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The neutrophil as a mediator of myocardial ischemia-reperfusion injury: time to move on

Abstract Granulocytes, especially neutrophils, are recruited in myocardium during the evolution of acute myocardial infarction. Because the neutrophil reaction is most intense during reperfusion and because these cells are a rich source of toxic oxidant species and proteolytic enzymes, it has become a widely held view that neutrophils are an important mechanism of myocardial injury extension during reperfusion. However, on close examination the evidence underlying this contention is equivocal. The basic experimental situation can be summarised thus. (1) All forms of reperfusion injury (i.e., cytotoxic or lethal cell injury, myocardial stunning, endothelial dysfunction, and reperfusion-induced arrhythmias) can be observed in neutrophil-free conditions. (2) "Anti-neutrophil" interventions (e.g., anti-inflammatory drugs, adenosine, anti-neutrophil antisera, leukocyte filters and inhibitors of the various pathways of neutrophil adhesion) do not consistently prevent reperfusion injury and they certainly do not consistently limit infarct size. (3) The time course of neutrophil accumulation in post-ischaemic myocardium may be different to the time course of injury. (4) Despite more than two decades of research, no double-blind, randomised controlled clinical trial assessing an anti-neutrophil therapy in myocardial infarction has yet reported a positive benefit that is attributable to inhibition of neutrophil recruitment. The evidence weighs against a pivotal role of neutrophils as a causal factor in most forms of ischemia-reperfusion injury. An exception may be microvascular injury and capillary plugging leading to the "no-reflow" phenomenon but even here the evidence suggests that the extent of neutrophil accumulation and microvascular injury is determined by, rather than a cause of, myocyte necrosis.

Key words Neutrophil – polymorphonuclear leukocyte – ischemia – reperfusion – myocardial infarction – stunning – adhesion molecule – endothelium

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Intravascular and tissue accumulation of neutrophils (polymorphonuclear leukocytes) is a characteristic component of the inflammatory response in many forms of tissue injury and infection. The intravascular margination and subsequent tissue infiltration of neutrophils is observed in myocardial ischemia-reperfusion injury. A widely-held view is that the neutrophil response in ischemic-reperfused myocardium is a progenitor of secondary inflammatory damage leading to patterns of injury now commonly termed "reperfusion injuries". These include lethal or irreversible reperfusion injury that may lead to extension of the infarcted zone, myocardial stunning resulting in prolonged depression of post-ischemic contractile function in myocardium that is not

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irreversibly injured, reperfusion-induced arrhythmias, and microvascular endothelial damage associated with the "no-reflow" phenomenon. While a contributory role of neutrophils to these reperfusion injuries may seem biologically plausible, the experimental evidence supporting this injurious role is, I would suggest, contentious or insufficiently reproducible to make a convincing case. Here, I will concentrate particularly on disputing the role of neutrophil-mediated cell injury in myocardial infarction. I shall also consider the role of the neutrophil in myocardial stunning, reperfusion arrhythmias, and in the mediation of the "no-reflow" phenomenon.

Neutrophil accumulation in myocardial ischemia-reperfusion

Granulocytic infiltration has long been recognised as a histological hallmark of recent myocardial infarction. The time course of histological changes, including neutrophil accumulation, in unreperfused human myocardial infarction was beautifully documented by Mallory et al. more than 60 years ago (40). The first signs of accumulation of granulocytes, predominantly neutrophils, were observed during the 24 hours after the onset of coronary occlusion. The intensity of the neutrophil infiltrate peaked at around 4 days and then practically completely disappeared by 14 days. Mallory et al. remarked

> *The exact function of the polymorphonuclear leucocytes is difficult to explain. … they produce no definite change in the muscle fibers that can be recognized histologically*

In experimental infarction, neutrophil accumulation in infarcted myocardium was observed histologically after 12 hours ischemia (34). In another study, 2 hours ischemia followed by 2 hours reperfusion was also associated with histologically detectable neutrophil accumulation (59). Intravascular margination of neutrophils, an early event preceding transmigration and interstitial accumulation, was histologically detectable within the infarcted zone after 4 hours of ischemia in a canine coronary artery occlusion model (57). Interestingly, reperfusion following a much briefer (40 min) period of coronary artery occlusion was found to accelerate the accumulation of neutrophils. Many subsequent experimental studies have contributed to a current view that neutrophils accumulate gradually during ischemia, especially at the peripheral edges of the infarcted area, and that reperfusion of the ischemic myocardium intensifies or accelerates neutrophil accumulation in infarcted myocardium (18, 19, 24, 42).

Thus, in myocardial infarction there is clear evidence of an intense intravascular neutrophil accumulation and tissue infiltration. In ascribing a purpose to the neutrophil response, it would seem reasonable to regard this as part of a concerted inflammatory reaction to necrosis. Key aspects of the molecular pathology of neutrophil chemotaxis have been elucidated in recent years. Activation of the complement system components during ischemia and reperfusion and the rapid upregulation or induction of several endothelium- and myocyte-borne adhesion factors including P-selectin, E-selectin, ICAM-1 and PECAM-1, set the scene for the first steps in neutrophil recruitment, i.e., margination and rolling (22, 26). The evolutionary origins of inflammation would suggest that the neutrophil recruitment is one feature of a coordinated response that promotes tissue healing and scar formation following necrosis. This is really not a point of controversy. More problematic are assertions that the neutrophil response is demonstrably deleterious and that this necessary inflammatory response accounts for major forms of secondary injury during reperfusion.

Controversy about the role of the neutrophil in ischemia-reperfusion injury stems from the notion that gained currency in the mid-1980s that the neutrophil influx, accelerated during reperfusion, might account for the free radical burst associated with the early phase of reperfusion (26, 39, 49). Activated and degranulating neutrophils are known to be a rich source of several toxic reactive oxygen and reactive nitrogen species, including superoxide, hydroxyl radical, hypochlorous acid and peroxynitrite anion, as well as lysosomal proteolytic enzymes. The idea that neutrophil infiltration and reperfusion injury go hand-in-hand has almost become an article of faith in some quarters. However, how good is the evidence to support a pivotal deleterious role of neutrophils in the pathophysiology of ischemia-reperfusion injury?

Anti-neutrophil interventions and infarct size

Duration of ischemia is the primary determinant of infarct size. The rate of evolution of infarction may be modified by secondary determinants such as the extent of collateral vessel formation and temperature. In humans and in large collateralised experimental species such as the dog, the rate of advance of the necrotic wavefront may be variable but substantial infarction, tending towards a transmural necrosis will be established within a few hours of coronary artery occlusion (47). In small experimental species relatively devoid of a native collateral circulation, such as the rat and rabbit, transmural myocardial infarction may be established within 30–60 minutes of the onset of coronary artery occlusion (63). The question of whether subsequent reperfusion causes further tissue injury beyond that sustained during ischemia is not definitively answered but in relation to

the role of the neutrophil, it is the area that has been most extensively researched.

The possibility that reperfusion may actually kill cells is highly controversial: we know that ischemic tissue has to be reperfused to salvage it. One school of thought is that some cells are irreversibly injured during ischemia and although "condemned to die" are not yet dead (31). The abrupt introduction of oxygen and calcium merely hastens the death of these cells. In other words, the process of necrosis that was initiated during ischemia is hastened and completed by reperfusion. The other school of thought is that the generation of free radicals, calcium overload and activation of proteolytic enzymes are so deleterious that they can overwhelm cells that were uninjured or only reversibly injured by ischemia (39). The further contribution of myocyte apoptosis as a mechanism of irreversible injury during reperfusion has been recognised more recently (21).

Early evidence supporting a causal role of neutrophil recruitment in the evolution of experimental infarction dates from the late 1970's. In the early 1970s, Hill and Ward (29) demonstrated that viper venom depletion of the C3 complement fraction was associated with a reduced myocardial granulocytosis 48 hours after permanent coronary artery ligation in the rat. Maroko et al. (41) later reported that complement depletion by cobra venom treatment limited the extent of infarction in a canine model of coronary artery occlusion and reperfusion. In 1982, Lucchesi's group (51) reported that treatment of dogs with the non-steroidal anti-inflammatory drug ibuprofen limited the extent of infarction due to 60 minutes left circumflex coronary artery occlusion and 24 hours reperfusion. Limitation of infarct size was associated with a substantial reduction in the accumulation of ^{111}In-labelled neutrophils in the infarcted tissue. This finding prompted the authors to conclude that suppression of the inflammatory response caused sparing of the ischemic myocardium. The following year, the same group (50) extended this observation by publishing a seminal study which at that time provided the most convincing evidence of a direct causal association between neutrophil recruitment and experimental myocardial infarction. Dogs rendered neutropenic (approximately 80% reduction in circuating neutrophil count) by anti-neutrophil antiserum were subjected to 90 min left circumflex coronary artery occlusion and 6 hours reperfusion. Infarct size in the neutropenic dogs was substantially smaller than that in untreated controls (47% of risk zone in control animals vs 27% of risk zone in neutropenic animals). This remarkable finding prompted the authors to conclude that

> *…neutrophil accumulation in ischemic myocardial tissue is an important determinant of the ultimate extent of tissue necrosis due to myocardial ischemic injury.*

Subsequently, throughout the 1980s and early 1990s many studies were undertaken to evaluate the association between infarct-limiting interventions and a reduction in neutrophil accumulation (commonly determined by either tissue myeloperoxidase activity within the infarct or border-zone or gamma-counting of ^{111}In-labelled neutrophils). Many of these studies would today be regarded as technically inappropriate and their conclusions suspect because they used permanent coronary artery occlusion models. No such studies are quoted here. A number of well-designed studies using appropriate models described experimental infarct size limitation and inhibition of neutrophil recruitment with a variety of interventions:

- non-steroidal anti-inflammatory drugs such as ibuprofen (11, 43, 51)
- neutrophil-depleting treatments such as anti-neutrophil antisera and leukocyte filters (5, 38, 50)
- monoclonal antibodies to endothelial and neutrophil adhesion molecules (3, 27, 55)
- inhibitors of neutrophil adhesion such as sialyl Lewis^x analogues (35)
- non-specific inhibitors of neutrophil adhesion such as adenosine and prostacyclin at reperfusion (30, 44, 45, 54, 65)

However, even with publication bias in favour of studies with a positive outcome, it is clear that infarct size limitation as a consequence of neutrophil depletion or inhibition is not a robustly reproducible phenomenon.

- Neutropenia induced in dogs by anti-neutrophil antiserum had no influence on infarct size (9, 13)
- in a variety of models, infarct limitation was not seen with non-steroidal anti-inflammatory drug treatment either pre-ischemically or during reperfusion (1, 48)
- in the canine open chest model of infarction, no protection was observed with anti-CD18 antibody (58). In the rabbit, anti-CD18 antibody limited infarct size when given at reperfusion following 30 min coronary artery occlusion but not when given after 45 min coronary artery occlusion (61)
- in the rabbit and dog, adenosine at reperfusion failed to show any infarct-limiting action (8, 25, 60)
- in the rabbit (6) and dog (23) sialyl Lewis^x analogues failed to show any infarct limiting action
- in the rat, a combined inhibitor of complement C5a with a sialyl Lewis^x analogue limited infarct size when given at reperfusion but this action was dissociated from any effect on neutrophil accumulation (64)

The reasons for this divergent and conflicting literature are difficult to discern. Arguments for and against the various influences of species differences, sub-total neutropenia, inadequate doses and timing of inhibitor compounds have been advanced but ultimately they are

unsatisfying. Latterly, we have witnessed the advent of genetically manipulated animals with ablation of components of the neutrophil adhesion mechanism, such as neutrophil or endothelium adhesion molecules. Mice with targetted deletion of P-selectin, ICAM-1 and CD18 have been reported to show reduced tendency to ischemia-reperfusion injury (see reference 32) but the interpretation of gene knockout studies may not always be straightforward. Caution is especially important in cases such as the present one where there is an exceptionally insecure backdrop provided by traditional technical approaches. There may well exist important and as yet unknown model-dependent reasons for the divergent literature upon which we can only speculate at present. The argument that duration of reperfusion employed in standard experimental models may be critical for the evaluation of neutrophil involvement in infarct expansion during reperfusion is worth considering briefly. In experimental infarct size studies, tetrazolium macrochemistry may be applied after 3 hours reperfusion to distinguish between necrotic and viable tissue. Prolonged periods of reperfusion (24–48 hours) do not increase the reliability of the technique. Characterisation of tetrazolium macrochemistry in experimental models has confirmed that the tetrazolium defect seen in infarcted tissuebetween 3–6 hours reperfusion corresponds with infarct size seen at 24–48 hours reperfusion using histological assessment. The idea that there is a "late" phase of reperfusion injury contributing to infarct size expansion, to which neutrophils might contribute, is simply not supported by experimental evidence.

Ultimately, the case for neutrophils playing any significant role in the evolution of infarction is substantially impaired by the clear demonstration of infarction in blood-free experimental preparations. A single example will suffice to illustrate this simple and easily overlooked point (62). In the anesthetised rabbit at physiological temperature (38 °C), 30 min coronary artery occlusion and 180 minutes reperfusion resulted in a tetrazolium-determined infarct size of 36% of the ischemic risk zone. In the same laboratory, the same group reported that in isolated, buffer-perfused rabbit hearts at 37 °C subjected to the same coronary occlusion insult resulted in infarct size 28% of the ischemic risk zone. The difference in infarct size in the two experimental preparations can be entirely explained by temperature (38 °C vs. 37 °C) since in the rabbit heart a 1 °C temperature change produces approximately 7% change in infarct size (10).

Neutrophils and myocardial stunning

Brief periods of ischemia, insufficient to cause irreversible cell injury, may render the myocardium susceptible to severe contractile dysfunction during subsequent reperfusion (28). For a short period, the role of neutrophils as mediators of myocardial stunning was researched by several groups. Evidence supporting a role of neutrophils was first advanced by Engler and Covell (16). Subsequent work detailing the time course of neutrophil recruitment in stunned myocardium (24) and work with a variety of neutrophil depleting interventions (33) defined the conclusion that neutrophils do not contribute to myocardial stunning (7).

Neutrophils and ischemia-reperfusion arrhythmias

The occurrence of tachyarrhythmias (ventricular premature beats, ventricular tachycardia and ventricular fibrillation) during coronary artery occlusion and reperfusion is a well-established clinical and experimental phenomenon. Life-threatening ischemia-induced arrhythmias may occur within minutes of the onset of coronary artery occlusion (phase 1 arrhythmias) or later during the evolution of myocardial infarction (phase 2 arrhythmias). Reperfusion-induced arrhythmias usually occur during the first minutes of reperfusion and in experimental models they are more likely to occur following brief periods of preceding ischemia. A wealth of evidence suggests that ischemia and reperfusion arrhythmias occur as a result of re-entrant electrical circuits in the ischemic-reperfused myocardium. The molecular basis of re-entry is still not fully understood but predisposing factors in ischemia-reperfusion include intracellular Ca^{2+} overload, extracellular K^+ and reactive oxygen species (12, 36). The contributory role neutrophils in mediating these arrhythmias *in vivo* has not been the subject of comprehensive investigation to date. However, it is clear that both ischemic arrhythmias and reperfusion arrhythmias occur very readily in isolated crystalloid buffer-perfused hearts.

The time of onset of phase 1 ischemic arrhythmias is inconsistent with a pathogenetic role of neutrophils since there is scant evidence of recruitment of these cells during the first hour of ischemia. The severity of infarct-associated phase 2 arrhythmias is determined by the extent of infarction. Because infarct size is probably a determinant of the extent of neutrophil recruitment, rather than *vice versa*, secure dissociation of the roles of irreversible tissue injury and neutrophil accumulation as determinants of phase 2 arrhythmias are impossible in most published studies where arrhythmia has been reported as an endpoint. Late-occurring ventricular arrhythmias are certainly a feature of regional infarction in isolated crystalloid-perfused heart preparations but no studies are available to clearly define a role of neutrophils *in vivo*. With regard to reperfusion-induced arrhythmias, two studies have suggested that neutrophils may contribute to reperfusion arrhythmogenesis (13,

14). However, a characteristic feature of these arrhythmias in experimental models is that they are most severe following a relatively brief episode of ischemia superseded by rapid reperfusion. Under these circumstances, the brevity of ischemia would tend to argue against an important role of neutrophil recruitment in the genesis of reperfusion arrhythmias.

Neutrophils and microvascular injury

A clinically-relevant manifestation of reperfusion injury is the "no-reflow" phenomenon associated with microvascular injury. No-reflow was initially described following cerebral and renal ischemia but the first comprehensive investigation of this pathology in myocardium was made by Kloner and colleagues in a canine coronary artery occlusion model (37). They reported that following prolonged ischemia (but not after brief ischemia), distinct areas of a perfusion deficit were detectable after restoration of epicardial coronary artery flow. Subsequent studies revealed characteristic ultrastructural defects in the microvascular endothelium in the areas of anatomical perfusion defect. Temporal analysis of the microvascular injury suggested that it occurred after irreversible cardiac myocyte injury and cardiac myocyte swelling, leading to compression of the microvessels. Engler et al. (17) elegantly demonstrated the contribution of capillary (but not arteriole or venule) plugging by neutrophils in the no-reflow areas of the previously ischemic region. Ambrosio and colleagues demonstrated that microvascular injury and no-reflow were related, at least in part, to events occurring during reperfusion since the development of the regional flow deficit occurred progressively during reperfusion (2). This gradual development of the perfusion defect was associated with significant accumulation of neutrophils in the areas of no-reflow. However, it is clear that the no-reflow phenomenon is a complex pathology with several contributory mechanisms of which intracapillary neutrophil plugging is but one (46). As with all the other reperfusion injuries, the severity of the microvascular endothelial injury is related to the duration of the preceding ischemia. Areas of no-reflow occur only within the infarcted tissue and follow the development of irreversible cardiac myocyte injury. While endothelial adhesion molecules are upregulated in the infarcted zone, facilitating neutrophil-endothelium interaction, it is arguably the case that microvascular injury begets neutrophil adhesion rather than *vice versa*. Although "anti-neutrophil" interventions have been reported in some experimental studies to attenuate the no-reflow phenomenon (38), the literature does not reveal a consistent pattern of protection with these treatments (6). The fact that areas of no-reflow can be demonstrated within crystalloid buffer-perfused hearts (53, 56) re-inforces the view that neutrophil plugging and neutrophil-mediated damage are, at best, secondary contributory mechanisms in the no-reflow phenomenon.

Clinical trials

In view of the inconclusive, or indeed negative, outcome of a substantial pre-clinical literature, it is little surprise that clinical trials of anti-neutrophil interventions in acute myocardial infarction have been consistently unrewarding. Anti-inflammatory drugs such as non-steroidal anti-inflammatory drugs or corticosteroids have no place in the management of acute myocardial infarction. Two clinical trials, FESTIVAL (52) and LIMIT-AMI (4), have been reported recently in which humanised anti-CD18 monoclonal antibodies were administered in acute myocardial infarction as adjunctive therapy to reperfusion. In neither study was there any significant difference in infarct size or clinical outcome. Ischemic stroke is a condition in which the molecular pathology of tissue injury is similar to acute myocardial infarction, and in which there is a similarly inconsistent preclinical literature with regard to neutrophil mediated injury. The recently reported EAST trial of enlimomab, a murine antibody to ICAM-1, as adjunct to conventional treatment in ischemic stroke showed no neurological or survival benefit (20). Indeed, patients treated with enlimomab had a significantly worse outcome and higher mortality.

Some commentators on the anti-CD18 trials have (perhaps unfairly) focused blame for their failure on the inadequacy of experimental animal models (15). I would argue strongly that the real cause of the failed trials lies in a failure to fully appreciate and properly weigh the preclinical literature. The experimental literature on the causal role of neutrophils in myocardial ischemia-reperfusion injury is clearly inconsistent. Negative pre-clinical studies say as much as, if not more than, the studies with positive outcomes. In such a scenario, wilfully ignoring carefully-conducted negative studies may not only be a high-risk and costly business strategy, but is also anti-scientific.

Conclusion

In summary, neutrophil infiltration occurs as an inevitable consequence of myocardial infarction but a direct causal association between neutrophils and myocardial cell death is unproven. Rather, it is conceivable, indeed probable, that the extent of myocardial injury determines the intensity of neutrophil infiltration rather than *vice versa*. Thus, cytoprotective interventions that limit the

extent of irreversible injury will result in a reduction in the number of recruited neutrophils. The assumption that limitation in infarct size is a consequence of neutrophil inhibition has often been made and is usually unjustified. The experimental evidence to support a role of secondary injury due to neutrophils, resulting in infarct extension during reperfusion, is not robustly reproducible. Modelling of acute myocardial infarction in crystalloid buffer-perfused hearts, devoid of any formed blood elements, demonstrates that myocardial ischemia-reperfusion causes infarction by primary pathological mechanisms intrinsic to the tissue. The search for effective therapeutic approaches in the treatment of myocardial ischemia-reperfusion injury continues with increasing urgency while a coronary artery disease pandemic threatens. Meanwhile, more than two decades of research have left a legacy of controversy, confusion, irreproducible data, clinical disappointment and financial loss. In the absence of evidence that is secure enough to convict the neutrophil beyond reasonable doubt, surely it is time to move on, to re-orient our attitude to leukocytosis in myocardial ischemia-reperfusion and to concentrate effort on elucidating the primary mechanisms of injury.

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