Leukocyte and endothelial cell adhesion molecules in a chronic murine model of myocardial reperfusion injury

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Received 16 March 1999; accepted in final form 13 June 2000

Jones, Steven P., Steven D. Trocha, Micah B. Strange, D. Neil Granger, Christopher G. Kevil, Daniel C. Bullard, and David J. Lefer. Leukocyte and endothelial cell adhesion molecules in a chronic murine model of myocardial reperfusion injury. Am J Physiol Heart Circ Physiol 279: H2196–H2201, 2000.—Expression of endothelial and leukocyte cell adhesion molecules is a principal determinant of polymorphonuclear neutrophil (PMN) recruitment during inflammation. It has been demonstrated that pharmacological inhibition of these molecules can attenuate PMN influx and subsequent tissue injury. We determined the temporal expression of α-granule membrane protein-40 (P-selectin), endothelial leukocyte adhesion molecule 1 (E-selectin), and intercellular cell adhesion molecule 1 (ICAM-1) after coronary artery occlusion and up to 3 days of reperfusion. The expression of all of these cell adhesion molecules peaked around 24 h of reperfusion. We determined the extent to which these molecules contribute to PMN infiltration by utilizing mice deficient (−/−) in P-selectin, E-selectin, ICAM-1, and CD18. Each group underwent 30 min of in vivo, regional, left anterior descending (LAD) coronary artery ischemia and 24 h of reperfusion. PMN accumulation in the ischemic-reperfused (I/R) zone was assessed using histological techniques. Deficiencies of P-selectin, E-selectin, ICAM-1, or CD18 resulted in significant (P < 0.05) attenuation of PMN infiltration into the I/R myocardium (MI/R). In addition, P-selectin, E-selectin, ICAM-1, and CD18 −/− mice exhibited significantly (P < 0.05) smaller areas of necrosis after MI/R compared with wild-type mice. These data demonstrate that MI/R induces coronary vascular expression of P-selectin, E-selectin, and ICAM-1 in mice. Furthermore, genetic deficiency of P-selectin, E-selectin, ICAM-1, or CD18 attenuates PMN sequestration and myocardial injury after in vivo MI/R. We conclude that P-selectin, E-selectin, ICAM-1, and CD18 are involved in the pathogenesis of MI/R injury in mice.

CORONARY ISCHEMIA and subsequent reperfusion induce an inflammatory state of the myocardium (8). Despite the necessity of reperfusion, it is paradoxically associated with the activation of multiple inflammatory cell types (4). Neutrophils and endothelial cells are two important cell types stimulated by myocardial ischemia-reperfusion (MI/R). Activated neutrophils can potentially have a tremendous impact on myocardial cell viability. Many previous studies of MI/R injury implicate neutrophils as pathogenic mediators of the damage associated with MI/R injury (18). Consequently, intense investigative efforts have focused on the precise mechanisms of neutrophil-endothelial cell interactions and consequent principal determinant of neutrophil (PMN)-mediated injury.

In general, the two known endothelial selectins, endothelial leukocyte adhesion molecule-1 (E-selectin) and α-granule membrane protein-40 (P-selectin), participate in the initial step of PMN-endothelial cell (PMN-EC) interactions: rolling. As PMNs circulate through the ischemic-reperfused (I/R) cardiac microvasculature, endothelial P-selectin and E-selectin form loose, transient bonds with PMN-bound P-selectin glycoprotein ligand-1 (PSGL-1) and sialyl Lewisα (SLα). These weak, selectin-mediated events slow the velocity of the neutrophil and allow endothelial-derived factors (i.e., platelet-activating factors, interleukin-8 (IL-8)) to affect PMN function. Although these selectin-mediated events are known to occur in the coronary microvasculature after MI/R, the magnitude of coronary selectin expression has not been quantitated in vivo after coronary ischemia and extended periods of reperfusion.

Slowing the velocity of the neutrophil also potentiates firm adhesive interactions between endothelial intercellular adhesion molecule-1 (ICAM-1) and CD18 complexes [lymphocyte functional antigen-1 (LFA-1); macrophage-1 (Mac-1); p150, 95] expressed on the PMN. With the use of monoclonal antibodies, it is apparent that blockade of ICAM-1 (11, 19, 30) or CD18 (1, 2, 12, 17, 20) attenuates myocardial reperfusion injury. Although the aforementioned studies provide important insight into the role of cell adhesion molecules in MI/R injury, quantitative studies of ICAM-1 over the course of in vivo MI/R are lacking.

Consequently, our present endeavor focuses on clarifying the role of endothelial cell adhesion molecules in...
the development of myocardial injury after regional, in vivo ischemia and reperfusion in mice. In the present study, we quantitatively examined the regional coronary expression of P-selectin, E-selectin, and ICAM-1 in wild-type mice during the first 72 h of reperfusion. In addition, we utilized mice deficient in P-selectin, E-selectin, ICAM-1, and CD18 to identify the role of adhesion molecule expression in myocardial neutrophil infiltration and cardiac necrosis after myocardial ischemia (MI) and prolonged reperfusion.

MATERIALS AND METHODS

All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 86–23, Revised 1985. Animal Resources Program, DRR/NIH, Bethesda, MD 20205] approved by the Council of the American Physiological Society, and with federal and state regulations. All experimental procedures were approved by the Louisiana State University Medical Center Animal Care and Use Committee.

Transgenic mice. Male mice containing homozygous gene-targeted mutations in the E-selectin, P-selectin, ICAM-1, or CD18 gene were used for these studies. The development of these mutant lines has been described previously (6, 7, 26, 29). All of these mutations were backcrossed for at least six generations onto a C57BL/6 background (Jackson Laboratory; Bar Harbor, ME). Inbred C57BL/6 males were used for controls. All mice were age and weight matched.

Surgical procedures. Wild-type (n = 20), E-selectin −/− (n = 8), P-selectin −/− (n = 8), CD18 −/− (n = 7), and ICAM-1 −/− (n = 11) mice were allowed free access to normal rodent chow, exposed to 12:12 h light-dark cycles, and housed in a climate-controlled room. The surgical protocol and infarct size determination were performed similar to methods described previously (13, 14) with several modifications because of the longer period of reperfusion in the present study. Briefly, the mice were anesthetized intraperitoneally with pentobarbital sodium (50 mg/kg) and ketamine (50 mg/kg). Through direct visualization, the mice were orally intubated with polyethylene-90 (PE-90) tubing. The animals were then connected to a rodent ventilator (model 683, Harvard Apparatus). Access to the heart was accomplished via a left anterior thoracotomy. The left anterior descending coronary artery was visualized and ligated with 7-0 silk suture. After 30 min of LAD occlusion, the ligature was removed and reperfusion was confirmed visually. The chest wall was closed with three interrupted sutures (4-0 silk) and the skin was approximated with a continuous suture (4-0 silk). The animals were given butorphanol tartrate (−0.08 mg/kg sc) for analgesia. The animals were given supplemental oxygen (100%) via a nasal cone and allowed to recover in a temperature-controlled area.

The next day (at the end of 24 h of reperfusion), a tracheostomy was performed and the mouse was connected to the ventilator. The right common carotid artery was cannulated for Evans blue infusion. The LAD was ligated and Evans blue (1.5 ml of 1.0% solution) was retrogradely infused into the carotid artery catheter to delineate the ischemic zone from the nonischemic zone. The heart was serially sectioned along the long axis. Incubation of the sections in 2,3,5-triphenyltetrazolium chloride (TTC) for 5 min at 37°C allowed differentiation between the viable and necrotic areas of the myocardium previously rendered ischemic. Each of the five 1-mm-thick slices was weighed, and the areas of infarction, risk, and left ventricle were assessed using computer-assisted planimetry (NIH Image 1.57) by a blinded observer.

Myocardial adhesion molecule expression. Radiolabeled P-selectin, E-selectin, ICAM-1, and isotype-matched control monoclonal antibodies were prepared with the use of the Iodo-Gen (Sigma) method as previously described (9). Wild-type (n = 48) mice were anesthetized intraperitoneally with pentobarbital sodium (50 mg/kg) and ketamine (50 mg/kg). The mice were instrumented with carotid artery and jugular vein catheters. Mice were randomly assigned to the following groups (n = 4/group/timepoint) for P-selectin, E-selectin, or ICAM-1 expression measurements: baseline expression (without MI), 30 min MI + 0.4 h reperfusion (R) (P-selectin only), 30 min MI + 4 h R (12 h R in ICAM-1 group), 30 min MI + 24 h R, or 30 min MI + 72 h R. At the appropriate time, radiolabeled antibodies (volume titrated to 200 μl with 0.9% NaCl) were injected through the jugular vein catheter. Monoclonal radiolabeled (125I) antibody directed against P-selectin (IB4, Pharmingen) or E-selectin (10E9.6, Pharmingen) or ICAM-1 (YN-1, Bayer) and a nonbinding radiolabeled (131I) antibody (P-23, Pharmacia-Upjohn) were slowly administered through the jugular vein catheter. After 5 min of circulation, a 50-μl plasma sample was withdrawn. The mouse was then perfused with 15 ml of warm (pH 7.4), heparinized bicarbonate-buffered saline (BBS), while being exsanguinated, to flush the excess monoclonal antibody and nonbinding control antibody. LAD reperfusion was followed by infusion of 1 ml of 1.0% Evans blue to delineate the ischemic zone from the nonischemic zone. The heart was then excised and serially sectioned into 1-mm slices. The ischemic and nonischemic zones were dissected, dried, weighed, and measured for radioisotopic activity. Cardiac radioactivity was measured using an automatic gamma counter (1480 Wizard, Wallac) to determine MI/R-induced ECAM expression. The following items were also measured with the use of the gamma counter: a 50-μl plasma sample, syringe, intravenous catheter, and 2 μl of the original antibody mixture. The gamma counts (counts/min, cpm) of these items were factored into the determination of endothelial cell adhesion molecule expression using the following equation

\[
\frac{[(125\text{I} \text{cpm/g})/(125\text{I} \text{cpm injected})]}{[(131\text{I} \text{cpm/g})/(131\text{I} \text{cpm injected})]}
\]

Assessment of myocardial neutrophil infiltration. Routine histological staining was performed on multiple sections of midventricular cardiac sections to determine the extent of PMN infiltration. Wild-type (n = 9), P-selectin −/− (n = 4), E-selectin −/− (n = 3), CD18 −/− (n = 4), and ICAM-1 −/− (n = 4) mice were subjected to 30 min of coronary occlusion and 24 h of reperfusion as described above. Midventricular tissue slices (1-mm in thickness) were prepared from hearts subjected to the myocardial I/R protocol after the completion of all experimental procedures. The tissue sections were immediately fixed and stored in a 10% neutral buffered formalin solution (Sigma Diagnostics). The tissue slices were then paraffin embedded and cut into 10-μm sections and placed on slides. The tissue specimens were then stained with Gill no. 3 hematoxylin and eosin. The slides were then viewed microscopically, and the number of PMNs per high-power field was determined. For each of the hearts examined, the number of PMNs was counted in six fields of three independent tissue sections by a blinded observer.

Statistical analyses. All data were analyzed with ANOVA. A Scheffé’s post hoc identification of group differences was used for the adhesion molecule expression data and infarct data. The neutrophil infiltration data were analyzed with a...
Fisher’s post hoc identification of group differences. All values are reported as means ± SE. Statistical significance was set at \( P < 0.05 \).

**RESULTS**

*Endothelial cell adhesion molecule expression.* P-selectin (Fig. 1A) and E-selectin (Fig. 1B) expression (in nanogram-radiolabeled monoclonal antibody/gram of cardiac tissue) under baseline conditions was low (3 ± 1 and 2 ± 1 ng/g, respectively). After 30 min of MI and 4 h of reperfusion, P-selectin and E-selectin expression significantly \( (P < 0.05) \) increased (47 ± 6 and 21 ± 4 ng/g, respectively). The expression of P-selectin and E-selectin was maximal and significant \( (P < 0.01) \) after 24 h of reperfusion (68 ± 20 and 36 ± 7 ng/g, respectively). After 3 days (72 h) of reperfusion, P-selectin and E-selectin expression were approaching \([P = \text{not significant} (\text{NS})]\) baseline values (17 ± 6 and 17 ± 6 ng/g).

The expression of ICAM-1 (Fig. 2) followed a slightly different profile than that of the selectins. ICAM-1 is constitutively expressed at high levels in the myocardium (257 ± 20 ng/g). After 30 min of MI and 12 h of reperfusion, ICAM-1 expression (563 ± 219 ng/g) was slightly \( (P = \text{NS}) \) elevated compared with baseline. After 24 h of reperfusion, ICAM-1 expression (933 ± 140 ng/g) was significantly \( (P < 0.01) \) elevated compared with baseline values. Interestingly, ICAM-1 was still significantly \( (P < 0.05) \) elevated after 72 h of reperfusion.

*Myocardial histology.* According to routine histological staining, deficiency of P-selectin, E-selectin, ICAM-1, or CD18 resulted in a 30–40% reduction \( (P < 0.05) \) in neutrophil infiltration into the I/R myocardium compared with wild-type mouse hearts (Fig. 3). The values for neutrophil infiltration \( \text{(PMNs/mm}^2) \) for the wild-type, P-selectin \(-/-\), E-selectin \(-/-\), ICAM-1 \(-/-\), and CD18 were 58 ± 3, 39 ± 6, 41 ± 1, 34 ± 3, and 42 ± 6, respectively.

*Myocardial infarct size determination.* As indicated in Fig. 4, all groups of animals were subjected to similar \( (P = \text{NS}) \) areas at risk per left ventricle (AAR/LV). The wild-type \((n = 20)\), P-selectin \(-/-\) \((n = 8)\), E-selectin \(-/-\) \((n = 8)\), ICAM-1 \(-/-\) \((n = 11)\), and CD18 \(-/-\) \((n = 7)\) mice AAR/LV measured 59 ± 3, 66 ± 3, 56 ± 3, 56 ± 3, and 63 ± 3%, respectively. When expressed relative to the AAR, the area of necrosis (INF/AAR) in adhesion molecule-deficient mice was significantly reduced by ∼40–60% \( (P < 0.05 \text{ vs. wild-type}) \). The actual values for the percent INF/AAR for P-selectin \(-/-\), E-selectin \(-/-\), ICAM-1 \(-/-\), and CD18 \(-/-\) mouse hearts was 43 ± 2, 27 ± 3, 24 ± 4, 26 ± 3, and 18 ± 5%, respectively.

**DISCUSSION**

We present data for the first time demonstrating the time course of P-selectin, E-selectin, and ICAM-1 in
mouse hearts after regional, in vivo MI/R. After the time of peak expression of P-selectin, E-selectin, and ICAM-1 in wild-type mice after MI/R was identified, mice with gene-targeted mutations of P-selectin, E-selectin, ICAM-1, or CD18 were subjected to MI/R to assess neutrophil influx and myocardial necrosis. Mice with any of the adhesion molecule deficiencies exhibited attenuated neutrophil infiltration compared with wild-type animals. In addition, adhesion molecule deficient mice demonstrated significantly less myocardial necrosis compared with their wild-type counterparts. Consequently, the present results of prolonged MI/R injury provide a clear understanding of the role of adhesion molecules in the propagation of myocardial reperfusion injury.

Previous studies investigated the role of selectin-mediated neutrophil injury in other animal models of MI/R. It is widely accepted that P-selectin and E-selectin are responsible for neutrophil rolling on the endothelium of inflamed tissue. Although studies of E-selectin antibody treatment per se in MI/R injury are uncommon, several important early studies of P-selectin blockade exist. Weyrich et al. (28) demonstrated the cardio- and vasculoprotective effects of immunoblockade of P-selectin in cats subjected to in vivo coronary ischemia and subsequent reperfusion. An additional study by Lefer et al. (15) corroborated these findings in a canine model of MI/R injury. Another approach to inhibiting selectin-mediated interactions involves indirect blockade of P-selectin and E-selectin using SLe^a-containing oligosaccharides (i.e., CY-1503) during MI/R. CY-1503 is a carbohydrate that blocks both P-selectin and E-selectin-mediated PMN adhesion. One such study (16) demonstrated reduction of myocardial infarct size in SLe^a analog-treated dogs. Similarly, another study (5) found diminished myocardial necrosis, improved coronary endothelial function, and preservation of myocardial contractility. Other investigators (21) found that immunoneutralization of L-selectin attenuated myocardial necrosis.

Although the majority of studies indicate cardioprotection associated with blockade of selectin-mediated...
neutrophil-endothelial cell interactions, some studies have shown negative results. One such study (3) used the same agent in rabbits (CY-1503) as used in dogs in a previous positive study (16) and found no effect on infarct size after MI/R. This discrepancy may be explained by the use of different species, dose of CY-1503, timing of administration, and the short half-life of CY-1503. Another negative study (10) in dogs undergoing prolonged reperfusion also used CY-1503 as a selectin blocking agent. Interestingly, the study by Gill et al. (10) demonstrated a 50% reduction in myeloperoxidase activity and 25% decrease in myocardial infarct size. However, neither parameter reached statistical significance ($P = 0.06$ for both parameters). Although this study (10) assessed infarct size after prolonged reperfusion, it may have been limited by the lack of followup doses during reperfusion to account for the short half-life of CY-1503. P-selectin and E-selectin may have been inhibited early during reperfusion but as CY-1503 was metabolized and excreted, the inhibition may have been lost thereby leaving the myocardium prone to further neutrophil-mediated injury. Ultimately, few conclusive and direct comparisons can be made among these three studies. This is precisely the issue the present study avoids. Although imperfect, genetic deficiency of an adhesion molecule is not associated with the aforementioned concerns about dosages, timing of administration, half-life, routes of administration, and species cross-reactivity.

The second phase (firm adhesion) of neutrophil sequestration involves adhesion of the neutrophil to the endothelium. This interaction involves endothelial-expressed ICAM-1 and neutrophil-expressed CD18. Consequently, many have studied the possible effects of blockade of either of these adhesion molecules in MI/R injury. Studies of pharmacological inhibition of ICAM-1 demonstrated cardioprotection in dogs (11) and rabbits (30). Similarly, many investigators found inhibition of CD18 (the common subunit to LFA-1, Mac-1, and p150,95) to protect the myocardium after ischemia and reperfusion. Studies in dogs (1) and booons (2) demonstrated improvement in contractile function in addition to decreased myocardial injury with CD18 blockade after MI/R. Attenuation of coronary vascular injury in dogs (12) and cats (20) has also been reported after treatment with an antibody directed against CD18. Similarly, treatment with an antibody directed against CD11b (Mac-1 only) attenuates myocardial injury in dogs (24, 25). Despite this wealth of data supporting cardioprotective effects of anti-CD18 therapy, other studies (27) have demonstrated no effect on myocardial injury.

One very interesting and novel finding in the present study is the attenuation of infarct size in E-selectin $−/−$ mice after MI/R. To our knowledge, this is the first demonstration of a role for E-selectin in the development of myocardial necrosis after ischemia and reperfusion in vivo. These data may suggest that E-selectin is an important contributor to PMN-mediated myocardial injury. However, the functional role of E-selectin may overlap with that of P-selectin. In future studies, mice with a deficiency of both P-selectin and E-selectin could be subjected to MI and reperfusion to at least partially address the issue of duplicate functions of the endothelial selectins in the coronary microvasculature.

Although the present data clearly demonstrate cardioprotection in the setting of genetic deficiency of adhesion molecules, several study limitations exist. Because all of the animals used in this study were mice, these results may not apply to other species. In addition, these findings are not intended to be extrapolated across all models of myocardial I/R injury. In fact, our lab (22, 23) has previously demonstrated results to this effect. In these studies (22, 23), P-selectin $−/−$, ICAM-1 $−/−$, and CD18 $−/−$ mice demonstrated cardioprotection after 30 min of ischemia and 2 h of reperfusion. When subjected to 60 min of MI and 2 h of reperfusion, these mice did not demonstrate cardioprotection despite deficiency of adhesion molecules. These previous findings (22, 23) imply that reperfusion therapy may be limited to a narrow therapeutic window.

Aside from our previous reports (22, 23), there are no other studies to our knowledge that describe the role of these leukocyte and endothelial cell adhesion molecules in the pathogenesis of MI/R injury in mice. There are several interesting comparisons to be made between these acute studies (22, 23) and the present data. When these acute studies are compared with the present study, we find that neutrophil infiltration continues to occur between 2 and 24 h of reperfusion in the wild-type and adhesion molecule-deficient mice hearts. Conversely, it appears that myocardial necrosis does not significantly progress between 2 and 24 h of reperfusion in any of the groups.

It is also clear from the present study that myocardial injury is not entirely dependent on any one adhesion molecule. Although significant injury occurs to the myocardium during reperfusion, a substantial amount of damage also occurs as a result of the ischemic event itself. The myocardium may be directly injured by edema, calcium overload, and oxygen-derived free radicals independent of PMNs. The possibility exists that neutrophil-independent processes may be occurring during reperfusion to further exacerbate myocardial injury or synergize with PMN-mediated injury. Furthermore, it is possible that other currently unknown adhesion molecules are involved in the propagation of PMN-induced myocardial injury during reperfusion.

In summary, the present study demonstrates enhanced expression of endothelial cell adhesion molecules after in vivo murine MI and reperfusion. These experiments indicate that endothelial cell expression of P-selectin, E-selectin, and ICAM-1 is maximal at 24 h of reperfusion. Knowing that P-selectin, E-selectin, and ICAM-1 are upregulated after ischemia and reperfusion, we found that deficiency of any of these molecules (or CD18) results in decreased neutrophil infiltration. Furthermore, deficiency of P-selectin, E-selectin, ICAM-1, or CD18 is associated with attenuation of infarct size. We conclude that expression and upregulation of cell adhesion molecules is responsible...
for a portion of myocardial cell injury after ischemia and reperfusion in this chronic murine model.

Surgical supplies were donated by the Willis-Knighton Medical Center in Shreveport, LA, DeRoyal Surgical in Powell, TN, and Ethicon Surgical in Somerville, NJ.

D. J. Lefer was supported by National Institutes of Health Grant RO1 HL-60849. D. J. Lefer and D.N. Granger were supported by National Institutes of Health Grant PO1 DK-43785.

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