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Reperfusion Injury After Myocardial Infarction: The Role of Free Radicals and the Inflammatory Response

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Development of thrombolytic therapy as a treatment for myocardial infarction has focused attention on the events that occur upon reperfusion of ischemic myocardial tissue. Although it is well documented that salvage of the ischemic myocardium is dependent upon timely reperfusion, it is likely that the very events critical for survival may, in fact, lead to further tissue injury. A widely recognized source of reperfusion injury is the generation of oxygen-derived free radicals. These reactive oxygen species, which are formed within the first moments of reperfusion, are known to be cytotoxic to surrounding cells. In addition, strong support exists for the involvement of the inflammatory system in mediating tissue damage upon reperfusion. Coincident with the recruitment of neutrophils and activation of the complement system is an increase in the loss of viable cells. Although a number of mechanisms are likely to be involved in reperfusion injury, this discussion focuses on the roles that oxygen-derived free radicals and the inflammatory system play in mediating reperfusion injury.

KEY WORDS: adhesion molecules; complement; inflammation; membrane attack complex (MAC); myocardial ischemia; neutrophil; oxygen paradox; oxygen radical; reperfusion.

Abbreviations

ATP	Adenosine triphosphate
CANP	Calcium-activated neutral proteases
O_2^-	Superoxide anion
H_2O_2	Hydrogen peroxide
OH^-	Hydroxyl radical
CD11b	Mo1
CD11a	LFA-1
CD11c	gp150
LTB4	Leukotriene B4
PAF	Platelet-activating factor
ICAM-1	Intracellular adhesion molecule-1
IL-1	Interleukin-1
TNF	Tumor necrosis factor
ELAM	Endothelial-leukocyte adhesion molecule
HOCl	Hypochlorous acid
RNHCl	Chloramine
rt-PA	Tissue plasminogen activator
DAF	Decay accelerating factor
HRF	Homologous restriction factor
CD59	Protectin
CR1	Complement receptor type 1
sCR1	Recombinant soluble CR1

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Introduction

Although early reperfusion of the ischemic myocardium is important for preservation of tissue viability, it now is apparent that reperfusion may in itself be harmful to the surrounding tissue [reviewed in (1–3)]. Thus, a paradoxical situation develops where reoxygenation, which is essential for survival of the tissue, may in fact be harmful. Damage due to the restoration of blood flow is termed "reperfusion injury." Simply defined, reperfusion injury is the conversion of reversibly injured cells to a state of irreversible injury due to the reintroduction of flow to an ischemic area (4). The detrimental effects of reperfusion injury have received greater attention in recent years due to the use of thrombolytic agents to manage patients with an evolving acute myocardial infarction. The use of lytic therapy has been shown to reduce mortality in patients undergoing an acute myocardial infarction (5); however, it is becoming increasingly evident that the events coincident with thrombolysis also are of importance.

The concept of reperfusion injury was first put forth by Hearse in 1977 (6). Before this time, it was assumed that the increase in cell death upon reintroduction of blood flow was due to the death of myocytes that were previously irreversibly injured. However, investigation into this phenomenon has suggested that the cells damaged upon reperfusion were in fact viable before the reintroduction of blood flow (3). Thus, although essential for survival, reperfusion may be associated with the risk of extending the area of myocardial injury beyond that originally attributed to the ischemic process. This conclusion suggests that a number of crucial events occurring during reperfusion induce or enhance cel-

lular injury. It should be pointed out, however, that the concept of reperfusion injury has yet to be accepted by all and that there are inconsistencies among the experimental results. A number of investigators believe that the reintroduction of flow to an ischemic area only increases the rate of death for cells that were irreversibly injured as a result of the ischemic insult (7). Conclusive proof for the existence of reperfusion injury would require experimental evidence indicating that cells that were viable before reperfusion are irreversibly injured upon or soon after the onset of reperfusion (3). Direct evidence of the conversion of ischemic cells to an irreversibly injured state upon reperfusion is lacking. Our laboratory has shown that the binding of a labeled monoclonal antibody to the intracellular protein, myosin, is significantly increased upon reoxygenation of the hypoxic rabbit isolated heart (8). The antimyosin antibody has been shown in previous studies to bind to myocytes that have decreased membrane integrity associated with irreversible injury (9). Thus, the results support the concept that sudden reoxygenation of the hypoxic heart is associated with extension of irreversible myocardial damage.

The ability of reperfusion to elicit additional cellular damage makes it imperative that the mechanism(s) of reperfusion injury be understood. Understanding these mechanisms may greatly augment the existing procedures that are currently administered to the patient undergoing an acute ischemic event. The quest to determine the mechanisms involved in reperfusion injury has led to the discovery of a number of causative factors. Having recognized the importance of minimizing the extent of reperfusion injury, a great deal of effort is being devoted to the development of therapeutic approaches to limit damage incurred during reperfusion. Although research into this area has focused on the myocardium, the process is not solely limited to this tissue. Any tissue or organ deprived of blood flow is subject to the events related with reperfusion injury (10). Therefore, this concept is of interest to physicians in a number of clinical settings including organ transplantation and any surgical intervention where blood flow to an organ is interrupted. This discussion will focus on the events that lead to an extension of cellular injury associated with reperfusion of the ischemic myocardium. Special attention will be placed on the roles of oxygen-derived free radicals and the inflammatory response in initiating reperfusion injury.

Oxygen and calcium paradoxes

The reintroduction of flow to an ischemic or hypoxic area has been associated with the development of two similar events known as the "oxygen paradox" and the "calcium paradox."

Both events have been characterized by the development of myocardial contracture, release of intracellular cytosolic enzymes, loss of mechanical activity, and changes in myocyte ultrastructure (11). One of the early indications that molecular oxygen was involved in the development of reperfusion injury was the observation that reperfusion of the ischemic heart with an oxygenated solution enhanced injury whereas reperfusion with an hypoxic solution did not increase myocardial injury (12). Both *in vitro* and *in vivo* studies have shown that the reintroduction of molecular oxygen to the ischemic myocardium is accompanied by the formation of oxygen-derived free radicals (13,14). The appearance of cytotoxic oxygen metabolites upon myocardial reperfusion suggests an important role for molecular oxygen as a mediator of myocardial tissue damage. It was suggested by Hearse *et al.* that the reintroduction of oxygen may induce injury through transmembrane calcium fluxes that result in intracellular calcium accumulation (15). This suggestion would aid in explaining the numerous similarities between the oxygen and calcium paradoxes.

The calcium paradox is characterized by a rapid increase in the intracellular free calcium concentration. Whereas an increased tissue calcium concentration is not seen during 60 min of global ischemia, there is a tenfold increase within the first 10 min of reperfusion (16). Several possibilities exist as to the route of calcium entry during the calcium paradox. It was widely assumed that calcium was derived from external sources including entry through voltage-sensitive calcium channels and sodium-calcium exchange mechanisms (17,18). However, the use of calcium-channel blockers in experimental models of ischemia-reperfusion have shown inconsistent results in protecting the ischemic heart (19,20). Supporting this observation is a study by Nayler *et al.* showing that high doses of calcium-channel blockers result only in a partial decrease in intracellular calcium levels (21). Currently, there is debate as to whether the influx of calcium into the cell is due to the ischemia-induced disruption of the membrane. Thus, the influx of calcium may be a result of free radical-induced membrane damage and not truly a cause of membrane injury (22). Intracellular sites have been mentioned as a potential source for the increase in intracellular calcium. It has been hypothesized that the formation of intracellular free radicals following reperfusion may cause leakage of calcium from intracellular stores such as the sarcoplasmic reticulum, although this view is not shared by all investigators (21). In addition, it is conceivable that the cell may lose the ability to extrude calcium or that the uptake of calcium by the sarcoplasmic reticulum is decreased (23).

A number of biochemical events have been postulated to occur upon calcium influx. Foremost is the activation of different groups of intracellular enzymes. Activation of phospholipases may lead to formation of cell-damaging arachidonic acid metabolites and depletion of adenosine triphosphate (ATP)

stores (24,25). Furthermore, a number of proteases are known to be activated in the presence of calcium. Among these proteases are the calcium-activated neutral proteases (CANP). This group of enzymes have been reported to mediate myofibrillar turnover and the degradation of various proteins including cytoskeletal filaments (26). A number of ultrastructural changes are associated with calcium influx. The development of contracture bands and the formation of large amorphous densities within the mitochondria are examples of the most dramatic changes in the cellular ultrastructure. The appearance of these ultrastructural markers is largely considered to be indicative of irreversible injury and cell death (27).

Effects of free radical generation in reperfusion injury

Oxygen-derived free radicals have been strongly implicated in the extension of tissue injury following reperfusion. This highly reactive and unstable group of compounds is formed as a result of the addition of an unpaired electron to the outer orbital of the molecule. Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical (OH^-) are the most prominently mentioned free radicals in the pathogenesis of reperfusion injury.

Evidence for the existence of free radicals in hearts subjected to reperfusion is derived from the use of electron-resonance spectroscopy and spin-trapping agents to detect free radicals in the ischemic zone following reperfusion (13,14). Perhaps though, the strongest evidence lies in the ability of free radical scavengers to limit infarct size in experimental models of myocardial infarction. A number of investigators have shown that radical scavengers including superoxide dismutase and peroxide-degrading agents such as catalase (Figure 1) are capable of eliminating the radicals thus protecting the reperfused myocardium (28,29). While naturally present in the myocardium, these protective agents may be limited and subsequently overwhelmed by the sudden generation of free radicals upon reperfusion (30). Thus, the addition of these substances prior to reperfusion may decrease the concentration of the newly generated radicals.

$$2O_2^{\cdot} + 2H^+ \xrightarrow{\text{Superoxide Dismutase}} H_2O_2 + O_2$$

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2\,H_2O + O_2$$

Figure 1 — Reactions catalyzed by superoxide dismutase and catalase. Generation of superoxide anion and its subsequent conversion to hydrogen peroxide ultimately results in the formation of water and oxygen through the action of these enzymes.

Mechanisms of free radical-induced damage

Adding to the destructive nature of oxygen radicals is their ability to attack and damage a number of critical cellular components. The cell membrane, composed primarily of lipid and proteins, is one of the most vulnerable areas to attack (31). Free radicals have the ability to alter membrane lipids in a number of different fashions. Unsaturated lipids containing double bonds are especially vulnerable to peroxidation, resulting in the formation of lipid peroxides, aldehydes, and lipid hydroperoxides (31). Membrane-bound proteins are another likely target for free radical damage. The ability of these radicals to oxidize sulfhydryl groups on methionine residues allows proteins to be altered in a number of different ways including conformational changes, denaturation, and enhanced susceptibility to hydrolysis (32,33). These groups are important in the transport of organic cations and are often located near active sites of enzymes. Amino acids such as tyrosine, proline, tryptophan, and phenylalanine are oxidized by free radicals. It is important to point out that alteration of membrane lipids may have an indirect effect by altering the surrounding environment, the net result being the loss of the protein's ability to function (34). Membrane proteins that serve enzymatic or receptor functions would be especially vulnerable to this type of alteration (34). In addition, intermediates of lipid peroxidation have been shown to alter the protein function through fragmentation or polymerization of proteins (35). Damage to critical membrane proteins and lipids would have serious repercussions on the ability of the cell to function normally, calling into question the cell's ability to survive.

Production of free radicals

The major source of free radicals during ischemia and reperfusion has yet to be fully elucidated. One commonly mentioned source is the enzyme xanthine oxidase, found primarily within the vascular endothelium (36). Following ischemia, xanthine dehydrogenase, which is responsible for the metabolism of hypoxanthine, is converted to xanthine oxidase. This enzyme is thought to produce O_2^- and H_2O_2 through the utilization of hypoxanthine as a substrate (37). Experimental evidence for the role of xanthine oxidase in reperfusion injury is seen in that allopurinol, an inhibitor of the enzyme, has been reported to afford protection in reperfused tissues (38). However, these studies were conducted in species, such as the canine, where xanthine oxidase is known to be present. In other species including rabbit and human, both xanthine dehydrogenase and xanthine oxidase are virtually absent, casting doubt that this is the predominant route of radical production in humans (39,40). Therefore, other routes of free radical production are likely to exist.

Cardiac myocytes and endothelial cells have been

implicated as a free radical source. A number of organelles including the mitochondria and nuclear membrane have been shown to generate free radicals. Thus, in response to an ischemic event, organelles may begin to produce reactive oxygen species. In this scenario, myocytes produce both H_2O_2 and superoxide by the cyclooxygenase and lipoxygenase pathways during the synthesis of arachidonic acid metabolites (41). The electron transport chain of mitochondria has been mentioned as a potential source of free radicals in both the endothelial cell and myocyte (42). Under normal physiologic conditions, mitochondria produce H_2O_2; thus, it is plausible that during ischemia, production is increased or the ability to dispose of H_2O_2 is lost (37). The latter explanation is supported by the observation that cellular elements that serve to protect the tissue from free radical attack such as glutathione, glutathione peroxidase, and superoxide dismutase are decreased during ischemia and reperfusion (43). Free radicals produced by intracellular organelles have the ability to cross biological membranes. Thus, radicals generated in an environment where defense mechanisms have been impaired have the ability to inflict damage throughout the entire cell and surrounding tissue.

Role of the polymorphonuclear leukocyte

A critical aspect of reperfusion injury is the infiltration of polymorphonuclear leukocytes into the ischemic zone [reviewed in (44,45)]. While the primary role of the neutrophil is to protect the host from infectious agents, the infiltration and subsequent activation of these cells may indeed prove detrimental to the surrounding myocardium. Infiltration of neutrophils into the ischemic zone begins within 60 min after the onset of ischemia and increases progressively for up to 90 min after reperfusion (46). Early evidence for the involvement of neutrophils in reperfusion injury is derived from a number of investigators. These studies showed that nonsteroidal, antiinflammatory agents such as ibuprofen could protect the myocardium in *in vivo* models of myocardial infarction (47,48). In addition, Romson and associates noted that the reduction in infarct size seen in canines treated with ibuprofen was associated with a reduction in neutrophil infiltration into the area at risk (48). Direct evidence for the role of the neutrophil in eliciting myocardial damage comes from studies showing that depletion or inhibition of neutrophil adhesion prior to ischemia decreased infarct size (49,50). Furthermore, monoclonal antibodies directed against leukocyte adhesion molecules have been shown to decrease infarct size. In one such study, Simpson *et al.* administered a monoclonal antibody directed against the CD11b/CD18 adhesion molecule (Mol) to an open chest dog 45 min after the start of a 90 min period of myocardial ischemia. Following 6 h of reperfusion, it was noted that the administration of this antibody decreased infarct size by 46% when compared to control (51). The ability of the Mol antibody to reduce infarct size not only provides a therapeutic approach to limiting damage, but illustrates the role of adhesion molecules in mediating this process.

Neutrophil recruitment: The role of adhesion molecules and chemotactic factors

A number of critical events must occur during and after an ischemic event in order for a sufficient number of neutrophils to accumulate and elicit myocardial damage. Chemotactic factors, derived from a variety of sources, serve to activate the neutrophil and amplify the inflammatory response. These factors include fragments of complement activation such as C5a and C3a; and arachidonic acid-derived products including leukotriene B_4 (LTB_4). In addition to the direct effects of free radical formation, it has been proposed that the superoxide anion may act as a chemoattractant for neutrophils (52). Concurrent with the formation and release of these chemotactic factors is the upregulation of a number of adhesion receptors, located on both the neutrophil and endothelial cell. Adhesion and subsequent migration of the neutrophil into the surrounding tissue is a complex process consisting of a number of steps including: rolling of the neutrophil along the endothelial cell surface; movement through the endothelium (diapedesis); and extravascular migration into the tissue. These steps involve a number of distinct groups of adhesion receptors and a number of cell-derived mediators.

The selectin family of adhesion molecules mediates the early events associated with neutrophil adhesion [Figure 2; (53,54)]. Upon activation of the endothelial cell, P-selectin (GMP-140, PADGEM), localized within intracellular granules, is mobilized to the cell surface. Another member of the selectin family, L-selectin (Mel-14, LECAM-1), in conjunction with P-selectin, is involved in the "rolling" of the neutrophil along the endothelium. L-selectin is shed from the neutrophil upon activation, coincident with the upregulation of CD11b (53). Von Andrian and colleagues (55) suggest a two-step model for adhesion: L-selectin and P-selectin act in concert to facilitate neutrophil recruitment into the microenvironment of the vasculature. Before movement out of the vasculature, a longer lasting adhesion, mediated *via* the leukocyte β_2 integrins (CD11/CD18 complex) is formed (55). Another molecule that may play a role in the early events of adhesion is platelet-activating factor (PAF). This biologically active phospholipid is formed in conjunction with the release of arachidonic acid and transported to the cell surface (56). PAF functions in a duel manner by direct activation of neutrophils and acting indirectly through upregulation of an endothelial adhesion molecule responsible for facilitating "rolling" of neutrophils on the endothelial surface (56–58). The rolling phenomenon permits the neutrophil to maintain a close attachment to the endothelial cell, allowing for activation and the establishment of a firm attachment to the endothelium. Rolling of the neutro-

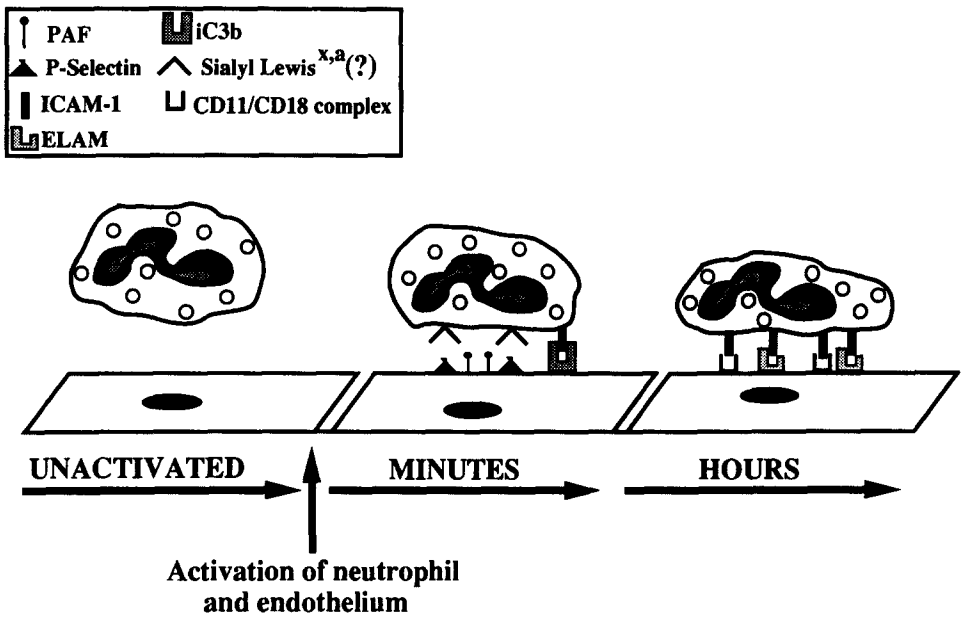

Figure 2 — Adhesion of neutrophils to the endothelium is a complex process requiring a number of steps. The early events (minutes) of adhesion are characterized by increased expression of P-selectin and PAF by the endothelial cell. Attachment of the neutrophil to the endothelium through these means allows the endothelial cell to upregulate different adhesion molecules including ICAM-1 and E-selectin.

phil is dependent upon the neutrophil receptor, L-selectin, and its endothelial cell-associated ligand, P-selectin. The adherent neutrophils undergo activation *via* interaction of their β_2 integrin adhesion-promoting receptors and the endothelial cell-associated ligands (59). Lorant *et al.* (60) have proposed that PAF, in conjunction with P-selectin, may act to adhere the neutrophil to the endothelial cell and signal the cell to increase expression of the CD11/CD18 integrins. Thus, the action of PAF and P-selectin would provide a means of securing the neutrophil to the endothelium during the upregulation of the CD11/CD18 complex (see Figure 2).

As mentioned previously, firm attachment of the neutrophil to the endothelial cell requires another group of proteins known as the CD11/CD18 β_2 integrins. These glycoproteins possess a common β subunit (CD18) noncovalently bound to a distinct alpha subunit designated as either LFA-1 (CD11a), Mol (CD11b, Mac-1), or gp150 (CD11c). Like P-selectin, Mol is stored in intracellular sites and is mobilized to the cell surface in response to the appropriate stimuli (*e.g.*, C5a). At the surface, the molecule serves as the receptor for complement-derived iC3b opsinized particles and is involved in not only adhesion, but also chemotaxis and spreading of the neutrophil (61,62). The importance of the Mol subunit in mediating neutrophil adherence and reperfusion injury is seen in the ability of antibodies directed against Mol to decrease the extent of ischemia/reperfusion injury in the canine myocardium (51,63). In addition to serving as the receptor for iC3b, Mol interacts with intracellular adhesion molecule-1 (ICAM-1) located on the endothelial surface. Inactive endothelial cells normally express low levels of ICAM-1. However, stimulation by cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) upregulate expression of ICAM-1 and endothelial-leukocyte adhesion molecule (ELAM, E-selectin), a member of the selectin family. The upregulation of these ligands is maximal within 4 to 6 h (64). Antibodies to ICAM-1 not only decrease adhesion of neutrophils, but reduce infarct size in the ischemic/reperfused rabbit heart (65,66). In summary, the sequence of events associated with adhesion involves a number of neutrophil–endothelial cell interactions that are expressed sequentially over the course of many hours ultimately culminating in the inflammatory response to injury. It is likely that the early events (minutes) of adhesion are mediated through a transient interaction between the neutrophil and endothelium mediated *via* the selectins (L-selectin, P-selectin), and the phospholipid molecule, PAF. The β_2 integrins Mol and LFA-1 are likely responsible for adhesion lasting longer periods of time (minutes to hours; see Figure 2). During this time, the neutrophil may become firmly attached to the endothelium before movement out of the vasculature into the surrounding tissue. During episodes of chronic inflammation the third member of the β_2 integrin family, Gp 150.95, is likely to become the primary mediator of neutrophil adhesion (67). Expression of the neutrophil β_2 integrin, Mo-1 (CD11/CD18), is related to the stimulation of the neutrophil by C5a and the endothelial cell ligand for Mo-1 is iC3b. The inflammatory response to injury, therefore, involves activation of the complement system that is responsible, in part, for orchestrating the carefully timed sequence of events associated with the recruitment of neutrophils to the site of injury and ultimately, the repair of the damage.

Mediators of neutrophil-induced myocardial injury

Activated neutrophils are able to elicit tissue injury through several different mechanisms including generation of oxygen-derived free radicals and release of cytotoxic lysosomal enzymes. Chemotactic factors including C5a, PAF, and cytokines are capable of activating neutrophils. Stimulation of the neutrophils by one or more of these factors elicits the "respiratory burst," characterized by a sudden increase in oxygen consumption and a release of reactive oxygen metabolites into the surrounding environment. Superoxide anion, hypochlorous acid (HOCl), OH^-, and chloramine ($RNHCl^-$) are the oxidants produced by the stimulated neutrophil. In addition, circulating PAF stimulates neutrophils to synthesize H_2O_2 (68) that has been shown to induce a PAF-dependent adherence of neutrophils to the endothelium (69). Thus, generation of H_2O_2 by the neutrophil may act as a positive feedback mechanism, allowing the neutrophil to recruit other circulating neutrophils to the injured tissue. Evidence for the role of reactive radicals in reperfusion injury, coupled with the ability of neutrophils to produce free radicals, implicates this mechanism as one way by which neutrophils elicit tissue damage.

Coinciding with the generation of oxygen-derived free radicals by the neutrophil is the release of a number of cytotoxic proteases stored in intracellular granules. A number of these granule products have the capacity to alter vascular permeability, thereby aiding the movement of the neutrophil into the surrounding tissue. Cationic proteins and neutral proteases serve to alter vascular permeability and disrupt the basement membrane of the vascular wall. Two metalloproteases, collagenase and gelatinase, when activated by HOCl, are capable of degrading collagen and lysing endothelial cells (70). Other important lysosomal enzymes released during activation include elastase and heparinase, the latter participating in degradation of heparin sulfate within the subendothelial matrix. An inhibitor of elastase, α_1-antiproteinase, is present in normal tissue to decrease the injurious effects of elastase. However, this inhibitor is sensitive to oxidation by neutrophil-derived HOCl, resulting in decreased affinity of the inhibitor for elastase (71). The role that protease release plays in reperfusion injury remains controversial. Some proteolysis inhibitors, such as aprotinin, have been shown to limit canine myocardial infarction, although the mechanism of this protection has yet to be substantiated (72,73). Furthermore, Bolli and co-workers found that the suppression of protease activity by a number of different inhibitors failed to decrease infarct size in the rat heart (74,75).

Controversy does arise when discussing which substances released from the neutrophil play a significant role in reperfusion damage. On that same note, it is not entirely clear whether the cells must emigrate from the endothelium into the surrounding tissue in order to elicit injury or if damage to the endothelial cell is sufficient. It is likely that the neutrophil causes deleterious effects in both the vasculature and surrounding tissue. Since neutrophils can form aggregates, small capillaries may become physically "plugged" and represent the underlying mechanism for the "no reflow phenomenon," where areas of the ischemic region are not properly reperfused (76). Neutrophils also may affect larger vessels such as arterioles and precapillary vessels. Release of vasoconstricting agents from the activated neutrophils are thought to decrease vessel diameter, resulting in decreased perfusion of the surrounding tissue (77). The decrease in perfusion may be exacerbated by release from the neutrophil of factors such as PAF, which serves to activate circulating platelets. The accumulation of platelets in the reperfused area would allow for an increase in vascular plugging in addition to release of platelet-derived factors that act upon the vasculature (78).

Complement-mediated injury of the myocardium

Recent attention has been focused on the role of the complement system in reperfusion injury (Figure 3). Like the neutrophil, the basic role of the complement system is in the defense of the host from microbial invasion. However, the complement system has been implicated in a number of adverse conditions including the exacerbation of reperfusion injury. First suggested by Hill and Ward (79), evidence has accumulated for the role of complement in reperfusion injury. A number of investigators have utilized the ability of cobra venom factor and other agents to decomplement experimental animals before the induction of myocardial infarction (80,81). Upon subsequent reperfusion, a decrease in infarct size was noted in addition to decreased neutrophil infiltration into the ischemic zone. These observations have resulted in numerous investigations to determine the effects of complement activation on the myocardium and the factors responsible for activating the complement cascade.

Two primary pathways have been described as to how complement activation elicits myocardial damage. The first, as mentioned previously, is through the generation of chemotactic factors and anaphylatoxins such as the cleavage products C3a, C4a, and C5a. These three products stimulate mast cells and basophils to release histamine, increasing vascular permeability [Figure 4; (82,83)]. In addition, both C3a and C5a act as chemotactic factors to amplify the inflammatory response by attracting neutrophils. Thus, the generation of these cleavage products play a role in the early events (increasing vascular permeability) and later events (recruitment of neutrophils) associated with reperfusion injury. Activation of the complement system also mediates direct myocardial damage through formation of anaphylatoxins and the membrane attack complex (MAC). While the anaphylatoxins, C3a, C4a, and C5a act primarily in an indirect manner, assembly

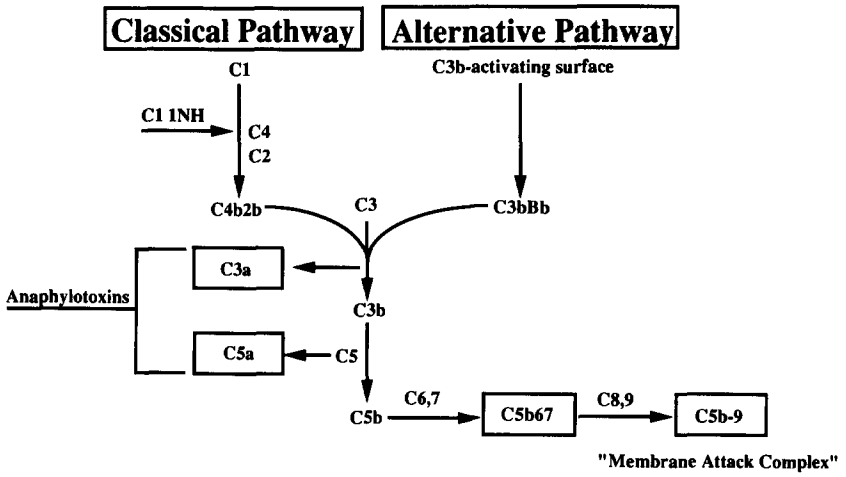

Figure 3 — Schematic outline of the complement system. Activation of complement ultimately results in the formation of the anaphylatoxins and the membrane attack complex (MAC).

and insertion of MAC within the membrane of both the endothelial cell and cardiomyocyte will lead to further tissue injury.

The MAC is an amphiphilic complex composed of complement proteins C5b–9. Activation of either the classical or alternative pathways of complement is associated with the formation of the C5b–7 trimolecular complex. This complex has the ability to associate with phospholipid membranes, most likely through an interaction of the membrane with C7, which contains phospholipid binding sites (84). Once this complex is inserted into the membrane of the target cell, proteins C8 and C9 become associated with the C5b–7 complex. Although the attachment of C8 to the C5b–7 complex is sufficient to cause cell lysis, it is the addition of multiple units of C9 that results in rapid cell destruction (85). The attachment of at least 12–18 C9 monomers to the C5b–8 complex within the membrane is associated with the formation of a circular pore or channel spanning the membrane (85).

Deposition of the MAC and other indicators of complement activation have been noted in areas of infarction while the surrounding normal tissue remains relatively free of complement components (86,87). Furthermore, concentrations of the soluble form of the MAC have been shown to increase in the plasma after an ischemic event (88,89). The effects of MAC deposition on nucleated cells varies widely. In sublytic amounts, deposition of the complex may act as a stimulatory signal leading to activation of both protein kinase C and G proteins, phospholipid

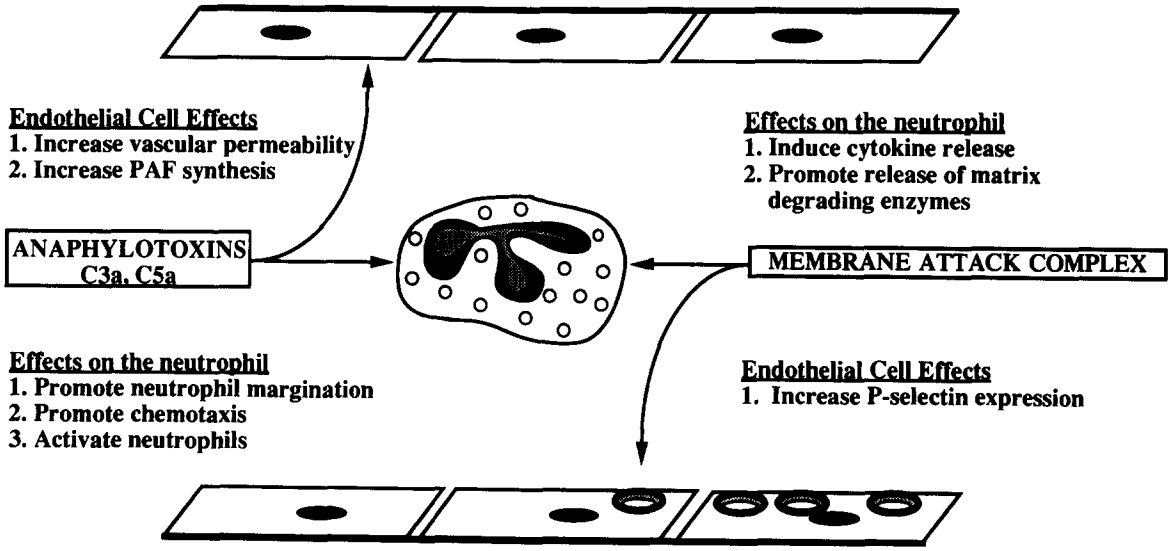

Figure 4 — Role of activated complement in mediating neutrophil adhesion to the endothelium. Products of complement activation, primarily consisting of the anaphylatoxins and the MAC, elicit their effects upon both the endothelium and the neutrophil. The anaphylatoxins C3a and C5a promote endothelial cells to synthesize PAF in addition to increasing vascular permeability. C3a and C5a also promote neutrophil migration and chemotaxis as well as activate the neutrophil. Formation of the MAC on the endothelium has been shown to lead to the expression of P-selectin. When formed on the neutrophil, the MAC may induce the release of cytokines and proteolytic enzymes.

turnover, and increased intracellular calcium (90,91). The MAC also may participate in initiating the inflammatory response by promoting neutrophil adhesion to the endothelium (Figure 4). Hattori *et al.* (92) have demonstrated that human endothelial cells exposed to sublytic amounts of C5b–9 protein rapidly express the neutrophil adhesion molecule P-selectin. Although the neutrophil is, for the most part, resistant to MAC formation, it is possible that deposition of the complex can form on the cell surface and once bound, the MAC may stimulate the neutrophil to release cytokines and matrix degrading enzymes, as has been previously noted in other cell types [see Figure 4; (93)]. Formation of the MAC also may directly injure endothelial cells and cardiomyocytes within the ischemic zone. When these cells have sustained "multiple hits" from the MAC, water, ions, and small proteins can move into and out of the cell. The free movement of these molecules may lead to disruption of cellular function and eventual cell lysis as a result of a loss of intracellular osmotic control.

The ability of the MAC to cause myocardial injury has been demonstrated recently in the functioning heart by Homeister *et al.* (94). In this study, human plasma was perfused through the rabbit isolated heat. Activation of the human complement system by the rabbit tissue resulted in cardiac damage, characterized by changes in cardiac function and release of intracellular enzymes. Evidence from this and subsequent studies revealed that deposition of the MAC was the primary agent responsible for the myocardial injury (95).

The pathway of complement activation during ischemia/reperfusion remains to be elucidated. The use of thrombolytic agents such as recombinant tissue plasminogen activator (rt-PA) has been associated with activation of complement (96). Plasmin, which is activated upon administration of rt-PA, is able to cleave component C1, resulting in activation of the entire cascade (97). Because activation of complement is associated with hearts that have not received rt-PA, it is likely that other mechanisms exist for complement activation after an ischemic event. A number of studies have shown that oxygen-derived free radicals have the ability to activate the complement cascade by converting C5 to a functionally active C5b-like form (98,99). In addition, Shingu and colleagues have shown that hydrogen peroxide and peroxide-like radicals released by activated neutrophils will activate complement (100).

Recent attention has been focused on the loss of the protective mechanisms that serve to guard cells from complement attack. Nucleated cells contain a number of protective mechanisms against complement activation [reviewed in (101)]. These defense mechanisms require the cell to be metabolically active (102). Thus, ischemia/reperfusion may metabolically impair the affected cells, leaving them susceptible to attack by complement. Damage to membrane proteins, possibly by free radical generation, also may play a role in complement-mediated damage. A number of cells express numerous regulatory proteins on their membrane that serve to protect the cell from attack by endogenous complement. These proteins include: decay accelerating factor (DAF), homologous restriction factor (HRF), and protectin [CD59; (103–106)]. At least one of these protective proteins, protectin, has been shown to be lost or damaged following an ischemic event (107). Another of these proteins, complement receptor type 1 (CR1) is thought to provide the greatest inhibitory effect. CR1 is a membrane protein found primarily on peripheral blood cells (84). A study by Weisman and colleagues (108) utilized a recombinant soluble form of the CR1 receptor (sCR1) to inhibit the effects of complement during ischemia and reperfusion. The sCR1 protein lacks the transmembrane and cytosolic domains, rendering it free to exert its protective effects in the soluble form (108). Addition of sCR1 to a rat infarct model resulted in a 44% reduction in infarct size and a decrease in neutrophil infiltration. Several additional reports have supported the role of sCR1 in protecting the myocardium (95,109,110). The protective effect of sCR1 not only provides additional evidence for the role of complement in myocardial injury, but may also provide a potential therapeutic avenue by which to decrease reperfusion injury.

Conclusions

Although the concept of reperfusion injury has become widely accepted, the question remains: to what extent is reperfusion involved in the genesis of myocardial injury and would therapeutic intervention be beneficial to the infarcted patient? There is no complete answer as yet, but it is suggested that reperfusion of the myocardium is associated with an increase in cell death. Furthermore, prevention of events associated with reperfusion, such as formation of oxygen-derived free radicals and prevention of neutrophil accumulation, has been shown to be beneficial in preserving the myocardium in a number of experimental models. The possibility remains that adjunct therapy administered at the time of thrombolysis may serve to decrease the detrimental effects of reperfusion and thus provide additional therapeutic benefits to the patient. As more knowledge becomes available regarding reperfusion injury, it becomes readily apparent that a number of contributing factors are involved. Recognition that the inflammatory response is associated with the pathogenesis of this type of injury has provided a number of additional avenues by which one may intervene in the progression of this type of injury. Inhibition of the complement system is particularly appealing in that inactivation of this pathway may afford protection against the actions of the MAC in addition to decreasing the infiltration of neutrophils into the ischemic zone. However, it is likely that any effective therapeutic intervention would require inhibition of a multitude of events, including both free radical formation and the inflammatory response.

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