Neutrophil-induced Myocardial Cell Damage and Active Oxygen Metabolites

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Free radicals derived from polymorphonuclear leukocytes (PMN) have been suggested to play an important role in myocardial ischemia-reperfusion injury. To define the mechanism by which activated PMN exacerbate ischemic myocardial damage, we investigated the extent of cell injury, free radical generation and lipid peroxidation in embryo mouse myocardial cells co-incubated with activated PMN. The generation of free radicals derived from PMN correlated with the extent of myocardial cell injury. Among the cell sheets preconditioned with hypoxic and glucose free medium, PMN-adhered myocardial cells were initially injured after adding PMN activator, extending to adjacent cells. Chemiluminescence emission and thio-barbituric acid reactive substance in the co-incubated cells were markedly increased and sustained compared with those in each cell mono-incubation. The augmented lipid peroxidation was related to the progression of myocardial cell injury. These results indicate that PMN-derived free radicals cause membrane disruption, contributing to the progression of myocardial injury.

ACCUMULATION of polymorphonuclear leukocytes (PMN) during the acute inflammatory response may exacerbate tissue injury through the release of active oxygen species, eicosanoids (arachidonic acid metabolites) and lysosomal enzymes. Recent experimental studies suggest that PMN, infiltrating in response to myocardial ischemia, play an important role in the propagation of myocardial necrosis resulting from coronary artery occlusion and reperfusion. Although the PMN-mediated reactions are important to the repair process in the irreversibly damaged myocardium, they may also result in the destruction of viable myocardial tissue. The detrimental effect of PMN can be supported by the evidence which shows that in experimental animals infarct size can be limited by anti-inflammatory agents that prevent PMN infiltration, as well as by depleting circulating PMN.

There are two possible pathways through which ischemic myocardial cell injury is enhanced by stimulated PMN: one is the indirect effect caused by coronary microcirculatory disorders, intravascular plugging of PMN; the other is the direct effect caused by the release of cytotoxic substances by PMN in the extravascular space. It has been postulated that the direct (cytotoxic) effect could be initiated by complements activated at the surface membrane of the ischemic myocardial cell.

To elucidate the mechanism by which stimulated PMN exacerbate ischemic injury, the role of PMN in myocardial cell injury will be investigated in detail.

Key words:
Free Radicals
Polymorphonuclear leukocytes
Myocardial cell injury

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myocardial injury, we investigated the correlation between the morphological deterioration of the myocardial cells and production of oxygen free radicals using cultured cardiac myocytes co-incubated with PMN. Results of these experiments indicate that oxygen free radicals derived from PMN could promote hypoxic myocardial cell damage leading to cell death with structural deterioration.

A Cellular Model of PMN-induced Myocardial Damage

Myocardial cells were obtained by trypsin treatment of embryo mouse (ddy strain) ventricles as described previously and were suspended in minimum essential medium containing 10% fetal bovine serum (3-5×10^4 cells/dish) at 37°C in humidified air with 5% CO_2 for 1 day. The cultured myocytes formed cell sheets consisting of 5—20 synchronously beating cells. The cultured cells were incubated in glucose free Hanks’ balanced salt solution (HBSS) for 60 min under hypoxia at 37°C in a closed chamber, into which was passed humidified hypoxic gas (95% N_2, 5% CO_2). After hypoxic incubation, the gas was changed to normal gas (95% air, 5% CO_2) and the medium was replaced by PMN suspension (3×10^6 PMN/ml HBSS/dish). PMN were prepared from heparinized peripheral blood of human volunteers by the Percoll gradient method.

The reoxygenated myocardial cells were incubated with PMN for 30 min, and thereafter phorbol 12-myristate 13-acetate (PMA,

![Fig. 1. Experimental protocol](image1)

![Fig. 2. Phase contrast microscopy of myocardial cells at same field before and after co-incubation with PMA activated PMN. Reoxygenated myocytes (a) showing no morphological change. These cells were co-incubated PMA activated PMN. After 60 min, these cells appeared rounded and retracted with bleb formation (b).](image2)
TABLE I INCIDENCE OF DAMAGED MYOCARDIAL CELLS (%)

<table>
<thead>
<tr>
<th></th>
<th>Control Cells</th>
<th>Ischemic Cells</th>
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<tr>
<td>PMN</td>
<td>2.5±0.4</td>
<td>3.6±0.5</td>
</tr>
<tr>
<td>PMA</td>
<td>2.1±0.5</td>
<td>3.8±0.5</td>
</tr>
<tr>
<td>PMN+PMA</td>
<td>57.4±8.0</td>
<td>89.6±4.0</td>
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*mean±SEM

Normal conditioned myocytes or reoxygenated myocytes were co-incubated with PMA (10 ng/ml) activated PMN (3×10⁶). Percent of injured myocardial cells was identified by erythrosine B exclusion.

PMN=polymorphonuclear leukocytes, PMA=phorbol 12-myristate 13-acetate.

10 ng/ml) was added. (Fig. 1). The myocardial cell damage was assessed by morphological changes seen by phase-contrast microscopy and electron microscopy.

When the cultured myocardial cells were incubated with glucose free HBSS under hypoxic conditions for 60 min (simulated ischemia in Fig. 1) all of the cells stopped beating but were without any morphological changes. Since these cells regained beating activity after 60—120 min incubation under the normal medium, the myocardial cells subjected to such transient hypoxia are judged to be reversibly injured cells, so-called “stunned cells”.

After the addition of PMA to the myocardial cells co-incubated with PMN, the myocardial cells were progressively damaged; the stunned myocardial cells exhibited transient irregular contractions and thereafter appeared rounded and retracted with bleb formation (Fig. 2). These damaged cells did not recover by replacing them in normal condition medium and most of the cells were erythrosine B positive. Thus activated PMN-induced myocardial injury was much more severe in the ischemic conditioned cells than in the control cells (Table I).

In electron micrographs, there was no morphological change in the reoxygenated myocardial cells co-incubated with PMN for 30 min, although several PMN adhered to the cells. At 20 min after PMN activation, there were injured cells as well as a large amount of cellular debris to which PMN adhered, although adjacent myocytes, which were not adherent to PMN, showed normal cellular morphology. The plasma membrane was intact and the mitochondria were generally dense and had numerous prominent matrix granules. At 40 min after PMN activation, however, the mitochondria of PMN non-adherent myocardial cells were also swollen. At 60 min, most of the cells had a ruptured plasma membrane, vacuole and swollen mitochondria with crystallization.

**Active Oxygen Metabolites as Cytotoxic Factors**

To further investigate the role of free radicals in such a PMN-mediated myocardial cell injury, we measured luminol dependent chemiluminescence¹⁰ and production of lipid peroxides from the myocardial cells co-incubated with PMA-activated PMN. Fig. 3 illustrates serial changes in the chemiluminescence of myocardial cells co-incubated with PMN with/without SOD or catalase.

Fig.3. Chemiluminescence in myocardial cells co-incubated with PMA activated PMN Free radical generation measured by chemiluminescence from myocardial cells co-incubated with PMN was inhibited by SOD or catalase.
Catalase inhibited myocardial cell injury by 52% and the chemiluminescence at 20 min after PMN-activation by 46%, while SOD showed less pronounced inhibition. A close correlation was found between the inhibitory effects of each enzyme on both the chemiluminescence and the myocardial cell injury. Production of lipid peroxides, as determined by TBA (thiobarbituric acid) reactive substance was potently enhanced in the co-incubation system compared with the monoincubation of PMN or myocytes (Fig. 4). Interestingly, enhanced production of lipid peroxides was seen at 20 min after addition of PMA when the chemiluminescence gradually decreased.

**DISCUSSION**

The present study demonstrates that activated PMN can accelerate myocardial cell damage from reversible to irreversible injury, through direct intercellular reactions. Oxygen-derived free radicals produced from PMN were found to be at least partly responsible for the aggravation of myocardial damage. The parallelism between the structural deterioration in the myocardial cells and the release of the cytotoxic chemical mediators suggests that oxygen-derived free radicals produced at the surface membrane of the myocardial cells trigger the chain reaction of membrane lipid peroxidation, resulting in the disintegration of myocardial cell membrane. In this experiment, we employed embryo mouse cardiac myocytes because the cultured cells maintained spontaneous beating under the oxygenated culture media and allowed us to have a cellular model of the reversible myocardial damage by modulating contents of oxygen and glucose in the media. By incubating with the glucose-free hypoxic media for 60 min, the myocardial cells became so-called stunned cells. The observation that these stunned cells were prone to irreversible damage by the reaction of PMN, strongly supports the view that infiltrating PMN may exacerbate ischemic myocardial cell injury.

Twenty minutes after PMN were activated at the surface membrane of myocardial cells, the sarcolemma and subcellular organella of the myocardial cells adhered to PMN were first to structurally deteriorate. At this time, the neighboring myocytes which were not adherent to PMN remained morphologically intact. During the initial 20 min, free radical production measured by chemiluminescence reached a maximum level and thereafter declined. At 20 min after addition of PMA, irreversible change, as viewed as round up of the myocardial cell sheets, was progressively increased. Therefore, oxygen-derived free radicals initially produced from activated PMN may directly contribute to morphological derangement of sarcolemma and subcellular membranes of a single myocyte adhered to PMN. Although the precise mechanism remained undefined, the initial reaction could lead to the progressive deterioration of myocardial cells. As a possible mechanism, oxygen-derived free radicals could trigger the chain reaction of membrane lipid peroxidation, the propagating membrane disintegration of neighboring myocardial cell injury.
dial cells. As shown in Fig. 4, lipid peroxidation (production of TBA reactive substance) in the co-incubation of myocardial cells and PMN was markedly enhanced compared with those in the mono-incubation.

In the present study, the PMN-induced myocardial cell damage was partially suppressed by treatment with SOD or catalase associated with a similar inhibition of luminol-enhanced chemiluminescence (Fig. 3). These findings further support the view that oxygen derived free radicals are, at least partly, responsible for the aggravation of myocardial cell damage. The observed partial inhibition of cell damage by free radical scavenging enzymes let us speculate the following issues. The other cytotoxic substances released from PMN such as protease enzymes and eicosanoids, could also participate in this model of myocardial cell injury. It is of interest that catalase was more effective in suppressing the myocardial damage than SOD. Previous studies suggested that hydrogen peroxide, hydroxyl radical, and hypochloric acid are cytotoxic by activated PMN. Therefore it is presumed that such radical species play a key role in the cytotoxic events in this model.

The present study indicates that when extravascular PMN were activated at the surface membrane of myocyte, oxygen radicals produced from PMN may induce myocardial cell injury through the chain reaction of lipid peroxidation on the myocardial cell membrane. If we can effectively inhibit the production of PMN-derived free radicals and/or PMN-adherence to myocytes, the ischemic myocardium would avoid the PMN-mediated insult. Further studies should provide more insights into the role of oxygen free radicals in determining the severity of myocardial cell damage.

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