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Anti-P-Selectin Antibody Protects Against Hepatic Ischemia–Reperfusion Injury

I. Singh, G.B. Zibari, H. Zizzi, D.N. Granger, L. Cruz, E. Gonsales, J.C. McDonald, and M.F. Brown

EARLY restitution of blood flow to ischemic tissue is essential to halt the progression of cellular injury associated with decreased oxygen and nutrient delivery. In recent literature, investigators have shown that reperfusion of ischemic tissues initiates a complex series of reactions that paradoxically injures tissues. Although several mechanisms have been proposed to explain the pathobiology of ischemic–reperfusion (I/R) injury, most attention has focused on the role of reactive oxygen metabolites and inflammatory leukocytes. A simple mechanism for I/R injury proposed by most researches is presented in Fig 1. The initial insult of I/R produces reactive oxygen metabolites which induces the production of cytokines. Cytokines not only activate NF$\kappa\beta$, a nuclear transcription protein, but also nonspecifically attract leukocytes to the injured tissue. NF$\kappa\beta$ then binds to the complimentary DNA promotor site and propagates the transcription of adhesion molecule messenger RNA. As the leukocytes enter the injured organ, the adhesion molecules now are responsible for the rolling, adherence, and emigration into the injured tissue.

In recent literature it has been implicated that P-selectin is the first adhesion molecule to be expressed during an inflammatory reaction. P-selectin is a transmembrane glycoprotein associated with α-granules in resting platelets.[1]

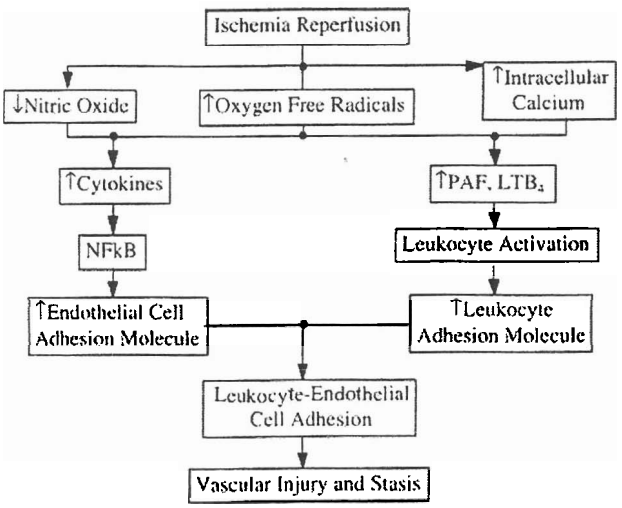

Fig 1. Ischemia–reperfusion cascade.

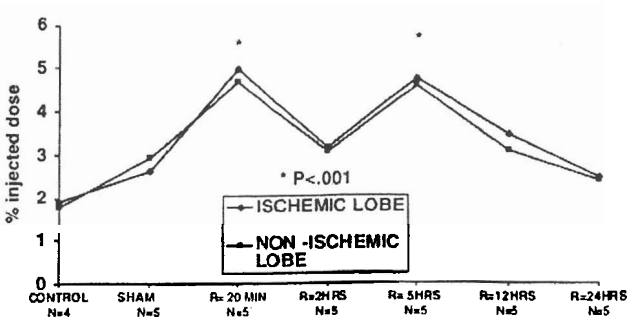

Fig 2. P-Selection expression in the liver.

P-Selectin is also found in granules of endothelial cells known as Weibel–Palade bodies.[2] We have shown that P-selectin is upregulated after an I/R injury insult and has a bimodal expression (Fig 2). P-Selectin is immediately expressed in liver at 20 minutes after an ischemic insult. Expression returns to baseline at 2 hours and then rises again at 5 hours. We believe the immediate expression is the preformed P-selectin that is mobilized from the Weibel–Palade bodies, and the 5-hr peak is due to the new translational P-selectin.[3]

There is substantial body of evidence that the leukocyte–endothelial cell interaction is largely responsible for the microvascular dysfunction induced by I/R. This work has led to the proposal that free radical ablation or inhibition of postischemic neutrophil infiltration may prove useful for therapeutic intervention in I/R injury. In light of these findings we hypothesized if P-selectin had any impact on tissue injury caused by I/ R. Therefore, we intend to show that pretreatment with anti-P-selectin antibody is protective against I/R injury.

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Role of P-Selectin in the Recruitment of Leukocytes in Mouse Liver Exposed to Ischemia and Reperfusion

G.B. Zibari, M.F. Brown, D.L. Burney, N. Granger, and J.C. McDonald

ISCHEMIA and reperfusion (I/R) injury are common mechanisms of injury in transplantation and may be an important part of the multiple organ failure that is associated with shock and sepsis. Organs subjected to ischemia ultimately undergo pathophysiologic changes leading to cell dysfunction and death unless reoxygenation occurs in a timely fashion.[1-3] Although there is a large body of evidence that implicates activated neutrophils in the pathogenesis of reperfusion injury, less is known about the mechanisms that underlie the recruitment of inflammatory cells into postischemic tissues. Recent evidence implicates several endothelial and leukocyte adhesion glycoproteins in the leukocyte recruitment elicited by I/R. These adhesion molecules include the β_2-integrins (CD11/CD18), members of the immunoglobulin supergene family (eg, intercellular adhesion molecule-1; ICAM-1), and the selectins (L-, P- and E-selectin). Adhesive interactions between CD11/CD18 on leukocytes and ICAM-1 on endothelial cells are thought to mediate firm adhesion of leukocytes to postcapillary venules after I/R.[2,4] The selectins, on the other hand, appear to mediate the low-affinity interactions that are manifested as leukocyte rolling and saltation.[4]

The technique of intravital videomicroscopy has been extensively employed to monitor and quantify the adhesive interactions between leukocytes and microvascular endothelium. This technology has also been used to characterize the factors that modulate the recruitment of leukocytes into postischemic tissues such as the mesentery and myocardium. Although there is substantial evidence implicating leukocytes in hepatic I/R injury, little is known about the specific adhesion glycoproteins that contribute to the leukocyte recruitment in the microvasculature of the postischemic liver. Hence, the objective of this study was to define the contribution of the endothelial cell adhesion molecule P-selectin to the leukocyte recruitment noted in the postischemic hepatic microvasculature. This objective was addressed by applying the technique of intravital videomicroscopy to livers of either wild-type mice receiving a P-selectin antibody[5] or to P-selectin deficient mice.[6]

METHODS

Anesthetized (Ketamine, 60 to 70 mg/kg and Xylazine, 5 to 7 mg/kg) male C57BI-6 (wild-type and P-selectin deficient) mice were placed supine on a heating pad and a midline laparotomy was performed. The liver was exposed and the arterial supply to the left lateral lobe occluded for 20 minutes using a microvascular clip. The clip was released and the abdominal incision was closed; the mouse was kept in a warm environment during recovery. After a reperfusion period of either 2, 5, 12, or 24 hours, the mouse was again anesthetized; a cannula was placed in the external jugular vein and the abdomen was re-entered. The ligamentous attachments to the liver from the diaphragm and the stomach were severed to minimize respiratory movement. The stomach and intestines were displaced from the liver using moist gauze. The mouse was placed in right lateral recumbency on a Plexiglas board so that the liver was in contact with a glass cover slip over a window in the center of the board. Sodium fluorescein (1 μmol/kg), which stains plasma and allows visualization of the sinusoids, and rhodamine 6G (0.1 μmol/kg), which stains leukocytes and platelets, were administered through the jugular cannula. The Plexiglas board was placed on the stage of the microscope (Nikon Diaphot 300 with epi-fluorescence attachment) with the liver centered over the objective.[7]

Postsinusoidal venules, 25 to 40 microns in diameter and at least 100 microns long, were located, and each was viewed and recorded (for 2 minutes) using a CCD camera that was connected to a monitor and video recorder. For the group treated with P-selectin monoclonal antibody, one venule was viewed and taped for 2 minutes, after which 30 mg of purified anti-mouse CD62P (P-selectin) (PharMingen) was administered via the jugular cannula. The same venule was recorded for 2 more minutes, along with 1 to 2 additional venules. The total time for viewing the liver was not more than 10 minutes in order to prevent hepatocyte damage from the fluorescence. On playback of each 2 minute segment, all leukocytes which rolled in continual contact with the venule wall for the entire 100 micron viewing distance were designated as rolling leukocytes. Saltating leukocytes are those cells that remain in the 100 micron segment for at least 2 seconds, either rolling or transiently adhering.

All data are expressed as mean ± SEM, with 5 mice per group. Comparisons among the different groups was based on a one-way analysis of variance. Significance was set at $P \leq .05$.

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Role of P-selectin expression in hepatic ischemia and reperfusion injury

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Abstract: Background. Researchers have shown that reperfusion of ischemic tissues initiates a complex series of reactions that paradoxically injure tissues. Although several mechanisms have been proposed to explain the pathobiology of ischemic/reperfusion (I/R) injury, much attention has focused on adhesion molecules. Our research is intended to show the kinetics of P-selectin in the liver in response to I/R injury.
Methods. Left-lobar hepatic ischemia was induced for 30 min in 35 C57BL-6 mice and 20 P-selectin-deficient (K-O) mice. P-selectin expression was measured in these mice at 20 min, 2, 5, 12 and 24 h reperfusion times, as well as in control and sham animals. The animals were injected with radio-labeled P-selectin monoclonal antibody and the organs were harvested for counts/g tissue, expressed as the percentage injected dose. Serum liver enzymes were measured and pathological sections of ischemic and control livers were performed. The unpaired *t*-test was used for statistical analysis.
Results. P-selectin expression showed two peaks in this animal model. The first peak was at 20 min and the second peak at 5 h of reperfusion ($p < 0.001$). We documented an 8-fold increase in aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) levels 10 h following I/R injury. Pathological specimens showed periportal necrosis consistent with an ischemic event. P-selectin K-O mice showed no up-regulation as a separate control group, and the liver enzymes were significantly lower than the wild-type mice at 10 h ($p < 0.001$).
Conclusion. P-selectin has a bimodal expression following hepatic I/R injury. The first peak is attributed to the Weibel–Palade bodies and the second to new translational P-selectin. We noted no difference in the up-regulation of P-selectin in the ischemic and non-ischemic liver lobes in the same animal.

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Key words: ischemia/reperfusion – P-selectin – transgenic mice

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Early restitution of blood flow to ischemic tissue is essential to halt the progression of cellular injury associated with decreased oxygen and nutrient delivery. Recognition of this fact provides the basis for the traditional view that minimizing ischemic time is the only important intervention for diminishing the extent of ischemic injury. However, recent studies have shown that reperfusion of ischemic tissues initiates a complex series of reactions that paradoxically injures tissues (1–3). Although several mechanisms have been proposed to explain the pathobiology of ischemic/reperfusion (I/R) injury, most attention has focused on the role of reactive oxygen metabolites and inflammatory leukocytes. The I/R injury has been associated with multiple factors including enhanced production of inflammatory mediators, increased rolling, adherence and emigration of leukocytes, and protein leakage in the post-capillary venules (17–19, 21, 25). There is a substantial body of evidence that leukocyte–endothelial cell adhesion is important for the microvascular dysfunction induced by I/R (2–4, 22–24, 26). This work has led to the proposal that free radical ablation or inhibition of post-ischemic neutrophil infiltration may prove useful in treating I/R injury. A simple mechanism

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P-SELECTIN CONTRIBUTES TO THE INITIAL RECRUITMENT OF ROLLING AND ADHERENT LEUKOCYTES IN HEPATIC VENULES AFTER ISCHEMIA/REPERFUSION

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ABSTRACT—We have recently reported that hepatic ischemia/reperfusion (I/R) is associated with a biphasic increase in the expression of P-selectin in the liver microvasculature, with peak expression levels observed at 20 min and 5 h after reperfusion. This I/R-induced upregulation of P-selectin expression is accompanied by leukocyte-endothelial cell adhesion in terminal hepatic venules (THV). The objective of this study was to determine whether the early expression of P-selectin contributes to the initial recruitment of rolling and adherent leukocytes in THV after liver I/R. Left hepatic lobe ischemia was induced for 30 min in anesthetized C57Bl/6 and P-selectin knockout (KO) mice. The number of rolling, saltating, and adherent leukocytes in THV was measured at 0, 15, 30, 60, and 120 min after reperfusion using intravital video microscopy. Hepatic I/R elicited significant increases in the number of rolling, saltating, and adherent leukocytes, with peak values observed at 30 min after reperfusion. All of these responses were absent in P-selectin KO mice and in C57B1/6 mice treated with a blocking antibody to P-selectin. Our findings suggest that P-selectin is the primary determinant of leukocyte-endothelial cell adhesion observed in hepatic venules in the initial period after I/R. Hence, this adhesion molecule may represent a target for therapeutic intervention in liver transplantation and other conditions associated with hepatic I/R.

INTRODUCTION

Despite intensive tissue preservation methods during hepatic transplantation and exhaustive efforts to maintain hepatic function during multiple organ failure, ischemia/reperfusion injury remains one of the foremost adversaries to liver viability during these stressful conditions. Ischemia/reperfusion (I/R) injury in several vascular beds appears to involve leukocyte-mediated tissue injury (1–4). This injury process is elicited by the generation of inflammatory mediators (oxidants, cytokines), which in turn elicits the upregulation of endothelial cell adhesion molecules (e.g., P-selectin) that mediate the recruitment, retention, and activation of leukocytes in the microvasculature (5, 6), where they contribute to tissue injury. Hepatocellular injury, as defined by a significant elevation of liver enzymes in plasma, has been shown to correlate with the duration of hepatic ischemia and the extent of leukosequestration in the liver microcirculation. The elevated liver enzymes appear to be temporally correlated with the expression of P-selectin in the post-ischemic liver in wild-type mice, with minimal changes in liver enzymes noted in P-selectin–deficient mice exposed to I/R (7).

P-selectin is a transmembrane glycoprotein which is normally stored in alpha granules of platelets and in Weibel-Palade bodies of endothelial cells (8–11). An increased expression of this adhesion molecule on endothelial cells can be elicited via mobilization of its storage granules to the cell surface (occurs in min) or by transcription-dependent induction of protein synthesis (occurs over hours). Histochemical localization studies have revealed that P-selectin expression is most intense in hepatic venules, with considerably less expression on sinusoidal endothelial cells in the inflamed liver (12). P-selectin could contribute to I/R-induced leukosequestration in the liver via several mechanisms: 1) increased expression on venular endothelial cells could mediate rolling and the subsequent firm adhesion (adherence) of leukocytes in terminal hepatic venules, 2) increased expression on sinusoidal endothelium could facilitate the trapping of stiffer, activated leukocytes within sinusoids, and 3) increased expression of P-selectin on platelets could promote platelet-leukocyte aggregation and the consequent trapping of leukocytes in sinusoids (13).

We have recently shown that hepatic I/R results in a bimodal expression of P-selectin, with an initial peak observed 30 min after reperfusion and a second peak observed at 5 h (7). It is assumed that the initial phase of P-selectin expression represents mobilization of the preformed pool, while the later phase reflects *de novo* synthesis. The importance of the second phase of P-selectin expression to leukocyte recruitment in the post-ischemic liver was demonstrated by the observation that the intense recruitment of rolling and adherent leukocytes in terminal hepatic venules at 5 h after reperfusion is absent in

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Role of superoxide in hemorrhagic shock-induced P-selectin expression

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[1]*Department of Pediatric Surgery, School of Medicine, Dokuz Eylül University, Izmir, Turkey 35340; Departments of* [2]*Surgery and* [3]*Molecular and Cellular Physiology, School of Medicine, Louisiana State University, Shreveport, Louisiana 71130;* [4]*Department of Pediatrics, University of California, San Francisco, California 94143; and* [5]*Department of Anatomy and Physiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506*

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Akgür, Feza M., Mark F. Brown, Gazi B. Zibari, John C. McDonald, Charles J. Epstein, Christopher R. Ross, and D. Neil Granger. Role of superoxide in hemorrhagic shock-induced P-selectin expression. *Am J Physiol Heart Circ Physiol* 279: H791–H797, 2000.—Superoxide has been implicated in the regulation of endothelial cell adhesion molecule expression and the subsequent initiation of leukocyte-endothelial cell adhesion in different experimental models of inflammation. The objective of this study was to assess the contribution of oxygen radicals to P-selectin expression in a murine model of whole body ischemia-reperfusion, i.e., hemorrhage-resuscitation (H/R), with the use of different strategies that interfere with either the production (allopurinol, CD11/CD18-deficient or $p47^{phox-/-}$ mice) or accumulation [intravenous superoxide dismutase (SOD), mutant mice that overexpress SOD] of oxygen radicals. P-selectin expression was quantified in different regional vascular beds by use of the dual-radiolabeled monoclonal antibody technique. H/R elicited a significant increase in P-selectin expression in all vascular beds. This response was blunted in SOD transgenic mice and in wild-type mice receiving either intravenous SOD or the xanthine oxidase inhibitor allopurinol. Mice genetically deficient in either a subunit of NADPH oxidase or the leukocyte adhesion molecule CD11/CD18 also exhibited a reduced P-selectin expression. These results implicate superoxide, derived from both xanthine oxidase and NADPH oxidase, as mediators of the increased P-selectin expression observed in different regional vascular beds exposed to hemorrhage and retransfusion.

ischemia-reperfusion; leukocyte-endothelial cell adhesion; xanthine oxidase; β_2-integrins

BOTH OXYGEN RADICALS AND LEUKOCYTES have been implicated in the pathogenesis of ischemia-reperfusion (I/R) injury (10, 11, 31). Evidence supporting a role for oxygen radicals is provided by studies demonstrating diminished I/R injury in animals treated with agents that either blunt the production of or scavenge oxygen radicals or in mutant mice that overexpress an oxygen radical-scavenging enzyme, such as superoxide dismutase (SOD) (8, 10, 11, 14, 31). Multiple experimental strategies have also been used to invoke a role for leukocytes in I/R injury. Animals rendered neutropenic or that receive monoclonal antibodies that prevent leukocyte-endothelial cell adhesion (LECA) (5, 10, 12, 23) as well as mice that are genetically deficient in adhesion molecules that mediate LECA (5, 12, 23) all exhibit a diminished injury response to I/R. Although the ability of both oxygen radical- and leukocyte-directed interventions to blunt I/R injury suggests that at least two distinct mechanisms are involved in the injury process, there is evidence supporting a link between the two mediators (oxygen radicals and leukocytes) of I/R injury (10, 11, 31). Published reports describing the attenuation of I/R-induced LECA in animals treated with radical-scavenging enzymes (14, 19, 29) or peptide inhibitors of neutrophilic NADPH oxidase (17) or in mutant mice that overexpress SOD (6, 14) suggest that oxygen radicals contribute to reperfusion injury by initiating the recruitment and activation of adherent leukocytes.

LECA is regulated by a number of factors, including cell adhesion molecules (CAMs) expressed on the surfaces of leukocytes and endothelial cells (5, 12, 23). Although it has been shown that both leukocyte and endothelial CAMs are upregulated in tissues exposed to I/R, the mechanisms that contribute to this increased expression of adhesion glycoproteins remain poorly defined. P-selectin, which is the first endothelial CAM that is upregulated after I/R (5, 12, 23), appears to be under the control of oxygen radicals. Both in vitro (25) and in vivo (4, 9) experiments have revealed that superoxide and hydrogen peroxide are potent stimuli for the rapid upregulation of P-selectin on vascular endothelial cells. However, the relevance of these observations to the condition of I/R, wherein oxygen radical production is linked to LECA, remains uncertain. It is conceivable that I/R-induced oxygen radical production, resulting from either the activation of endo-

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Persantine Attenuates Hemorrhagic Shock-Induced P-Selectin Expression

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Ischemia/reperfusion (I/R), a phenomenon that is associated with conditions such as organ transplantation, trauma, vascular disease, and stroke, involves the recruitment of activated and adherent leukocytes that subsequently mediate tissue injury. Endothelial cell adhesion molecules such as P-selectin mediate I/R-induced leukocyte recruitment and allow the adherent leukocytes to damage the vascular wall and parenchymal cells. This study examines the influence of dypiridamole (persantine) on hemorrhagic shock (H/S)-induced P-selectin expression. H/S was induced in C57BL/6 mice by withdrawing blood to drop the mean arterial blood pressure to 30 to 35 mm Hg for 45 minutes. The mice were resuscitated by infusing the shed blood and Ringer's lactate (50% shed blood volume). *In vivo* P-selectin expression was determined using a dual monoclonal antibody technique in the heart, lung, liver, kidneys, stomach, small bowel, and colon of a control group, a hemorrhagic shock group, and a hemorrhagic shock group that was pretreated with Persantine (Boehringer, Ingelheim, Ingelheim, Germany). H/S significantly ($P < 0.01$) increased P-selectin expression in all regional vascular beds of untreated mice. Persantine treatment largely prevented the H/S-induced P-selectin expression in the same vascular beds. Persantine significantly attenuates the upregulation of P-selectin in the hemorrhagic shock model.

ISCHEMIA AND REPERFUSION (I/R) injury is a commonly encountered event in trauma, transplantation, and vascular disease that can lead to tissue dysfunction and plays an important role in multiple organ dysfunction syndrome. If reoxygenation does not take place in a timely fashion ischemia leads to cell injury and death.[1–3] Reperfusion of ischemic tissue has also been shown to start a complex cascade that leads to a nonspecific inflammatory process with local organ injury and/or systemic inflammatory reaction with remote organ injury. Neutrophils have been implicated in the pathogenesis of reperfusion injury; however, less is known about the recruitment mechanisms of inflammatory cells into postischemic tissues. The adhesion of leukocytes to endothelial cells is known to be a major component in I/R injury.[3,4] The key initiating event in the leukocyte-endothelial adhesion is the upregulation of the endothelial cell adhesion molecule. The endothelial adhesion molecules are divided into several categories: the β_2-integrins (CD11/CD18), the selectins (L-, P-, and E-selectin), and the immunoglobulin supergene family (ICAM-1), of which P-selectin is the first to be upregulated.[5,6]

Short periods of tissue ischemia have been shown to provide protection against future I/R injury. The exact mechanism of the protection is unknown; however, it is thought to be related to increased levels of adenosine.[7,8] Increased levels of adenosine at the endothelial surface have been shown to decrease the leukocyte-endothelial adhesions and the expression of adhesion molecules.[9] Dipyridamole (Persantine; Boehringer Ingelheim, Ingelheim, Germany) inhibits adenosine uptake and increases its concentration at the endothelial surface.[10] The purpose of this study is to investigate the effects of dipyridamole on hemorrhagic shock induced P-selectin expression.

Materials and Methods

Monoclonal Antibodies

The monoclonal antibodies (MAbs) used for the *in vivo* assessment of P-selectin were RB40.34, a rat immunoglobulin G_1 (IgG_1) against mouse P-selectin (Pharmigen Inc., San Diego, CA), and P-23, a nonbinding murine IgG_1 directed against human P-selectin.

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