function [*23*].

Recent studies have also shown that endothelial cells may be an important source of a specific neutrophil chemotactic factor [*24*]. Treatment of endothelial cells with either interleukin-a (IL–1) or tumor necrosis factor α (TNF-α) will induce the synthesis and secretion of a neutrophil chemotactic factor similar in structure to a human monocyte derived neutrophil activating factor (NAF). NAF will not only promote neutrophil chemotaxis but also induce an increase in intracellular calcium, oxidative burst, and granule exocytosis via a GTP-binding protein dependent pathway; a response similar to that observed with other chemotactic peptides (e.g., C_{5a}, formyl-methionyl-leucyl-phenylalanine) [*25*]. The potential role that endothelial cell derived mediators may play in activating neutrophils following ischemic injury has not been investigated but represents a potentially important mechanism for the activation of circulating neutrophils in ischemic myocardium.

The role of adherence-promoting cell surface glycoprotein complexes

The surface of the PMN exhibits a family of heterodimeric glycoproteins possessing a common β subunit of 95 kDa (CD18) associated noncovalently with separate α subunits of 177, 165 and 150 kDa which are designated as LFA–1 (CD11a), Mol or Mac–1 (CD11b) and gp150 (CD11c) respectively. The Mol glycoprotein heterodimer is involved in the process of cellular adhesion and serves as the receptor for C3bi opsonized particles. It is believed that distinct stimuli mobilize Mol from intracellular sites and lead to its expression on the cell surface. Mol participates in adherence of the leukocyte to the endothelial cell and to other PMN's as well as in chemotaxis and spreading on cell surfaces. An increase of up to 2-fold in Mol cell-surface expression has been observed therefore suggesting that at least 50% of the total population of Mol receptors is located intracellularly. The increased expression of Mol would increase neutrophil adherence to cell surfaces and facilitate chemotaxis. The increase in Mol receptor expression is coupled to the release of neutrophil gelatinase which participates in the degradation of connective tissue thereby allowing for the passage of neutrophils

through capillary walls and into the extravascular space. The up-regulation of Mol, which is the receptor for C3bi, would allow for enhanced recognition and attachment to endothelial surface which have been "opsonized" (Fig. 1). Prior endothelial injury resulting from ischemia and reoxygenation promotes neutrophil adherence to the cell surface. PMN adherence may be due to the uncovering on the endothelium of specific cell surface molecules that directly interact with neutrophil CD18 molecules or receptors for C3bi; the latter serving as the receptor for Mol expressed on the surface of the neutrophil. *In vitro* studies have demonstrated enhanced binding of neutrophils to endothelial surfaces secondary to the interaction of neutrophil CD18 molecules with intercellular adhesion molecule-1 (ICAM-1) on the endothelial cell membrane [*26*]. Enhanced ICAM-1 expression on endothelial cell surface, can be induced by multiple inflammatory mediators including bacterial endotoxin and the cytokines IL-1, interferon-γ (INF-γ), and TNF-α [*27*]. Similarly, a distinct endothelial leukocyte adhesion molecule (ELAM-1) that promotes neutrophil binding to endothelium can be transiently induced by specific cytokines [*28*, *29*]. Whether similar increased expression of ELAM-1, ICAM-1 or ICAM-1 like molecules occurs in ischemic endothelium is not known, but potentially may play an important role in leukocyte localization to ischemic myocardium.

These studies in conjunction with the recent observation that specific cytokines will induce the synthesis and secretion of a neutrophil chemotactic factor by endothelial cells provide further evidence for an active role of the endothelial cell in modulating neutrophil ac-

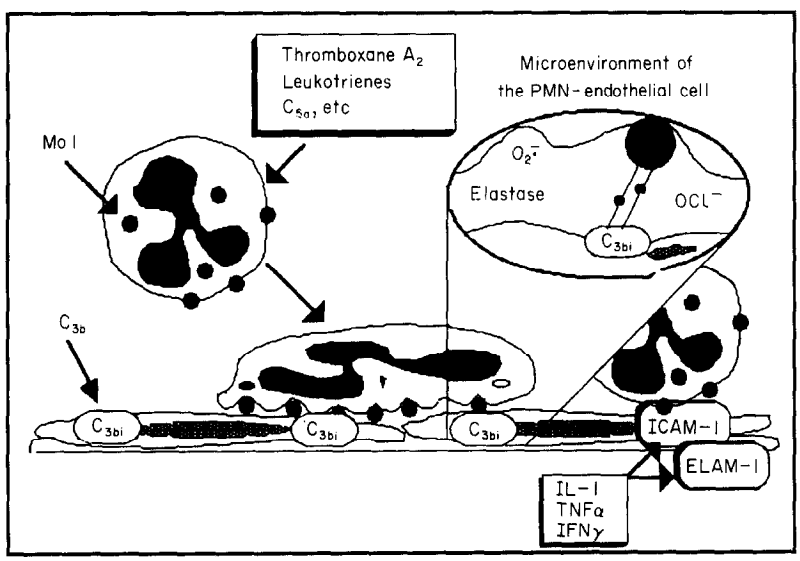

FIGURE 1. Schematic representation of the leukocyte–endothelial cell interaction in response to injury. Endothelial injury secondary to ischemia and reoxygenation promotes neutrophil adherence to the cell surface. Specific endothelial cell surface molecules interact with neutrophil CD18 glycoprotein adhesion promoting receptor. C3bi derived from the activation of the complement system in response to injury serves to "opsonize" the endothelial cell and is the receptor for Mol expressed on the surface of the neutrophil. Expression of the neutrophil CD11b/CD18 heterodimeric complex (Mol) on the plasma membrane of the neutrophil is a response to inflammatory stimuli (C5a, leukotrienes, thromboxane, etc.).

Neutrophil CD18 molecules can interact with intracellular adhesion molecule-1 (ICAM-1) on the endothelial cell membrane thereby serving as an alternate mechanism for the attachment of inflammatory cells to a target tissue. ICAM-1 and ELAM-1 (endothelial leukocyte adhesion molecule) are expressed in response to a variety of cytokines acting on the endothelial cell. In addition, the cytokines, interleukin-1 (IL-1), tumor necrosis factor (TNF) and interferon γ (IFN γ) will induce synthesis and secretion of a neutrophil chemotactic factor by endothelial cells thereby permitting the endothelium to participate actively in the recruitment and attachment of inflammatory cell mediators to the tissue which has been "targeted" as a result of injury. Whether or not the sequence of event described occurs in the heart which has been subjected to ischemia and reperfusion remains a topic of further study.

tivation and recruitment to ischemic tissues rather than a "passive" participant in the interaction of inflammatory cells with the vascular wall. The ability of endothelial cells and PMN's to attach to each other is required for an effective chemotactic response and for some forms of neutrophil mediated vascular injury and represents an important initial step in the sequence of events associated with the acute inflammatory response. A variety of agonists involved in acute inflammation, including thrombin, PAF and leukotriene C_4, are known to facilitate the adherence of neutrophils to the endothelial surface. The neutrophil CD18 glycoprotein molecules may participate in other functions aside from cell–cell adhesion and chemotaxis. The glycoproteins may be important in the transduction of signals from the cell surface to intracellular enzymatic regulatory systems as suggested by the presence of an extensive intracellular domain providing sites for interaction with cytoplasmic proteins and cytoskeletal elements.

Monoclonal antibodies specific for either the α or β subunits of Mol have been demonstrated to inhibit certain neutrophil/monocyte functions: (a) the binding of C3bi-opsonized particles, and (b) adhesive interactions of neutrophils and monocytes including neutrophil aggregation, monocyte/neutrophil adhesion and spreading on substrates (including vascular endothelium), and chemotaxis. The leukocytes of patients who are genetically deficient in their expression of the Mol glycoprotein exhibit an impairment of C3bi-particle binding and leukocyte adhesive interactions. It is suggested therefore that the Mol glycoprotein has a domain which functions as the plasma membrane receptor for C3bi(CR3). The critical role of Mol in the inflammatory response of activated neutrophils is suggested by the inhibitory effect of anti-Mol monoclonal antibody on neutrophil-mediated endothelial cell injury in a rat lung model of adult respiratory distress syndrome [*30*] and neutrophil-dependent vascular injury in skin [*31*]. A monoclonal antibody (904) that binds to the Mol leukocyte cell adhesion-promoting glycoprotein, CD11b/CD18, was administered to open chest anesthetized dogs 45 minutes after the induction of regional myocardial ischemia. Ischemia was produced by occluding the left circumflex coronary artery for 90 mins followed by reperfusion for 6 hours. There was no difference between control and antibody treated groups with respect to arterial blood pressure, heart rate or coronary blood flow. Administration of antibody produced no observable effect on circulating neutrophil counts, suggesting that antibody-bound neutrophils were not cleared from the circulation. The mean size of myocardial infarct expressed as percentage of the area at risk of infarction that resulted was reduced by 46% with anti-Mol treatment (Fig. 2). The area at risk of infarction was similar between groups. Accumulation of neutrophils within the myocardium was reduced significantly with anti-Mol antibody treatment [*32*]. Similar protective effects were obtained in subsequent studies with the anti-Mol F(ab')2 fragments in which antibody treatment was maintained for 48 h into the reperfusion period and infarct size determined at 72 h postreperfusion [*33*]. Furthermore, regional myocardial blood flow determinations indicated that both groups had equal degrees of ischemia in the inner 2/3 of the myocardium during the period of coronary artery occlusion. The results with the anti-Mol antibody indicate that the adhesive interactions of the neutrophil may play a central role in neutrophil mediated damage and that inhibition of neutrophil adhesive interactions can reduce the extent of irreversible myocardial damage associated with reperfusion. The results are in accord with previously mentioned studies in which myocardial reperfusion injury was reduced by a variety of interventions which shared the one common property of altering the ability of the neutrophil to react with its target tissue: the ischemically altered myocardial cell [*14, 12, 16, 19*].

The data from the above mentioned studies show that inhibition of neutrophil adhesion reduces the myocardial reperfusion injury and provided additional evidence for the important role of inflammatory cells in extending myocardial injury beyond that caused by the ischemic process itself. The data also support the hypothesis that a significant number of myocardial cells in the region at risk are viable after a 90-min inschemic period, but then progress to an irreversible state of injury upon reperfusion and the accumulation of polymor-

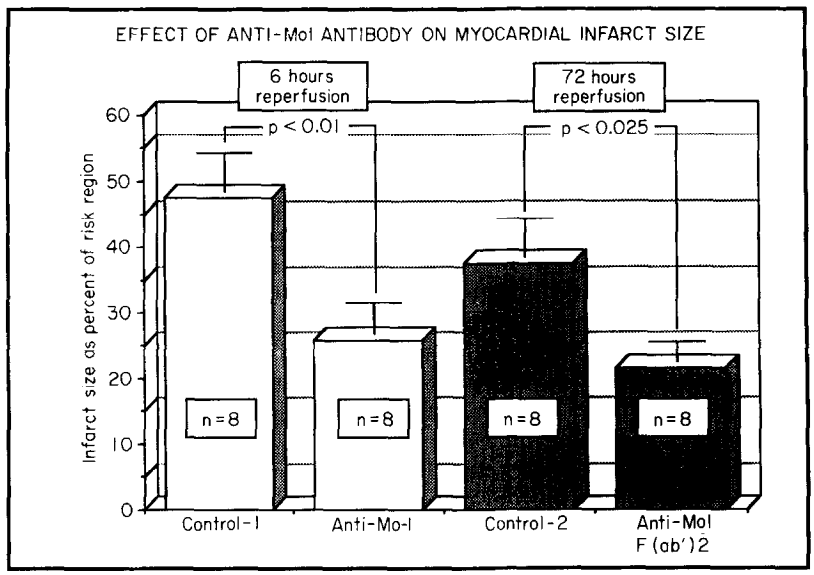

FIGURE 2. Myocardial infarct size in the canine heart expressed as a percentage of the risk region in a control group and in a group of animals treated with a murine monoclonal anti-Mol antibody. The two bars on the left represent the results from a study in which coronary artery occlusion was maintained for 90 mins and reperfusion lasted for 6 hours after which infarct size was determined. Treatment consisted of the administration of 1 mg/kg intravenously of Mol antibody given over 10 mins starting 45 mins after coronary artery occlusion. Inhibition of the Mol receptor by the anti-Mol antibody treatment resulted in a 46% reduction in myocardial infarct size as compared to a vehicle treated group.

The two bars on the right represent data taken from a similar study in which the F(ab'2) fragment of the Mol antibody was used in a dosing regimen of 1 mg/kg over 5 minutes starting 45 mins after coronary artery occlusion with repeated 0.5 mg/kg doses at 12, 24, 36 and 48 h after reperfusion. Infarct size was determined 24 h later (72 h after reperfusion). Despite the withdrawal of the antibody treatment, infarct size was reduced when determined at 72 h post-reperfusion. The data suggest that a sustained reduction in infarct size can be achieved by the modulation of neutrophil reactivity.

phonuclear leukocytes. Recent studies from this laboratory [*34*] have identified a specific "time window" in which neutrophils participate in the extension of myocardial infarction after ischemia and reperfusion. The inhibition of neutrophil activation and accumulation in the myocardium over a prolonged period of time (< 48 h) is necessary to ensure a beneficial effect on the ultimate extension of irreversible myocardial injury. Therefore, interventions which have limited pharmacologic half-lives while effective over brief periods of observation, may not show protective benefits if given in a single initial dose while assessment of infarct size is made at a period far removed from the point of administration of the treatment under study. Failure to recognize the pharmacokinetic properties of various interventions along with the dynamic and progressive nature of myocyte injury may account for many of the discordant results in the literature.

Neutrophils, oxygen radicals and myocardial injury due to ischemia and reperfusion

The observations enumerated above provide support for the hypothesis that under some conditions of regional myocardial ischemia and reperfusion, neutrophils adhere to the vessel wall at sites of inflammation and release toxic products capable of damaging the adjacent endothelium as well as myocytes within the reperfused region. It is well recognized that neutrophils can release cytotoxic products extracellularly without themselves undergoing destruction. Neutrophils can release a variety of mediators capable of promoting tissue injury, including proteolytic enzymes,

platelet-activating factor, arachidonic acid metabolites and activated species of oxygen. Among the last mentioned group of neutrophil derived products, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and hypochlorous anion (OCl^-) may be considered to be the most significant cytotoxic products derived from the metabolism of molecular oxygen upon perturbation of the PMN. It is proposed that the generation of the cytotoxic metabolites of oxygen generated within the micro environment formed between the adherent activated PMN's and altered endothelial cells leads to an increase in vascular permeability and myocyte damage which is "explosive" upon reperfusion as neutrophils are directed and attracted to the reperfused region under the influence of the local accumulation of chemoattractants. During an extended period of regional ischemia of 40 mins or longer [9], the stage is set for the neutrophil mediated inflammatory response and injury which involves cells which were normal or reversibly injured and is accelerated by reperfusion of the ischemic region. Thus, reperfusion injury involves cells which were viable or recoverable up until the time that reperfusion was instituted. It is suggested that the extension of cell death upon reperfusion of the ischemic myocardium is mediated, in part, by the neutrophil derived cytotoxic products of oxygen metabolism. It should be obvious that in the absence of reperfusion, all cells which are ischemic will undergo irreversible changes and that reperfusion is essential for survival of the myocytes which are viable up until the time of reperfusion. However, a portion of the still viable myocardial cells will undergo further injury due to the cytotoxic actions of the reactive species of oxygen.

The first suggestive evidence for the *in vivo* participation of oxygen radicals in myocardial reperfusion injury developed from the work of Jolly *et al.* [35] who demonstrated that the administration of superoxide dismutase plus catalase to anesthetized dogs subjected to a 90-min interval of regional myocardial ischemia followed by reperfusion reduced ultimate infarct size when the scavenger enzymes were administered either before the induction of regional myocardial ischemia or 15 mins before myocardial reperfusion. In contrast, the enzymes were without beneficial effect if they were infused 40 mins after reperfusion had been instituted. These observations suggested that an extension of myocardial injury, in addition to that associated with the ischemia-induced myocardial cell death, was occurring very early during reperfusion and that the myocardial damage could be attenuated by pretreatment with the free-radical metabolizing enzyme, superoxide dismutase. Subsequent studies have demonstrated that superoxide dismutase without the addition of catalase was equally effective in preventing the extension of myocardial injury, thereby implicating superoxide anion as the primary mediator of the myocardial damage associated with reperfusion. The relatively short pharmacologic half-life (6–10 mins) of superoxide dismutase represents a drawback to the study of the scavenger in experimental protocols which extend several days post-reperfusion. Neutrophil chemoattraction to the ischemically injured myocardium and the local formation of neutrophil derived reactive species of oxygen, will continue to effect a cytotoxic influence upon the viable myocardial cells in the reperfused tissue. The free radical induced injury, therefore, occurs soon after reperfusion and is sustained, until yet to be elucidated mechanisms bring about an arrest to the damaging effects of the inflammatory response.

The inability of recent studies to demonstrate a cardioprotective effect of superoxide dismutase may be attributable to the long interval between the administration of the enzyme, which has a brief half-life (6–10 mins), and the assessment of infarct size 4 days [36] or 7 days [37] later. The validity of this concept was examined using a modified form of superoxide dismutase in which the enzyme is conjugated to polyethylene glycol (PEG-SOD) [38]. The conjugated form of SOD has a pharmacologic half-life of 30 h. Significant plasma SOD activity can be demonstrated in the dog up to 7 days after a single intravenous administration of 1000 U/kg of PEG–SOD. Two protocols differing in the mode of administration and duration of the reperfusion interval were used. Dogs were subjected to occlusion of the circumflex coronary artery for 90 mins, then reperfused for either 6 h (Protocol A) or for 4 days (Protocol B). The animals received either polyethylene glycol conjugated

to albumin (PEG–ALB) or PEG–SOD 1000 U/kg. In Protocol A, treatment was administered starting 15 mins before coronary artery occulusion and continued for 2 h, terminating 15 mins after reperfusion. Infarct size was determined 6 h later. In Protocol B, the conjugated proteins were given 15 mins before reperfusion and ended simultaneously with reperfusion. Infarct size was measured after 4 days. A significant reduction in myocardial infarct size, expressed as a percent of the area at risk, was observed in Protocol A in the group of animals which received PEG–SOD as compared to the PEG–ALB treated controls. Likewise, in the extended protocol in which infarct size was determined 4 days after reperfusion, the PEG–SOD treated group exhibited a significant reduction in ultimate infarct size (Fig. 3). Hemodynamic variables did not differ during the period of coronary artery occlusion. The respective collateral flood flows to the inner two thirds of ischemic myocardium determined 60 mins after occlusion did not differ between the groups. Infarct size was related inversely to collateral blood flow in the PEG–ALB treated group. The regression line describing the relationship between the extent of irreversible myocardial injury and collateral blood flow was shifted downward (analysis of covariance, $P = 0.017$) in the group of animals treated with PEG–SOD. Using the mean collateral blood flow to the inner two thirds of the ischemic myocardium as a covariate, infarct size expressed as a percentage of the area at risk was smaller for the PEG–SOD treated group than for the PEG–ALB treated group ($P < 0.05$). The data demonstrate that unlike unmodified superoxide dismutase [*36*, *37*], PEG–SOD can achieve a reduction in ultimate infarct size when quantitation of myocardial injury is done after 4 days of reperfusion, a time when the plasma superoxide dismutase activity is maintained well above that found in the PEG–ALB control group. These data are consistent with studies noted above demonstrating the necessity to suppress neutrophil functions up to 48 h to effect a reduction in infarct size at 72 h [*34*] and support the hypothesis that there exists a critical "time window" during which antineutrophil and/or antioxidant therapy must be maintained to effect a

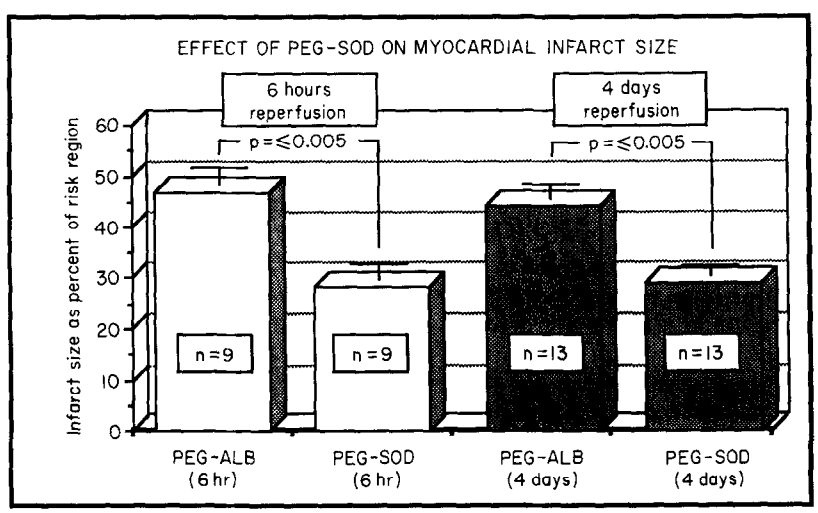

FIGURE 3. The effect of polyethylene-glycol conjugated superoxide dismutase (PEG–SOD) on myocardial infarct size due to a 90 min occlusion and subsequent reperfusion of the left circumflex coronary artery in the canine heart. A single dose of PEG–SOD of 1000 U/kg was administered before reperfusion in each of the two studies. The control groups received polyethylene-glycol conjugated to albumin so that the administered amount of PEG and conjugated protein was comparable in all animals. Infarct size in the two groups of dogs depicted on the left was determined after 6 h of reperfusion. The two groups of dogs shown on the right had infarct measurements made 4 days after reperfusion. The data illustrate that PEG-SOD with a 30 h plasma half-life is able to provide sustained protection against the cytotoxic effects of superoxide anion derived in part from the infiltration of inflammatory cells to the reperfused myocardium.

reduction in the ultimate extent of the infarcted myocardium following periods of ischemia and reperfusion. The potential beneficial effect of PEG–SOD, as compared to the native form of the enzyme, is demonstrated in a recently published study [*39*] in which the sustained presence of the oxygen radical scavenger prevented rather than delayed the development of irreversible myocardial cell injury.

The superior efficacy of PEG–SOD compared to the native enzyme may relate to both its continued presence in the circulation and its ability to adhere to the surface of the vascular endothelium. Polyethylene glycol conjugation of superoxide dismutase enhances cell association and uptake of the enzyme in a manner that renders the vascular endothelium more oxidant resistant. The vulnerability of the endothelium to injury from reactive species of oxygen may be related to a number of factors, which include the anatomical proximity to activated and invading inflammatory cells and localization of xanthine oxidase activity within endothelial cells. Vascular endothelial cells subjected to an ischemic equivalent of anoxic incubation followed by reoxygenation become effective generators of oxygen free radicals as determined by electron paramagnetic resonance measurements with a spin trap agent, DMPO [*40*]. Superoxide dismutase or catalase abolished the radical signal, suggesting that oxygen is sequentially reduced from O_2^- to H_2O_2 to $^{\bullet}OH$. Attenuation of the free radical signal by oxypurinol suggested that xanthine oxidase was a major source of the superoxide anion. It is conceivable that upon reperfusion of the ischemic myocardium, the endothelial cells as well as the adherent and infiltrating neutrophils may provide the initial source of cytotoxic species of reduced oxygen and that the subsequent inflammatory process involving the continued migration and accumulation of PMN's to the injured myocardium provides a sustained source of oxygen radicals which diminished with time as reparative processes supervene.

Regardless of the cellular site for their generation (endothelial cell, neutrophil, or other cellular loci) the role of reactive species of oxygen in mediating cellular injury associated with reperfusion has gained wide support through a number of studies which have been reviewed recently [*5, 41, 4, 3*]. The literature has not always agreed with the concept that reperfusion of the ischemic myocardium may result in an extension of injury beyond that which has resulted from the ischemic insult itself. It is our belief that the inconsistent observations among laboratories are related to important differences in the models employed with respect to instrumentation of the heart in chronic animal studies, the duration of occlusion (90 mins vs 40 min or 3 h), and the duration therapy relative to the duration of reperfusion. Superoxide dismutase, neutrophil depletion, and ibuprofen, which inhibits neutrophil-mediated injury, did not limit the extent of myocardial injury in studies that employed a coronary occlusion lasting 3 h [*42, 43, 44*]. Most important is the need to consider the pharmacokinetic properties of the respective interventions employed to modify the extent of reperfusion injury in view of the fact that the neutrophil mediated component of reperfusion injury may be operative for several days [*34*]. Previously reported negative studies with native SOD may be related to the short half-life of its free radical scavenging capacity, which compromises the chances of observing a protective effect after 4 days of reperfusion. On the other hand, protocols which involve a 40 min ischemic interval may not be of sufficient duration to allow for recruitment of the inflammatory response. Thus, the resulting tissue damage may be related primarily to the ischemic insult. In the latter instance, it would be difficult to detect a beneficial response to an intervention directed against cell damage related to reperfusion which we believe is mediated in large part by the cytotoxic action of invading inflammatory cells.

The progression of tissue injury and cell death is a time dependent phenomenon involving cooperative interactions resulting in the activation of the complement system, formation of chemotactic factors, cytokine mediated stimulation of the endothelium and expression of cell surface adhesive receptors, all of which play an active role in mediating the inflammatory response. The complex sequence of events, once initiated, are likely to continue, albeit for a limited duration which may be measured in days rather than minutes. The answer to the question of whether inter-

ventions administered before or at the time of reperfusion can limit the ultimate extent of tissue injury remains unanswered. Efforts must be devoted towards eliminating differences among those studies which report disparate results. This essay has been developed with the intent of calling attention to some of the multiple factors which can mediate tissue injury. It is our hope that the reader will be stimulated toward further study, especially concerning those immunologic aspects of the problem which have as yet not been explored thoroughly in the setting of myocardial reperfusion injury.

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