K. M. MONSON
M.S.
2004

This is to certify that the
thesis entitled

HEPATIC ISCHEMIA/REPERFUSION INJURY AND THE ROLE OF P-SELECTIN AND INTERCELLULAR ADHESION MOLECULE-1

presented by

Keith M. Monson, MD

has been accepted towards fulfillment
of the requirements for the

Master of Science degree in Surgery

Major Professor's Signature

July 6, 2004

Date

HEPATIC ISCHEMIA/REPERFUSION INJURY AND THE ROLE OF P-SELECTIN AND INTERCELLULAR ADHESION MOLECULE-1

By

Keith M. Monson, MD

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Surgery

2004

ABSTRACT

HEPATIC ISCHEMIA/REPERFUSION INJURY AND THE ROLE OF P-SELECTIN AND INTERCELLULAR ADHESION MOLECULE-1

By

Keith M. Monson, MD

Hepatic ischemia-reperfusion (I/R) injury is mediated by neutrophils and thought to be facilitated by their interaction with adhesion molecules expressed on endothelial cells and hepatocytes. A role for P-selectin and intercellular adhesion molecule-1 (ICAM-1) in hepatic I/R injury has been implicated. We proposed that hepatic I/R injury would be attenuated in transgenic mice deficient in ICAM-1 and P-selectin (P/I null). The P/I null mice and wild-type controls underwent 90 minutes of partial hepatic ischemia followed by reperfusion times of 6 and 15 hours, and a survival study. Sham controls underwent laparotomy and dissection but no ischemia. Hepatic injury was assessed by plasma alanine aminotransferase (ALT) levels and by liver histopathology. Neutrophil infiltration of liver was assessed using liver myeloperoxidase (MPO) levels and immunohistochemical staining for neutrophils. Hepatic I/R induced significant liver injury when compared to shams. No statistically significant differences were seen between wild-type and P/I null mice in liver injury (ALT and histopathology), hepatic neutrophil content (MPO and immunohistochemistry), or survival, although there was a trend towards increased survival in the P/I null group. This study suggests that ICAM-1 and P-selectin are not critical for neutrophil infiltration and liver injury following hepatic I/R.

I would like to dedicate this thesis to my chairman and mentor Dr. Richard E. Dean whose long career of educating, encouraging and caring for his surgical residents has inspired me to strive for excellence in all aspects of academic surgery. Enjoy your retirement.

ACKNOWLEDGEMENTS

I would like to thank Richard E. Dean, MD, FACS, Chairman and Program Director of the Department of Surgery for everything he has done for me as a surgical resident in general and with respect to this project specifically. I will always be in your debt.

I would also like to thank my major professor, Elahe Crockett-Torabi, PhD, Director of Basic Science Research, for her assistance with all aspects of this research project, patience, encouragement and hours of teaching.

I would like to recognize and thank the other members of my thesis committee including Alan T. Davis, MD who was always available and taught me what statistics are all about, Douglas F. Naylor, MD, FACS who has always provided endless support and education for all of his residents, and Andrew Saxe, MD, FACS whose dedication to academic surgery and leadership is a very welcome addition to our department.

I would especially like to thank Jack Harkema, DVM, PhD, Veterinary Pathologist, for performing the histopathologic analysis in this project and for spending time out of his busy schedule to discuss the results with me.

I would like to extend my sincere gratitude to Crystal Remelius, BS, Research Assistant, and Karen Hess, MS, Research Assistant whose technical assistance was invaluable and much appreciated.

Finally, I would like to thank my colleague Curtis S. Young, MD who paved the way for this project and often took time out of his extremely busy schedule to answer my questions and provide me with support and encouragement.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLE VI

LIST OF ABBREVIATIONS VIII

INTRODUCTION 1

OVERVIEW 1
REVIEW OF THE LITERATURE 3
Xanthine Oxidase-Mediated Generation of Reactive Oxygen Species Occurs After Hepatic Ischemia and Reperfusion *4*
Kupffer Cells Mediate The Early Stage of Hepatic Ischemia-Reperfusion Injury *8*
Nuclear Factor-κB Is Activated By Inflammatory Mediators and Regulates Transcription of Additional Inflammatory Mediators *9*
Complement Factors Are Activated By ROS and Promote Further Inflammation *10*
Neutrophils Mediate The Late Phase of Hepatic Ischemia-Reperfusion Injury *10*
Cellular Adhesion Molecules Are Involved In The Process of Neutrophil Sequestration, Transendothelial Migration, And Binding To Hepatocytes *14*
Hepatic Ischemia-Reperfusion Injury Can Result in Systemic Manifestations and Mortality *17*
Previous Studies Suggest P-selectin and ICAM-1 May Play A Role in Neutrophil-Mediated Hepatic Ischemia-Reperfusion Injury *18*

MATERIALS AND METHODS **21**

ANIMALS 21
MODEL OF PARTIAL HEPATIC ISCHEMIA/REPERFUSION INJURY 21
IMMUNOHISTOCHEMISTRY FOR ICAM-1 AND NEUTROPHILS 25
DETERMINATION OF PLASMA ALANINE AMINOTRANSFERASE LEVELS 27
HISTOLOGICAL ASSESSMENT OF HEPATOCELLULAR DAMAGE 28
DETERMINATION OF HEPATIC MYELOPEROXIDASE LEVELS 28
STATISTICAL ANALYSIS 30

RESULTS **31**

VERIFICATION OF ICAM-1 DEFICIENCY IN P/I NULL MICE 31
DEMONSTRATION OF HEPATOCELLULAR INJURY BY QUANTIFYING PLASMA ALT LEVELS 34
DEMONSTRATION OF HEPATOCELLULAR INJURY BY HISTOPATHOLOGY – HEMATOXYLIN AND EOSIN STAINING 38
DEMONSTRATION OF LIVER NEUTROPHIL CONTENT BY QUANTIFYING HEPATIC MYELOPEROXIDASE LEVELS 45
DEMONSTRATION OF NEUTROPHIL INFILTRATION USING IMMUNOHISTOCHEMISTRY 48
SURVIVAL STUDY 54

DISCUSSION **56**

REFERENCES **68**

LIST OF FIGURES AND TABLE

Figure 1:	Xanthine Oxidase Pathway	7
Figure 2:	Model of Partial Hepatic Ischemia	24
Figure 3A:	ICAM-1 Staining In Wild-Type Mouse Liver	32
Figure 3B:	ICAM-1 Staining In P/I Null Mouse Liver	33
Figure 4:	Plasma ALT After 90 Minutes of Hepatic Ischemia and 6 Hours of Reperfusion	36
Figure 5:	Plasma ALT After 90 Minutes of Hepatic Ischemia and 15 Hours of Reperfusion	37
Figure 6:	Histopathologic Tissue Section Stained With H+E From Sham-Operated Mouse	40
Figure 7A:	Histopathologic Tissue Section Stained With H+E From Wild-Type Mouse After 6 Hours of Reperfusion	41
Figure 7B:	Histopathologic Tissue Section Stained With H+E From P/I Null Mouse After 6 Hours of Reperfusion	42
Figure 8A:	Histopathologic Tissue Section Stained With H+E From Wild-Type Mouse After 15 Hours of Reperfusion	43
Figure 8B:	Histopathologic Tissue Section Stained With H+E From P/I Null Mouse After 15 Hours of Reperfusion	44
Figure 9:	Hepatic MPO After 90 Minutes of Hepatic Ischemia and 6 Hours of Reperfusion	46
Figure 10:	Hepatic MPO After 90 Minutes of Hepatic Ischemia and 15 Hours of Reperfusion	47
Figure 11A:	Immunohistochemical Staining For Neutrophils In Sham Mice	49
Figure 11B:	Immunohistochemical Staining For Neutrophils In Wild-Type Mice After 6 Hours of Reperfusion	50

Figure 11C: Immunohistochemical Staining For Neutrophils In P/I Null Mice After 6 Hours of Reperfusion 51

Figure 12A: Immunohistochemical Staining For Neutrophils In Wild-Type Mice After 15 Hours of Reperfusion 52

Figure 12B: Immunohistochemical Staining For Neutrophils In P/I Null Mice After 15 Hours of Reperfusion 53

Figure 13: Kaplan-Meier Survival Curve 55

Table 1: Summary of Plasma ALT and Hepatic MPO Levels in Wild-Type and P/I Null Mice 35

LIST OF ABBREVIATIONS

Alanine Aminotransferase.....ALT

Adenosine Monophosphate.....AMP

Adult Respiratory Distress Syndrome.....ARDS

Adenosine Triphosphate.....ATP

Central vein.....CV

Gadolinium Chloride.....$GdCl_3$

Hematoxylin and Eosin.....H+E

Hexadecyltrimethylammonium Bromide.....HTAB

Intercellular Adhesion Molecule-1.....ICAM-1

Interleukin-1.....IL-1

International Units.....IU

Ischemia-Reperfusion.....I/R

Lymphocyte Function-Associated Antigen.....LFA-1

Monoclonal Antibodies.....mAbs

Multiple Organ Dysfunction Syndrome.....MODS

Myeloperoxidase.....MPO

Messenger Ribonucleic Acid.....mRNA

Nicotinamide-Adenine Dinucleotide.....NAD^+

Nuclear Factor-κB.....NF-κB

Platelet-Activating Factor.....PAF

Platelet Endothelial Cell Adhesion Molecule-1.....PECAM-1

Portal vein.....PV

P-selectin and ICAM-1 Deficient.....P/I Null

P-selectin Glycoprotein Ligand-1.....PSGL-1

Reactive Oxygen Species.....ROS

Revolutions Per Minute.....rpm

Systemic Inflammatory Response Syndrome.....SIRS

Tetramethylbenzidine.....TMB

Tumor Necrosis Factor α.....TNF-α

Vascular Cell Adhesion Molecule-1.....VCAM-1

INTRODUCTION

Overview

This study investigated the role of the adhesion molecules P-selectin and intercellular adhesion molecule-1 (ICAM-1) in hepatic ischemia-reperfusion (I/R) injury. There is evidence that these molecules may be important for neutrophil-mediated hepatic injury, namely, neutrophil sequestration in the liver, transendothelial migration through the microvasculature, and adherence-dependent killing of hepatocytes. We hypothesized that the cellular adhesion molecules P-selectin and ICAM-1 are important for neutrophil adhesion mechanisms and hepatic injury following I/R.

Elucidating the roles of these adhesion molecules in hepatic I/R injury will help us to understand the specific mechanisms of the process. By understanding these mechanisms we can potentially design specific therapeutic interventions to prevent hepatic I/R injury. Such prevention will benefit patients undergoing hepatic transplantation or resection as well as patients subjected to shock or temporary hepatic ischemia as occurs after portal triad cross-clamping in hepatic trauma surgery. Successful therapeutic interventions can prevent not only the hepatic injury but also the adult respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS) that often occurs with it. Additionally, a better understanding of the mechanisms of hepatic I/R injury can provide valuable insights into the process of I/R injury in other organs. Heart attacks, strokes, and ischemic events of the mesentery and extremities are all very important clinical I/R problems.

Mice with deletions of the genes encoding for P-selectin and ICAM-1 were employed to determine the importance of these molecules in hepatic I/R injury. Three

groups of mice were studied. The experimental group included male mice with genetic deficiencies in P-selectin and ICAM-1 (P/I null mice) that were subjected to 90 minutes of partial hepatic ischemia followed by variable reperfusion times. The positive control group included male wild-type mice that similarly underwent 90 minutes of partial hepatic ischemia followed by the same variable periods of reperfusion. The negative control group (sham group) consisted of both P/I null and wild-type mice that underwent laparotomy and hepatic mobilization without ischemia, followed by closure after 90 minutes and the same variable periods of reperfusion. The periods of reperfusion examined were 6 hours, 15 hours, and a survival study in which surviving mice were sacrificed after 3 weeks.

Hepatocyte injury was assessed by monitoring serum alanine aminotransferase (ALT) and liver histopathology. Histopathologic changes in the hepatic tissue were examined using standard hematoxylin and eosin (H+E) stained tissue under light microscopy. Neutrophil infiltration into the hepatic parenchyma was assessed using immunohistochemical stains specific for mouse neutrophils. Neutrophil activity within the hepatic tissue was also assessed by quantifying hepatic myeloperoxidase (MPO) levels.

Review of the Literature

Hepatic I/R injury is an important clinical problem in surgery. It can occur during surgical resection or transplantation of the liver, after portal triad cross-clamping for control of hemorrhage in hepatic trauma, or after shock. In these situations, after a period of ischemia, the liver's endothelial cells and hepatocytes can be significantly injured upon reperfusion. If severe enough, this can lead to liver failure, the systemic inflammatory response syndrome (SIRS), ARDS, and MODS which are associated with high rates of morbidity and mortality.[1;2] The clinical consequences of I/R injury are well described but many of the specific mechanisms involved remain to be elucidated.

Studies with endothelial cell monolayers have shown that hypoxia followed by reoxygenation results in activation of xanthine oxidase, increased production of reactive oxygen species (ROS), increased expression of adhesion molecules on the endothelial cell surface, and a reduction in the barrier function of the endothelial cell.[(reviewed in 3)] In addition to the alterations that occur in endothelial cells, I/R activates Kupffer cells which generate ROS[4] and release various toxic mediators[5;6] that further the inflammatory cascade. Therefore, hepatic ischemia and subsequent reperfusion results in the early activation of endothelial cells and Kupffer cells. These are the first steps in the complex pathway that culminates in a neutrophil mediated hepatic I/R injury and its concomitant systemic manifestations.

Xanthine Oxidase-Mediated Generation of Reactive Oxygen Species Occurs After Hepatic Ischemia and Reperfusion

Experiments with hepatic I/R in the late 1980s showed that after a period of ischemia, upon reperfusion with blood, the liver sustained injury. This injury was not seen in livers exposed to 60 minutes or 20 hours of ischemia without reperfusion. Moreover, it appeared that the non-parenchymal cells, i.e. endothelial and Kupffer cells, were injured to a significantly greater extent than the parenchymal cells.[7;8] It was also shown that explants perfused with nitrogen-saturated perfusate prior to implantation were spared the reperfusion injury while explants perfused with oxygen-saturated perfusate suffered significant injury. Similar injury had been found after I/R in other organs including the heart,[9] small intestine,[10] and kidney.[11] Numerous studies using antioxidants such as superoxide dismutase, catalase, glutathione and α-tocopherol or inhibitors of oxy-radical formation such as allopurinol (which acts by inhibiting xanthine oxidase) showed an attenuation of hepatic I/R injury.[12] These studies all support the concept that the liver is injured by reperfusion after a period of ischemia and that oxygen radicals play an important role in this injury.

Studies of I/R in a number of organs have demonstrated that upon reperfusion of hypoxic tissues, free radicals are generated via the enzyme xanthine oxidase in an oxygen-dependent reaction. Xanthine oxidase is distributed throughout normal tissues. It is synthesized as xanthine dehydrogenase which accounts for 90 percent of its total enzymatic activity in normal tissue. Xanthine dehydrogenase serves to reduce nicotinamide-adenine dinucleotide (NAD^+) and is not capable of transferring electrons to

oxygen molecules to form ROS. However, xanthine oxidase uses molecular oxygen as a substrate rather than NAD^+ and catalyzes the production of ROS (see Figure 1). Under hypoxic conditions such as ischemia, xanthine dehydrogenase is converted to xanthine oxidase. Ischemia deprives tissues of the oxygen needed to generate adenosine triphosphate (ATP). Without an adequate ATP supply cells become unable to maintain the proper transmembrane ion gradient. An elevation of calcium levels in the cytosol occurs and results in the activation a protease that converts the dehydrogenase to the oxidase form. Additionally, ATP depletion results in increased levels of adenosine monophosphate (AMP) which is catabolized to adenosine, inosine, and finally hypoxanthine. Hypoxanthine is a substrate for the xanthine oxidase catalyzed production of superoxide radical and hydrogen peroxide. The other substrate that is required for this reaction is molecular oxygen. Therefore, ischemia results in an increase in the activity of xanthine oxidase relative to xanthine dehydrogenase and also provides an abundant supply of hypoxanthine, one of the required substrates for this reaction. Reperfusion supplies the second required substrate, molecular oxygen, resulting in the generation of superoxide radicals and hydrogen peroxide.[13;14]

It was originally presumed that the intracellular ROS generated by the xanthine oxidase pathway caused the cellular damage seen in I/R injury via lipid peroxidation. However, several lines of evidence have since suggested that this mechanism was not sufficient to induce the observed damage. Significant oxidant stress could not be detected in reperfused livers in a leukocyte-free system.[15] Additionally, hepatocytes have a tremendous capacity to scavenge intracellular ROS.[16] Finally, the amount of lipid peroxidation required to cause significant liver cell injury is much higher than that seen

after I/R.[17] It appears, therefore, that intracellular generation of ROS via the xanthine oxidase pathway does not significantly contribute to liver cell injury after I/R via direct mechanisms such as lipid peroxidation. However, indirect mechanisms resulting from the xanthine oxidase mediated generation of ROS cannot be ruled out. Such mechanisms may include the activation of transcription factors (e.g., nuclear factor-κB) that promote the upregulation of pro-inflammatory molecules such as cytokines or adhesion molecules.

Although the xanthine oxidase pathway may not play a role in direct injury via the generation of intracellular ROS, it may contribute significantly to the formation of extracellular ROS. It has been shown that xanthine oxidase is released into the circulation after hepatic I/R, in sufficient quantities to generate enough ROS to cause endothelial cell injury.[18] The plasma is a relatively anti-oxidant poor compartment and prolonged periods of ischemia are likely to deplete plasma anti-oxidants. Therefore, the potential for cellular injury caused by ROS generated by this pathway is greater than that found in the intracellular compartment. Additionally, circulating xanthine oxidase may play a role in the systemic tissue injury that affects multiple organs after hepatic I/R injury.

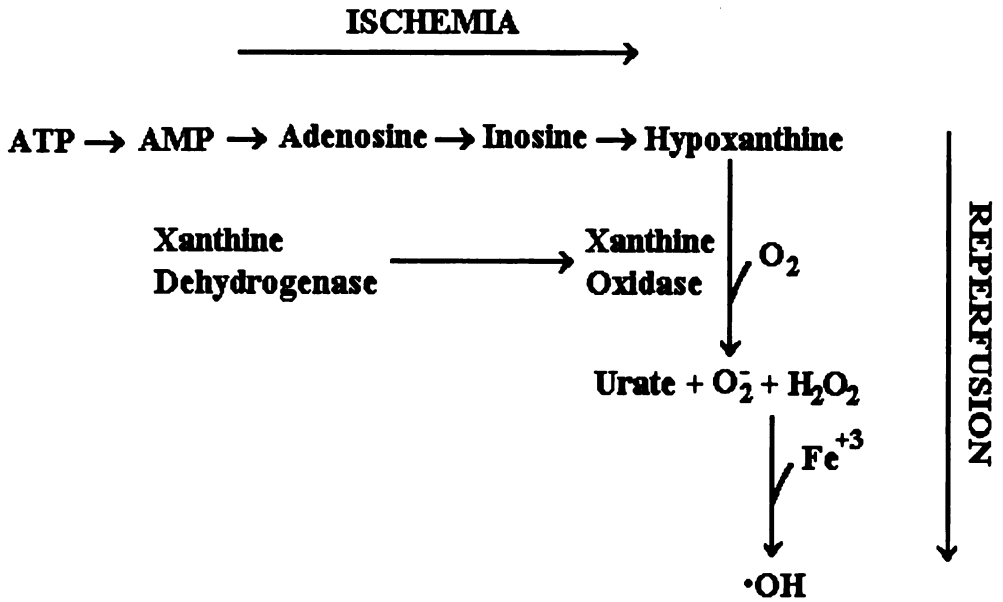

Figure 1. The Xanthine Oxidase Pathway

Kupffer Cells Mediate The Early Stage of Hepatic Ischemia-Reperfusion Injury

Resident macrophages in the liver, or Kupffer cells, are another source of ROS after I/R. Upon hypoxia and reoxygenation, cultured rat Kupffer cells generated large amounts of superoxide radical.[4] Other studies demonstrated that hepatic I/R caused morphologic changes in Kupffer cells such as swelling, ruffling, and increased number of pseudopodia and lamellapodia indicating an activated state.[19] There was an increased uptake of carbon particles by the Kupffer cells after hepatic I/R which is an indication of increased Kupffer cell activity.[20] Ischemia/reperfusion also resulted in marked increases in the production of tumor necrosis factor- α (TNF-α) and interleukin-1 (IL-1) which are known to be secreted by activated Kupffer cells.[21] Hence, numerous studies have demonstrated both morphological and functional evidence that I/R results in the activation of Kupffer cells.

Activated Kupffer cells release a variety of toxic mediators including superoxide anion radical, proteases, leukotrienes, and cytokines such TNF-α and IL-1.[4-6] The contribution of Kupffer cells to I/R injury has been shown to be important in a number of studies utilizing Kupffer cell suppressing agents such as gadolinium chloride ($GdCl_3$) or methyl palmitate. Pretreatment with these agents in rat models of hepatic I/R injury resulted in significantly reduced hepatic microcirculatory disturbances, decreased generation of free radicals, decreased TNF-α levels, reduced hepatic neutrophil infiltration, and reduced hepatocyte death.[22-26] This initial Kupffer cell mediated phase of hepatic I/R injury causes hepatocyte damage within the first hour of reperfusion, most likely through direct injury caused by Kupffer cell release of ROS and proteases. A later,

more severe phase occurs between 6 and 24 hours after the initiation of reperfusion and is mediated predominately by neutrophils.[27]

Kupffer cells are activated by I/R. Activated Kupffer cells then contribute to hepatic I/R injury both directly, through additional generation of ROS and release of proteases, and indirectly, through the release of a variety of inflammatory mediators. Among the most important of these mediators released by activated Kupffer cells are the cytokines TNF-α and IL-1. The primary source of TNF-α and IL-1 production is the macrophage. There is an approximately two-hour delay between the induction of tissue injury and the rapid increase in TNF. This suggests that this cytokine is not stored in cells but is synthesized and secreted upon cell activation. TNF induces the production of IL-1 and IL-6 by endothelial cells as well as platelet-activating factor (PAF) which is chemotactic for neutrophils. IL-1 induces production of TNF,[28] neutrophil chemotactic factor and monocyte chemoattractant protein-1. TNF-α and IL-1 also promote the expression of adhesion molecules on endothelial cells and neutrophils which are thought to play a role in the later neutrophil-mediated stage of I/R injury.[29;30] These effects occur through TNF-α and IL-1 induction of the transcription factor nuclear factor-κB (NF-κB).[31]

Nuclear Factor-κB Is Activated By Inflammatory Mediators and Regulates Transcription of Additional Inflammatory Mediators

NF-κB is a ubiquitous transcription factor that is responsible for activating the transcription of a number of genes encoding for pro-inflammatory proteins. Several conditions present during reperfusion favor the activation of NF-κB. These include oxidative stress, and the presence of TNF-α, IL-1, or leukotriene B4. Once activated by

these stimuli, NF-κB initiates the transcription of pro-inflammatory cytokines and adhesion molecules which promote the chemotaxis, sequestration and transendothelial migration of neutrophils.[32]

Complement Factors Are Activated By ROS and Promote Further Inflammation

The activation of Kupffer cells is potentiated by activated complement factors. Experiments in rat models showed that the macrophage-mediated oxidative stress was enhanced by activation of complement factors and reduced by depletion or blocking of these factors.[33] Complement can be activated by ROS.[34] It is therefore not surprising that the complement cascade is activated during reperfusion after ischemia. Complement depletion or blocking reduced Kupffer cell generation of superoxide anion but did not prevent it. Other factors such as phagocytosis of cellular debris and ischemia itself can activate Kupffer cells. It seems that complement factors are not the sole cause of Kupffer cell activation but they do contribute to their activation. Additionally, activated complement factors induce the release of cytokines TNF and IL-1 establishing a positive feedback loop with these cytokines.[(reviewed in 35)] Finally, complement factor C5a is a potent chemotactic factor for neutrophils and has been shown to induce neutrophil-mediated endothelial cell injury both *in vitro* and *in vivo*.[36] Complement factor C5a is involved in the upregulation of adhesion molecules and the activation of neutrophils.[37;38]

Neutrophils Mediate The Late Phase of Hepatic Ischemia-Reperfusion Injury

As discussed previously, the early phase of hepatic I/R injury is mediated by Kupffer cells and can be attenuated using agents that block Kupffer cell activity. Plasma

ALT levels can be used as a marker of hepatocyte injury as ALT contained within hepatocytes is released into the plasma during hepatocyte injury. Plasma ALT levels rise within the first hour of reperfusion but then do not change significantly until after six hours of reperfusion. This early rise is secondary to the release of proteases and ROS by Kupffer cells[22] as well as the generation of ROS through the xanthine oxidase pathway[18] and by mitochondria.[39]

Neutrophils begin to accumulate in the post-ischemic liver lobes within the first six hours of reperfusion but do not significantly contribute to the early phase injury. This is evidenced by experiments showing injury in this early phase is not affected whether neutrophils are present or absent.[27] Additionally, the injury is initiated before an increasing number of neutrophils are detected in the liver.[22] After six hours of reperfusion however, a significant rise in plasma ALT level occurs which can be attenuated by depleting the animal of neutrophils. This second phase of injury is more severe than the first, begins after 6 hours of reperfusion, and continues for at least 24 hours. Neutrophil accumulation peaks between 6 and 24 hours after reperfusion.[27]

Neutrophil-mediated I/R injury requires several sequential steps. The first step is neutrophil recruitment to and sequestration in the area of injury. As mentioned previously, activated endothelial and Kupffer cells release a variety of inflammatory mediators in the early stages of reperfusion. Several of these factors initiate neutrophil recruitment and activation including TNF-α,[40] IL-1,[41] activated complement factors,[33] and PAF.[42] A number of studies using in vitro systems and intravital microscopy have shown that neutrophil velocity slows in response to inflammation, as the neutrophils begin to roll along the endothelium of the post-capillary venules.[43-45] This rolling occurs

as a result of weak interactions between selectins and their ligands expressed on neutrophils and endothelial cells. Such rolling of neutrophils puts them in close contact with the endothelium and exposes the neutrophils to high concentrations of locally released inflammatory mediators. This stimulates neutrophil activation and the upregulation of various cellular adhesion molecules on the surface of the neutrophils. After rolling, neutrophils tightly adhere to the post-capillary endothelial cells and are thus sequestered in the reperfused organ. Although the importance of neutrophil rolling and adherence has been demonstrated in I/R injury in several organs including skeletal muscle,[46 47;48] mesentery,[49 50] heart,[51 52;53] lung,[54] and brain,[55] their role in hepatic I/R injury is less certain.

In a number of organs, neutrophil sequestration is dependent on interaction between adhesion molecules expressed on the surface of endothelial cells and on the neutrophils themselves. Such adhesion molecules are upregulated in response to I/R. Preventing this interaction, through the use of monoclonal antibodies (mAbs) against various adhesion molecules, has resulted in an attenuation of injury. This has been demonstrated after I/R in the heart,[51;52] intestine,[49;56] lung,[54;57] skeletal muscle,[47;58] and brain.[55] The primary site of neutrophil adherence and transendothelial migration in these organs is the post-capillary venules. Neutrophil adhesion and transmigration in the post-capillary venules results in extravasation of plasma proteins with increased fluid filtration, edema, and impairment of tissue perfusion.[59] In the liver, however, the neutrophils responsible for the late phase of I/R injury are sequestered in, and transmigrate through the sinusoids rather than the post-sinusoidal venules.[60]

With the first step of neutrophil-mediated I/R injury being neutrophil sequestration and adherence, the second step is neutrophil transmigration through the endothelial cell lining of the sinusoids, or *transendothelial migration*. In response to neutrophil chemoattractants, neutrophils adherent to endothelial cells extend pseudopodia between endothelial cells and pull themselves out of the vessel and into the interstitial space.[61-63] As mentioned previously, transendothelial migration after I/R in the liver occurs only through the sinusoids as opposed to the post-sinusoidal (or post-capillary) venules as they do in other organs.[60] This step is dependent upon interactions between adhesion molecules expressed on the surface of both neutrophils and endothelial cells. It has been suggested that this step, although important for I/R injury to occur in other organs systems, may not play as critical a role in hepatic I/R injury. Unlike endothelial linings in other areas of the body, the hepatic sinusoids are fenestrated rather than continuous. Additionally, since severe I/R injury can results in the damaging or even removal of, sinusoidal endothelial cells, neutrophils may have free access to regions of the hepatic parenchyma without transendothelial migration.[64;65]

The third step in neutrophil-mediated I/R injury, after transendothelial migration, is adherence-mediated hepatocyte injury. After extravasating, neutrophils bind directly to hepatocytes in a process which is again mediated by cellular adhesion molecules. Adherence of neutrophils to hepatocytes results in a significant increase in both the quantity and duration of cytotoxic mediators generated by the neutrophils.[66;67] Activated neutrophils bound to hepatocytes generate ROS and proteases that kill hepatocytes. Experiments using antioxidants[12;68] and protease inhibitors[69;70] have shown an attenuation of the neutrophil-mediated hepatic I/R injury.

Cellular Adhesion Molecules Are Involved In The Process of Neutrophil Sequestration, Transendothelial Migration, And Binding To Hepatocytes

Tumor necrosis factor-α, IL-1, C5a, and PAF released during the early stage of I/R injury act, in part, by upregulating the expression of adhesion molecules on the surface of endothelial cells, Kupffer cells, and hepatocytes. Neutrophils likewise upregulate cell surface adhesion molecules which act as ligands to those upregulated in the liver. The interactions between these adhesion molecules facilitate neutrophil sequestration in the liver, transendothelial migration, binding to hepatocytes, and ultimately hepatocyte injury and death.[reviewed in 71]

There are three main families of cellular adhesion molecules involved in inflammation. These include the selectins, the immunoglobulin gene superfamily, and the integrins. [reviewed in 72;73] The selectin family includes the three molecules L-selectin, E-selectin, and P-selectin. L-selectin is constitutively expressed on leukocytes, including neutrophils, and is shed from the cell surface upon activation of the neutrophil in response to inflammatory stimuli. E-selectin is not normally expressed in the liver but its expression on the luminal surface of large liver vessels, and to a lesser extent on the sinusoidal endothelium, is upregulated in response to a variety of inflammatory stimuli.[reviewed in 71] P-selectin (CD62P) is stored in the α-granules of platelets and the Weibel-Palade bodies of endothelial cells. In response to inflammatory mediators including TNF-α and IL-1, these vesicles fuse with the plasma membrane resulting in a rapid expression of P-selectin on the cell surface.[74] P-selectin is not constitutively expressed in any of the cells of the liver. However, in response to inflammatory stimuli, P-selectin expression in the liver increases on the surface of the endothelial cells in the

larger vessels but not in the sinusoids.[75-77] Sinusoidal endothelial cells lack Weibel-Palade bodies.[78]

As mentioned previously, the initial contact between neutrophils and the endothelium occurs as a result of interactions between the selectins and their ligands. The selectins in general bind to sialyl-Lewis x and other fucosylated carbohydrates.[(reviewed in 73)] P-selectin and E-selectins bind to a sialyl Lewis x glycoprotein called P-selectin glycoprotein ligand-1 (PSGL-1) which is expressed on the surface of neutrophils.[79;80] These low-affinity interactions are formed and then quickly break, repetitively, resulting in the rotational motion of neutrophils along the endothelial surface referred to as rolling.[81-84] Selectin-mediated rolling of neutrophils results in neutrophil activation and the firm adherence of neutrophils to the endothelial cells which is mediated by the other two families of adhesion molecules. Additionally, P-selectin contains nine complement-binding short consensus repeats. There is evidence that the expression of P-selectin may provide a binding site for complement thus activating the complement cascade. Complement activation can injure endothelial cells through the formation of complement membrane attack complexes. Complement components also serve as chemotactic factors for neutrophils.[85]

The immunoglobulin gene superfamily includes the intercellular adhesion molecules (ICAM-1, ICAM-2, and ICAM-3), platelet endothelial cell adhesion molecule-1 (PECAM-1), and vascular cell adhesion molecule-1 (VCAM-1).[(reviewed in 72)]

Intercellular adhesion molecule-1 is constitutively expressed on the surface of sinusoidal endothelia and Kupffer cells in normal livers but is absent on hepatocytes.[75] The inflammatory stimuli cause a greatly increased expression if ICAM-1 on the surface

of sinusoidal endothelia as well as on endothelia of portal vein, artery, and central vein. Expression of ICAM-1 is also increased on hepatocytes in response to specific inflammatory stimuli.[77] The cytokines TNF-α and IL-1, which are released by Kupffer cells during hepatic I/R, are both sufficient stimuli to induce this upregulation of ICAM-1.[41] Such upregulation of ICAM-1 requires transcriptional activation and increased ICAM-1 messenger ribonucleic acid (mRNA) can be detected in hepatic endothelial cells, Kupffer cells, and hepatocytes.[75]

Integrins are membrane glycoproteins which assemble in dimers consisting of an α and a β subunit. There are six known families of integrins each with a common β subunit (β_1- β_6). The β_2-integrin Mac-1 (CD11b/CD18) is of particular interest because it is upregulated primarily on the surface of neutrophils, monocytes, and macrophages (e.g. Kupffer cells). (reviewed in 72) In these cells, Mac-1 is stored in granules that fuse with the cell membrane in response to inflammatory mediators such as TNF-α, IL-8 and activated complement factor C5a. Complement factor 5a incites the initial Mac-1 upregulation while TNF- α maintains its expression for prolonged periods.[86] This results in a rapid increase in the expression of Mac-1 that, unlike ICAM-1, does not require immediate protein synthesis. Increased expression of Mac-1 on neutrophils has been shown in both hepatic[87] and muscular[88] models of I/R. Intercellular adhesion molecule-1 is a ligand for Mac-1 and there is evidence that the interaction between the two is necessary for both transendothelial migration of neutrophils and binding to hepatocytes after certain inflammatory insults.[89] Various studies using blocking antibodies against either ICAM-1 or Mac-1 have shown decreased hepatocellular injury after I/R.[40;87;90;91]

Hepatic Ischemia-Reperfusion Injury Can Result in Systemic Manifestations and Mortality

Multiple organ dysfunction syndrome is the leading cause of death in critically ill patients. It is a documented consequence of ischemia and reperfusion to multiple organs including the liver.[92-94] As previously discussed, hepatic I/R results in the release of inflammatory mediators such as TNF-α and IL-1 from Kupffer cells in the early stages of injury.[23;28] When released in large quantities, these mediators travel systemically through the circulation. These systemic mediators can then activate the vascular endothelium in multiple organs throughout the body inducing the expression of various adhesion molecules including P-selectin and ICAM-1.[95-97] At the same time, these inflammatory mediators activate circulating leukocytes which also upregulate expression of adhesion molecules on their surfaces.[98;99] Thus, leukocyte-endothelial cell interactions can occur in the vascular beds of organs throughout the body resulting in endothelial cell injury, capillary leakage, leukocyte transmigration, and parenchymal injury.

Compromise of the intestinal mucosal barrier, which may occur in conjunction with the primary ischemic injury or as a consequence of it, may result in the translocation of enteric bacteria and lipopolysacchaaride to the mesenteric lymph nodes and portal blood.[100-102] These bacterial challenges can activate monocytes resulting in their release of further inflammatory mediators thus adding to the systemic inflammation and tissue injury.[103] Widespread generation of ROS from circulating xanthine oxidase[18] and systemic activation of the complement system[34] may also contribute to systemic injuries resulting from hepatic I/R injury. The lung is the most frequently injured organ in MODS and it can suffer various degrees of injury ranging from acute lung injury to ARDS.[(reviewed in 104)] These conditions are associated with high rates of mortality.

Previous Studies Suggest P-selectin and ICAM-1 May Play A Role in Neutrophil-Mediated Hepatic Ischemia-Reperfusion Injury

Experiments using monoclonal antibodies to P-selectin in murine models of hepatic I/R injury have been performed. Blockade of P-selectin in these models resulted in decreased hepatic tissue injury and improved survival.[105;106] Additionally, intravital video microscopy in this model revealed that I/R caused significant increases in the number of neutrophils rolling along and adhering to endothelial cells in the terminal hepatic venules. Blocking P-selectin resulted in attenuation of this neutrophil behavior.[107] Blocking P- and E-selectins using soluble P-selectin glycoprotein ligand-1 in a murine model of hepatic I/R similarly suppressed hepatic neutrophil infiltration and injury and improved survival.[108] More recent studies have utilized gene-targeted mice deficient in P-selectin in hepatic I/R models. These studies showed decreased leukocyte rolling and adherence in terminal hepatic venules[107] and decreased liver injury in the P-selectin knockout mice when compared to wild-type controls.[109;110]

Multiple experiments using monoclonal antibodies to block ICAM-1 in models of murine hepatic I/R injury showed that hepatocellular injury was significantly reduced in the animals receiving the anti-ICAM-1 antibody.[90;111-114] Decreased adherence of neutrophils to the venular endothelial cells was observed in animals receiving the antibody but the antibody did not affect sinusoidal leukocyte adherence[91]or hepatic neutrophil infiltration in general.[90] Additionally, in a hepatic transplant model, blocking ICAM-1 with monoclonal antibodies did not improve early graft function.[115]

Several recent studies have examined the combined roles of selectins and ICAM-1 in various murine models. A recent hepatic I/R study employing mice deficient in

either ICAM-1, L-selectin, or both molecules showed that decreased injury and improved survival occurred in the L-selectin deficient mice and the combined L-selectin/ICAM-1 mice but not in the ICAM-1 deficient mice. This study suggests that L-selectin plays a more dominant role than ICAM-1 in mediating hepatic injury.[116] Kamochi et al investigated an endotoxin-induced model of hepatic injury in mice deficient in both P-selectin and ICAM-1.[117] These mice had decreased hepatic neutrophil activity and reduced vascular permeability relative to wild-type mice. Mortality in the double mutant mice was delayed but overall survival was unaffected. Separate studies have utilized both blocking antibodies and genetic deletions to assess the role of ICAM-1 and P-selectin in myocardial I/R injury. Lefer et al showed that blocking antibodies for either ICAM-1 or P-selectin resulted in improvement of post-ischemic myocardial blood flow and reduction in neutrophil accumulation. [118] However, the co-administration of both antibodies simultaneously did not improve these parameters over administration of the antibodies individually. In mice genetically deficient in P-selectin and ICAM-1 a significant reduction in neutrophil infiltration was observed. However, there was no difference in infarct size in these animals relative to wild-type controls.[119] Bullard et al investigated genetic knockout mice deficient in P-selectin and ICAM-1 receiving intraperitoneal injections of *Streptococcus pneumoniae*.[120] They found that these mice had a complete loss of neutrophil emigration into the peritoneal space while wild-type controls had extensive neutrophil emigration. These same double knockout mice, when subjected to *S. pneumoniae* induced pneumonia, had completely normal neutrophil emigration into the alveolar spaces when compared to wild-type controls.[120] All of these studies demonstrate that there is a great deal of diversity in neutrophil response to selectins and ICAM-1.

These responses are dependent on the etiology of the inflammation (e.g. endotoxin, chemical irritation, I/R), the method of neutralizing the adhesion molecules (i.e. blocking antibodies, soluble ligands, genetic deletions), and the nature of the affected organ.

A role for the adhesion molecules ICAM-1 and P-selectin in hepatic I/R injury has been suggested. The combined roles of P-selectin and ICAM-1 in hepatic I/R injury have not been previously characterized. Also, the majority of previous studies examining these molecules in hepatic I/R injury employed blocking antibodies to neutralize these adhesion molecules. However, such antibodies may not be completely specific for the molecules they seek to block. The use of such antibodies may result in the non-specific suppression of other undetermined molecules that may play a role in inflammation.[121] Because of this uncertainty, genetic manipulation to delete the P-selectin and ICAM-1 molecules would be preferable to blocking antibodies in investigating the roles of these molecules in hepatic I/R injury.

We hypothesized that the cellular adhesion molecules P-selectin and ICAM-1 are necessary for neutrophil-infiltration and hepatic injury following I/R. In this study we used mice with deletions of the genes encoding for P-selectin and ICAM-1 to determine the importance of these molecules in hepatic I/R injury.

MATERIALS AND METHODS

Animals

Gene-targeted mice deficient in P-selectin and ICAM-1 (P/I null), C57BL/6-Icam1^{tm1Bay}Selp^{tm1Bay}, were purchased from the Jackson Laboratory (Bar Harbor, ME). These mice demonstrate an increased number of neutrophils in the blood and a complete loss of neutrophil emigration into the peritoneum during *Streptococcus pneumoniae*-induced peritonitis.[120] Homozygotes are viable and fertile with no obvious phenotypic abnormalities. Male double-knockout mice were either bred under the guidance of the University Laboratory Animal Resources, Michigan State University, or purchased directly from the Jackson Laboratory. Wild-type mice were C57BL/6 from Charles River Laboratories, Portage, MI. Mice were maintained on 12-hour light and 12-hour dark cycles. The animals had access to food and water *ad libitum*. All experimental procedures were reviewed and approved by the Michigan State University Animal Use and Care Committee.

Model of Partial Hepatic Ischemia/Reperfusion Injury

A murine model of lobar hepatic ischemia/reperfusion was utilized.[23;122-125] All surgical procedures were performed at a controlled room temperature (22-24° C) under aseptic conditions using sterilized instruments. Mice were anesthetized with 1-2 ml of methoxyflurane (Schering-Plough, Union, NJ) applied to gauze in a face cone, which was fashioned from a 50-ml Corning polypropylene disposable centrifuge tube (Corning, NY). Once adequate anesthesia was obtained, as determined by a negative foot pinch reflex, an intraperitoneal injection (15 mg/kg) of pentobarbital sodium (50mg/ml, Abbott

Laboratories, North Chicago, IL) was administered. The animal was restrained in the supine position on the operating bed. The face cone was re-applied as needed to maintain anesthesia. The abdomen was shaved and prepped with povidone-iodine (Betadine®, Purdue Frederick Company, Norwalk, CT). A midline incision was made from the xiphoid process to the pubis. The small and large bowel were eviscerated and wrapped in normal (0.9%) saline moistened gauze (2 in. x 2 in., The Kendall Company, Mansfield, MA). The liver was exposed completely. The ligamentous attachments of the left lateral and median lobes were carefully divided. The left lateral and median lobes were freed. The portal circulation to both of these lobes was carefully dissected. The portal vein and hepatic artery supplying the median and left lateral lobes was then interrupted with the application of an atraumatic vascular clamp (Accurate Surgical and Scientific Instruments Corporation, Westbury, NY) to the vascular pedicle. The left lateral lobe was also rotated 180 degrees counter-clockwise on its vascular pedicle to eliminate any potential perfusion that might occur with an imperfect clamp occlusion. The caudate and right lateral lobes, as well as the papillary and quadrate processes of the liver were perfused to prevent mesenteric congestion and its potential to allow bacterial translocation. This results in the induction of ischemia to approximately 70% of the liver.[124;125] The small and large bowel were replaced into the abdominal cavity. One ml of sterile normal saline was dripped into the abdominal cavity to replace insensible fluid losses and prevent desiccation. During hepatic ischemia, the abdomen was covered with plastic wrap (S. C. Johnson & Son, Inc., Racine, WI) to prevent evaporation. After 90 minutes of partial hepatic ischemia, the clamp was removed, the left lateral lobe was rotated 180 degrees clockwise to return it to its anatomic position, and reperfusion was initiated. The midline

laparotomy was closed in a single layer using 5-0 nylon suture on an FS-2 needle (Ethicon, Inc., Somerville, NJ). Sterile Lactated Ringers solution (Abbott Laboratories, North Chicago, IL), 0.6 ml, was administered subcutaneously to compensate for operative blood and fluid losses. Sham mice underwent an identical procedure but without vascular occlusion and rotation of the left lateral lobe. Mice were euthanized after 6 and 15 hours of reperfusion. Additionally, a survival study was performed which included a control group using wild-type mice and an experimental group using P/I null mice but no sham group. Mice in the survival study were observed after surgery and the length of survival from the time reperfusion was initiated was recorded. Mice surviving three weeks were euthanized at that time. The vascular clamp placement and areas of induced ischemia are illustrated in Figure 2.

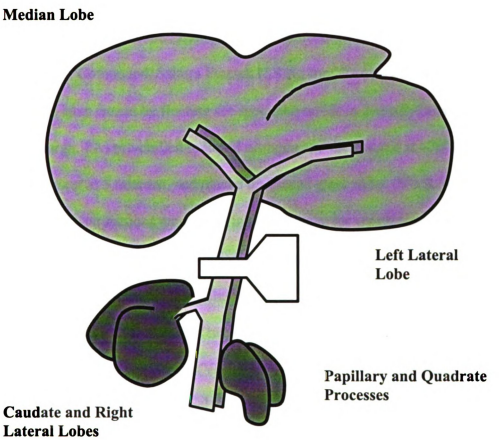

Figure 2. Model of Partial Hepatic Ischemia

The blood supply to the median and left lateral lobes is interupted with an atraumatic microvascular clamp as shown. The total ischemia time is 90 minutes, followed by various reperfusion times.

Immunohistochemistry for ICAM-1 and Neutrophils

ICAM-1 expression was analyzed by immunohistochemistry using an Armenian hamster IgG anti-mouse primary mAb (3E2) specific for ICAM-1 (CD54) (01541D, PharMingen, San Diego, CA). Biopsies of the ischemic median lobe were placed in intermediate Cryomolds (Miles Inc., Elkhart, IN) and embedded in embedding medium (Tissue-Tek® O.C.T. Compound, Sakura Finetek U.S.A., Inc., Torrance, CA). The samples were then snap frozen by floating the specimen molds in 2-methylbutane (Fisher Scientific, Pittsburgh, PA) pre-chilled in liquid nitrogen and then stored at –70° C. Cryosections were cut at 5 microns, and placed on frosted glass slides (VWR Scientific, Chicago, IL). The tissue sections were fixed in cold acetone for ten minutes and air dried. Slides were then placed in a humidified chamber. The slides were rinsed with Tris-buffered saline (TBS)(BioRad, Hercules, CA), pH 7.4, and incubated in TBS for 5 minutes. This was repeated for a total of two washes. To prevent nonspecific binding, the slides were incubated in goat serum (Vector Laboratories, Inc., Burlingame, CA)/hydrogen peroxide (Sigma Chemical Company, St. Louis, MO) for 10 minutes. The slides were then rinsed twice with TBS for 5 minutes per rinse. Next the slides were incubated with Avidin-D (Vector Laboratories, Inc., Burlingame, CA) and incubated for 15 minutes, followed by a wash with TBS. Biotin Blocking Reagent (Vector Laboratories, Inc., Burlingame, CA) was then applied to the slides and incubated for 15 minutes. This procedure was done to block the endogenous biotin activity present in liver tissue. Slides were again washed with TBS for 5 minutes and the excess was removed. Next 100 µl of the primary antibody, anti-mouse ICAM-1 antibody (10µg/ml), was applied to the tissue and incubated for 60 minutes at room temperature. The slides

were then rinsed three times with TBS for a total of 10 minutes and the excess was wiped away. Next 100 µl of the secondary antibody, biotin-conjugated mouse anti-hamster IgG mAb (12102D, PharMingen, San Diego, CA) was applied to the tissue and incubated for 30 minutes at room temperature. A 10-minute TBS rinse was again performed. Vectastain ABC Reagent (Vector Laboratories, Inc., Burlingame, CA) was applied to the tissue and incubated for 30 minutes, followed by a 10-minute TBS rinse. The 3,3'-Diamino benzidine tetrahydrochloride (DAB) enhancing solution (Pierce Chemical Company, Rockford, IL) was then applied and incubated for 10 minutes. The slides were then rinsed with 0.05 M sodium bicarbonate (J. T. Baker Chemical Company, Phillipsburg, NJ) and incubated for 10 minutes. The DAB enhancing solution was applied for 5-10 seconds per slide and rinsed with distilled water.

The tissues were then stained in hematoxylin (Gill's Formula, Vector Laboratories, Inc., Burlingame, CA) for 5 minutes. The slides were then rinsed with distilled water until the effluent was clear. Next the slides were dipped ten times in 2% acetic acid (EM Science, Gibbstown, NJ), followed by rinsing with distilled water. The slides were then placed in a bluing solution (1.5 ml NH_4OH (EM Science, Gibbstown, NJ) and 98.5 ml 70% EtOH) for one minute. Slides were then dipped ten times in distilled water and allowed to air dry. Finally, a coverslip was applied with a drop of Dako Mounting Media (Dako Corporation, Carpinteria, CA)

Immunohistochemical staining for neutrophils was performed on biopsies of the ischemic lobes embedded in OCT as described above for ICAM-1. The primary antibody was a rat IgG_{2a} anti-mouse neutrophil mAb (7/4) (CL8993AP, Cedarlane, Accurate Chemical & Scientific Corporation, Westbury, NY); while the secondary antibody was

the biotin-conjugated mouse anti-rat IgG mAb (PharMingen, San Diego, CA). The procedure was otherwise as described above.

Determination of Plasma Alanine Aminotransferase Levels

Plasma levels of ALT were utilized as an established marker of hepatic I/R injury.[110] Blood samples were obtained from the right ventricle via a midline sternotomy at the time of sacrifice. The blood was collected in a sterile heparinized (50 µl, 100 USP Units/ml, Abbott Laboratories, North Chicago, IL) 3 cc syringe (Becton Dickinson & Co., Franklin Lakes, NJ) with a 25G needle (Becton Dickinson & Co., Franklin Lakes, NJ) and placed in an ice bath. The blood was immediately centrifuged (Eppendorf Model 5415C, Brinkmann Instruments, Inc., Westbury, NY) at 14,000 rpm in 18-20° C for 15 minutes. The plasma fraction was then transferred to two sterile 1.5 ml eppendorf microcentrifuge tubes (Brinkmann Instruments, Inc., Westbury, NY) using a sterile transfer pipette (Sarstedt, Inc., Newton, NC). The plasma was then stored at –70°C until processing of the ALT assay.

Measurement of plasma ALT was performed using a diagnostic kit from Sigma Chemical Company (St. Louis, MO). This assay is based on colorimetric measurement of transaminase in plasma by formation of 2, 4-dinitrophenylhydrazones of the keto-acids produced by the enzymes.[126] Briefly, 1.0 ml of Alanine-α-KG Substrate (Sigma Chemical Company, St. Louis, MO) was pipetted into 15 ml disposable glass culture tubes (VWR Scientific, Chicago, IL). Plasma samples were diluted in 0.9% saline. Next 200 µl of each diluted plasma sample was added to each tube and vortexed (Vortex Genie 2, Fisher Scientific, Pittsburgh, PA) for 10 seconds. The samples were then incubated in

a 37° C water bath (Lauda, Model M20, VWR Scientific, Chicago, IL) for 30 minutes. Next, 1.0 ml of Sigma Color Reagent was added to the reaction tube and vortexed and incubated at room temperature for 20 minutes. The reaction was stopped with 10 ml of 0.40N sodium hydroxide (J. T. Baker Chemical Company, Phillipsburg, NJ) solution. The tubes were then covered with Parafilm® "M" (American National Can, Menasha, WI) and the contents mixed using gentle inversion. After 5 minutes, the absorbance was measured at 500 nm using a Spectramax Plus Microplate Reader (Molecular Devices, Sunnyvale, CA). A standard curve was generated from a plot of the O.D. values at 500 nm versus concentrations of the standard sample (Sigma Chemical Company, St. Louis, MO) with a known amount of ALT activity per ml (Sigma-Frankel Units/ml). The plasma concentration of ALT activity in the experimental samples was then calculated from the standard curve. Results were expressed in International Units per liter (IU/L).

Histological Assessment of Hepatocellular Damage

Portions of the ischemic and non-ischemic liver lobes were fixed in buffered 10% formalin and embedded in paraffin. Sections were cut to 5 microns, which underwent routine staining with hematoxylin and eosin (H+E). The tissues were examined by a board certified veterinary pathologist (J.H.) who was blinded from the experimental treatment of the individual mice.

Determination of Hepatic Myeloperoxidase Levels

Tissue levels of MPO were utilized as a specific marker of the hepatic neutrophil content.[127] This assay is based on the oxidation of tetramethylbenzidine (TMB) by the

enzyme MPO, which is specific to neutrophils. The reagent TMB turns blue in its oxidized form.

Hepatic tissue harvested from the animal was snap frozen in liquid nitrogen, wrapped in aluminum foil, and stored at –70°C until processing for determination of tissue MPO levels. Between 100 and 500 mg of tissue was weighed and placed in 10 ml of cold homogenization buffer (0.02 M potassium phosphate buffer, pH 7.4 with 0.1 mM EDTA) kept on ice. Tissue was then homogenized using a Tissue Tearor® (model 985-370, Biospec Products, Inc., Bartlesville, OK) set to level 2 until the tissue was liquefied. The solution was then transferred to a new 10 ml high speed centrifuge tube and centrifuged at 9500 revolutions per minute (rpm) for 5 minutes at 4° C. The supernatant was discarded using a plastic transfer pipette and the pellet was gently dislodged. Hexadecyltrimethylammonium bromide (HTAB) suspension buffer (0.05 M potassium phosphate buffer, pH 6.0 with 0.5% HTAB) was added to the tissue in a ratio of 200 μl of buffer to 100 mg of tissue. The solution was frozen at –70° C and then allowed to thaw. The samples were sonicated at 25% per second for 40 seconds using an ultrasonic processor (Sonicator,® Misonox, Inc., Farmingdale, NY). Samples were again frozen at –70° C and then allowed to thaw before being sonicated again. Samples were then incubated at 60° C for 2 hours[128] and centrifuged at 9500 rpm for 5 minutes at 4° C. The supernatant was then transferred to a 1.5 ml eppendorf tube and centrifuged at 14,000 rpm for 1 minute at 4° C. The new supernatant was divided between two 1.5 ml eppendorf tubes and stored in a freezer at -70° C. Sample, control, and standard solutions were pipetted in duplicate into a 96 well microtiter plate in a volume of 25 μl per well. TMB substrate (Cat#34021ZZ, Pierce, Rockford, IL) was added in a volume of 100 μl

and the plate was allowed to develop at room temperature for 8 minutes. The reaction was stopped with 100 μl of 2.0 M sulfuric acid. The absorbance of the plate was then read at 450 nm using the Spectramax Plus Microplate Reader. A standard curve was generated from a plot of the optical density values at 450 nm versus the standard control sample (Cat#34021ZZ, Pierce, Rockford, IL) with a known amount of MPO activity per μl. The tissue concentration of MPO activity in the experimental samples was then calculated from the standard curve.

Statistical Analysis

A Kruskal-Wallis One-Way Analysis of Variance followed by a Bonferroni test were utilized to test for differences in ALT and MPO between sham, wild-type, and P/I null mice. Survival data was assessed using the Kaplan-Meier log rank test. Analysis was performed using the Number Cruncher Statistical System (Number Cruncher Statistical Systems, Kaysville, UT). A p value less than 0.05 was considered statistically significant.

RESULTS

Verification of ICAM-1 Deficiency in P/I Null Mice

ICAM-1 expression was assessed *in situ* by specific immunohistochemical staining for ICAM-1 (CD54) utilizing a specific monoclonal antibody to mouse ICAM-1. ICAM-1 was demonstrated to be expressed in wild-type mice after I/R as indicated by intense brown staining along the endothelium of the central vein, sinusoids and portal vasculature (Figure 3A). In P/I null mice after I/R, ICAM-1 deficiency was confirmed by the lack of staining along the endothelium (Figure 3B). It should be noted that this image, and all images in this thesis are presented in color.

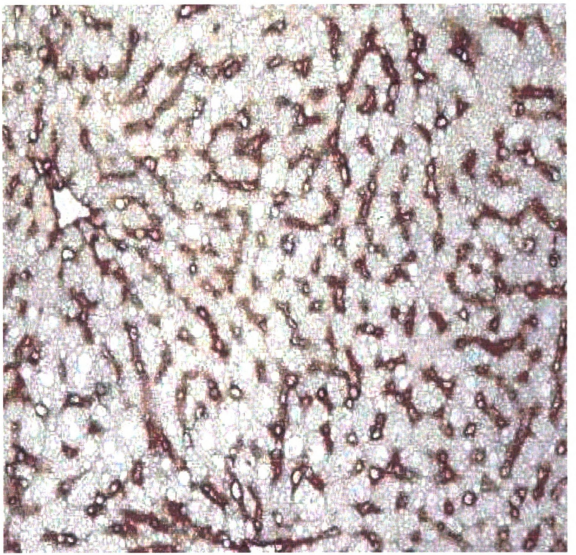

Figure 3A. ICAM-1 Staining In Wild-Type Mouse Liver

Immunohistochemical stain for ICAM-1 appears dark brown along the endothelial margins in this wild-type mouse liver after 90 minutes of ischemia followed by 6 hours of reperfusion.

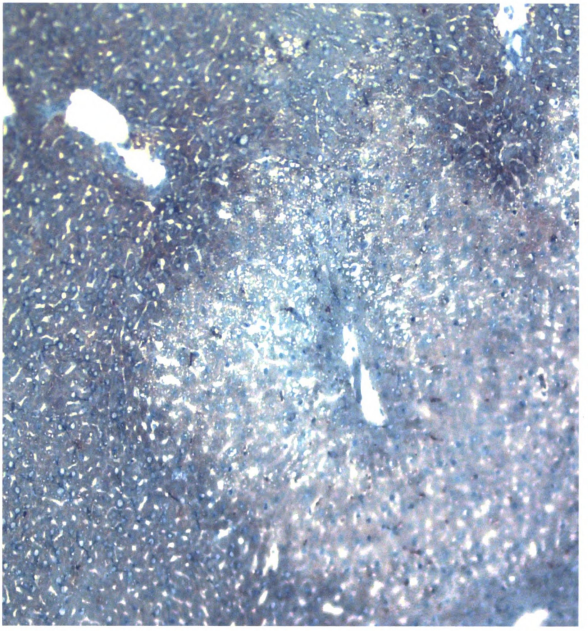

Figure 3B. ICAM-1 Staining In P/I Null Mouse Liver

Absence of the dark brown immunohistochemical stain in this tissue section of P/I null mouse liver after 90 minutes of ischemia followed by 6 hours of reperfusion confirms the absence of ICAM-1 expression in these animals.

Demonstration of Hepatocellular Injury By Quantifying Plasma ALT Levels

Hepatic I/R caused significant hepatocellular damage as demonstrated by plasma ALT levels. The plasma ALT levels of both wild-type and P/I null mice after 90 minutes of ischemia followed by 6 and 15 hours of reperfusion were significantly elevated compared to the sham-operated mice at the same time points. However, there was no statistically significant difference in ALT levels between the wild-type and P/I null mice at either time point (Table 1, Figure 4, Figure 5). The sample size was 7 mice for each of the ischemia groups and 6 mice for each of the sham groups.

Reperfusion Time	Assay	Sham	Wild-Type	P/I Null
6 h	ALT (IU/L)	45.3 ± 52	3211.1 ± 441*	3313.2 ± 269*
	MPO (IU/mL)	0.36 ± 0.02	0.67 ± 0.06*	0.60 ± 0.05*
15 h	ALT (I/U/L)	43.7 ± 6	1820.7 ± 351*	1095.6 ± 292*
	MPO (IU/mL)	0.49 ± 0.05	0.76 ± 0.05*	0.71 ± 0.07

Table 1. Summary of Plasma ALT and Hepatic MPO Levels in Wild-Type and P/I Null Mice

Values are expressed as the mean ± SEM with n = 6 for each sham group and n = 7 for each of the IR groups. *Indicates groups with a statistically significant difference compared to shams.

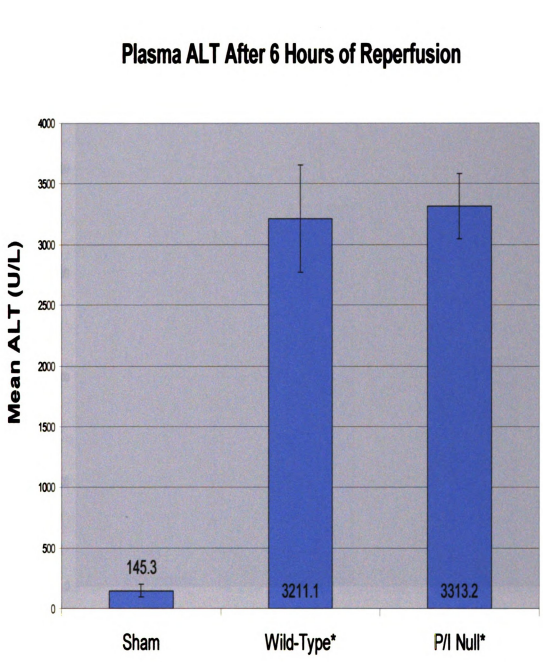

Figure 4. Plasma ALT After 90 Minutes of Hepatic Ischemia and 6 Hours of Reperfusion

Values are expressed as the mean with the error bars representing $\pm$ SEM. * Indicates statistically significant difference from the sham group. Wild-type ischemic mice were not significantly different from P/I null mice.

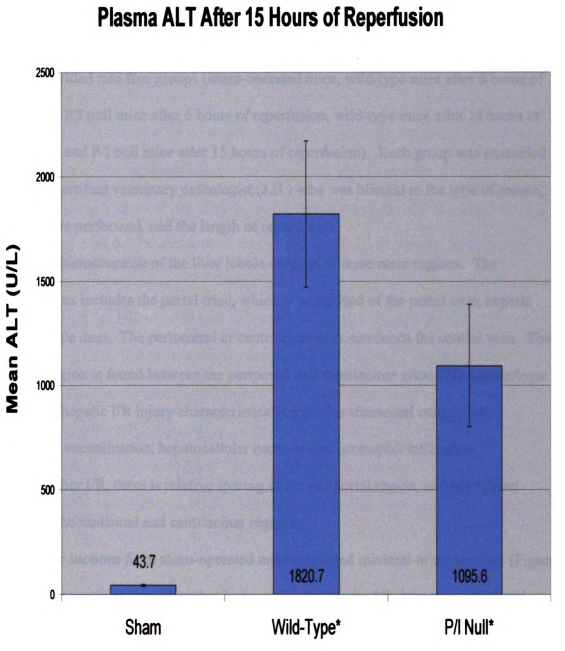

Figure 5. Plasma ALT After 90 Minutes of Hepatic Ischemia and 15 Hours of Reperfusion

Values are expressed as the mean with the error bars representing ± SEM. * Indicates significant difference from sham group. Wild-type ischemic mice were not significantly different from P/I null mice.

Demonstration of Hepatocellular Injury by Histopathology – Hematoxylin and Eosin Staining

Tissue sections harvested from the post-ischemic lobes of liver were stained with H+E and divided into five groups (sham-operated mice, wild-type mice after 6 hours of reperfusion, P/I null mice after 6 hours of reperfusion, wild-type mice after 15 hours of reperfusion, and P/I null mice after 15 hours of reperfusion). Each group was examined by a board certified veterinary pathologist (J.H.) who was blinded to the type of mouse, the procedure performed, and the length of reperfusion.

The histostructure of the liver lobule consists of three main regions. The periportal area includes the portal triad, which is comprised of the portal vein, hepatic artery and bile duct. The pericentral or centriacinar area surrounds the central vein. The midzonal region is found between the periportal and centriacinar areas. Histopathologic evidence of hepatic I/R injury characteristically includes sinusoidal congestion, cytoplasmic vacuolization, hepatocellular necrosis and neutrophil infiltration.[129] Typically, after I/R, there is relative sparing of the periportal region, and significant necrosis in the midzonal and centriacinar regions.

Liver sections from sham-operated mice displayed minimal or no necrosis (Figure 6). The liver sections from mice that had undergone hepatic I/R, however, all shared similar histopathologic characteristics (Figures 7A, 7B, 8A, and 8B). In these mice focal areas of hepatocellular degeneration and coagulative necrosis with associated sinusoid congestion, hemorrhage and accumulation of neutrophils (i.e., acute neutrophilic inflammation) were observed. Often sinusoidal microthrombi and larger fibrinous thrombi in the lumens of adjacent central veins were also present in the most affected regions of the liver. Necrotic areas in the hepatic parenchyma varied in size from small

foci restricted to midzonal or centriacinar regions in widely scattered acini to large coalescing areas of midzonal and centriacinar necrosis bridging several acini. Often periportal regions were spared, but in some areas of severe (massive) necrosis these regions of the acinus were also affected.

The only difference detected in the hepatic histopathology among the four I/R groups was the extent of the hepatic parenchyma undergoing necrosis. The wild-type group undergoing 6 hours of reperfusion generally had larger areas of coagulative necrosis when compared to P/I null mice after 6 hours of reperfusion, wild-type mice after 15 hours of reperfusion, and P/I null mice after 15 hours of reperfusion. The 6 hour wild-type group generally had massive areas of necrosis involving more than 75% of the liver section (Figure 7A) while the other three groups had moderate hepatocellular necrosis involving approximately 20 to 50% of the hepatic parenchyma (Figures 7B, 8A, and 8B). Other characteristics among the four I/R groups were similar. The reasons as to why the wild-type group undergoing 6 hours of reperfusion should have larger areas of coagulative necrosis are not clear but it is possible these differences are due to small liver tissue sample sizes.

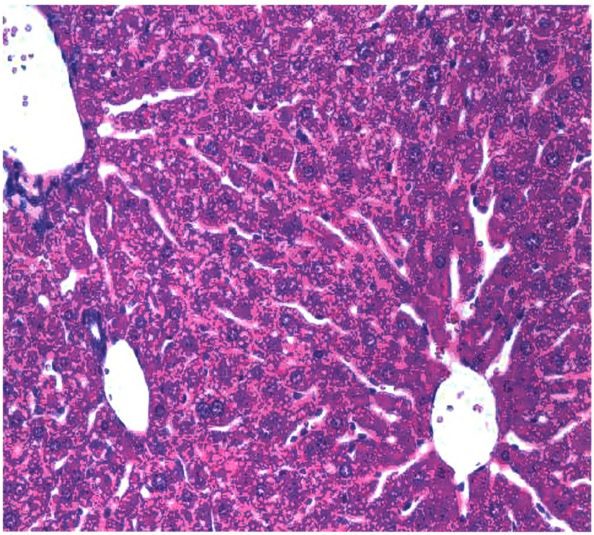

Figure 6. Histopathologic Tissue Section Stained With H+E From Sham-Operated Mouse

Little or no hepatocellular necrosis with minimal neutrophil infiltration was observed in this group.

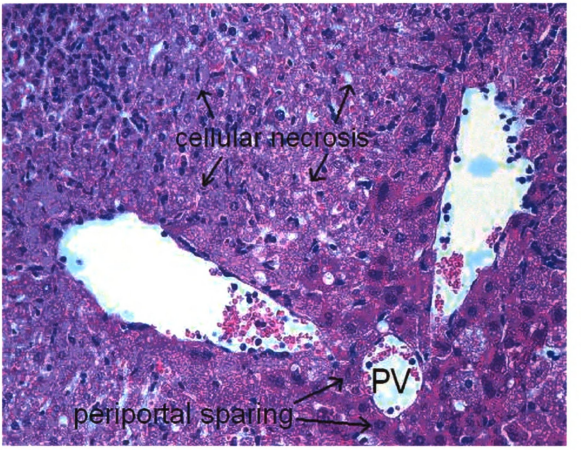

Figure 7A. Histopathologic Tissue Section Stained With H+E From Wild-Type Mouse After 6 Hours of Reperfusion

Large areas of hepatocellular degeneration and coagulative necrosis with associated sinusoid congestion, hemorrhage and accumulation of neutrophils were seen in this group. This group had massive areas of necrosis often involving more than 75% of the liver section. Cells closest to the portal vein were consistently spared from necrosis. (PV = portal vein)

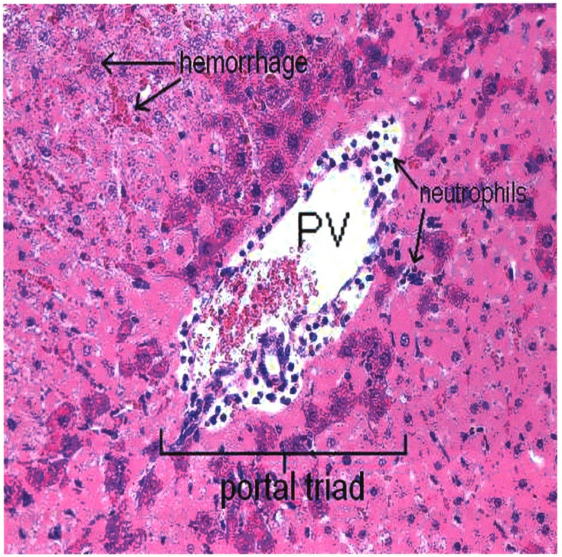

Figure 7B. Histopathologic Tissue Section Stained With H+E From P/I Null Mouse After 6 Hours of Reperfusion

Large areas of hepatocellular degeneration and coagulative necrosis with associated sinusoid congestion, hemorrhage and accumulation of neutrophils were seen in this group. This group had large areas of necrosis often involving 20-50% of the liver section. (PV = portal vein)

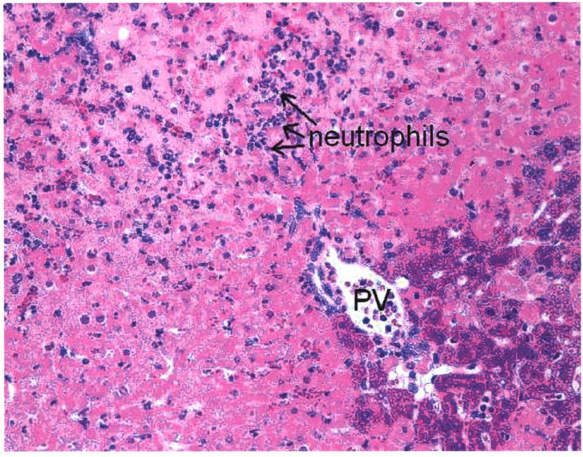

Figure 8A. Histopathologic Tissue Section Stained With H+E From Wild-Type Mouse After 15 Hours of Reperfusion

Large areas of hepatocellular degeneration and coagulative necrosis with associated sinusoid congestion, hemorrhage and accumulation of neutrophils were seen in this group. This group had large areas of necrosis often involving 20-50% of the liver section. (PV = portal vein)

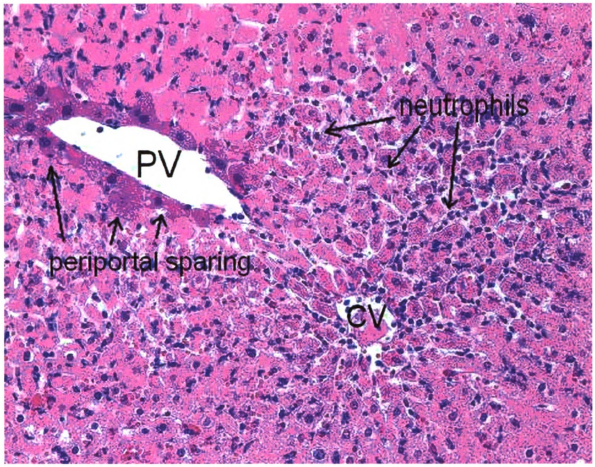

Figure 8B. Histopathologic Tissue Section Stained With H+E From P/I Null Mouse After 15 Hours of Reperfusion

Large areas of hepatocellular degeneration and coagulative necrosis with associated sinusoid congestion, hemorrhage and accumulation of neutrophils were seen in this group. This group had large areas of necrosis often involving 20-50% of the liver section. (PV = portal vein; CV = central vein)

Demonstration of Liver Neutrophil Content By Quantifying Hepatic Myeloperoxidase Levels

Myeloperoxidase is an enzyme contained specifically in neutrophils. Hepatic tissue sections were processed to quantify their MPO content in wild-type and P/I null mice to compare the neutrophil content of both sham-operated and ischemic groups. Hepatic I/R caused significant neutrophil infiltration into the liver as demonstrated by hepatic MPO levels. The hepatic MPO levels of both wild-type and P/I null mice after 90 minutes of ischemia followed by 6 and 15 hours of reperfusion were significantly elevated compared to the sham-operated mice at the same time points. However, although the liver MPO contents of P/I null mice were elevated after 15 hours of reperfusion, it did not reach a statistical significance when compared to the sham values (Figure 10). There was no statistically significant difference in MPO levels between the wild-type and P/I null mice at either time point (Table 1, Figure 9, Figure 10).

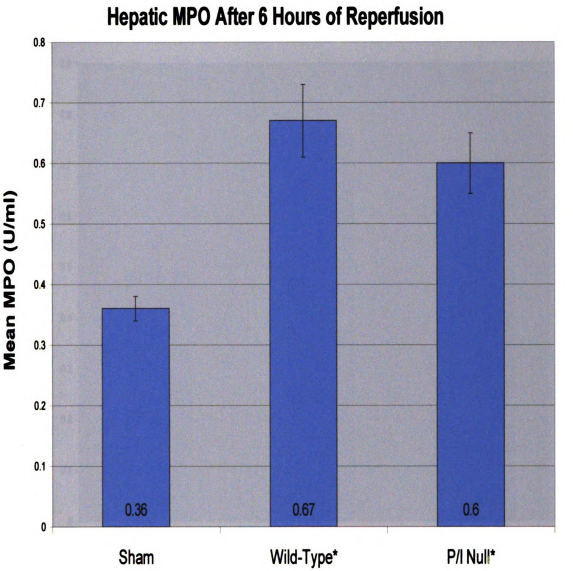

Figure 9. Hepatic MPO After 90 Minutes of Hepatic Ischemia and 6 Hours of Reperfusion

Values are expressed as the mean $\pm$ standard error of the mean. * Indicates significant difference from sham group. The wild-type ischemic group was not significantly different from the P/I null group.

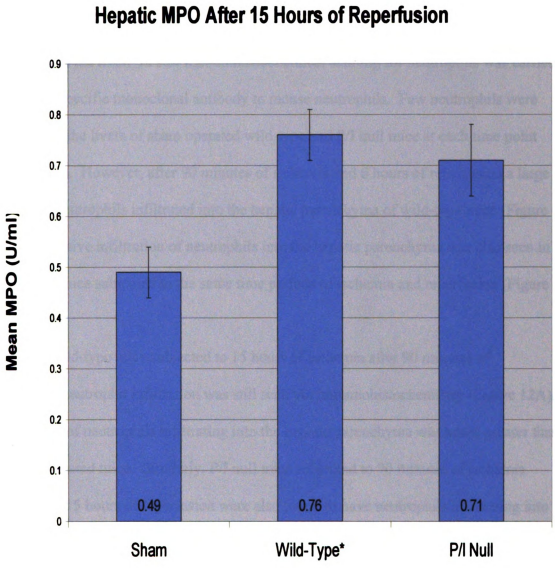

Figure 10. Hepatic MPO After 90 Minutes of Hepatic Ischemia and 15 Hours of Reperfusion

Values are expressed as the mean $\pm$ standard error of the mean. * Indicates a significant difference compared to the sham group. The wild-type ischemic group was not significantly different from P/I null group.

Demonstration of Neutrophil Infiltration Using Immunohistochemistry

Immunohistochemical staining for neutrophils was utilized to visualize neutrophil infiltration in the liver. *In situ* immunohistochemical staining for neutrophils was carried out using a specific monoclonal antibody to mouse neutrophils. Few neutrophils were identified in the livers of sham operated wild-type and P/I null mice at each time point (Figure 11A). However, after 90 minutes of ischemia and 6 hours of reperfusion a large number of neutrophils infiltrated into the hepatic parenchyma of wild-type mice (Figure 11B). Extensive infiltration of neutrophils into the hepatic parenchyma was also seen in the P/I null mice subjected to the same time periods of ischemia and reperfusion (Figure 11C).

In wild-type mice subjected to 15 hours of ischemia after 90 minutes of reperfusion neutrophil infiltration was still seen via immunohistochemistry (Figure 12A). The number of neutrophils infiltrating into the hepatic parenchyma was again greater than in sham-operated mice. Similarly, P/I null mice subjected to 90 minutes of ischemia followed by 15 hours of reperfusion were also found to have neutrophils infiltrating into the hepatic parenchyma greater than in sham-operated mice (Figure 12B).

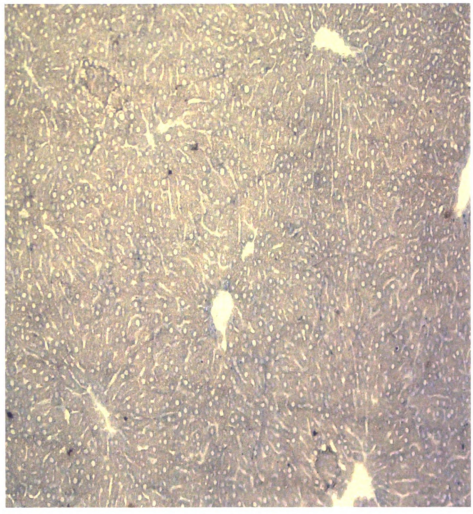

Figure 11A. Immunohistochemical Staining For Neutrophils In Sham Mice

This hepatic tissue section represents the typical sparse neutrophil infiltration seen in sham-operated mice. Neutrophils stain dark brown. This particular section is from a wild-type sham-operated mouse.

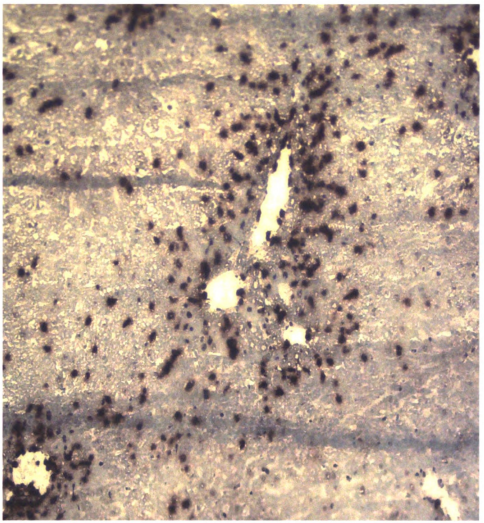

Figure 11B. Immunohistochemical Staining For Neutrophils In Wild-Type Mice After 6 Hours of Reperfusion

This hepatic tissue section represents the typical extensive neutrophil infiltration seen in wild-type mice after 90 minutes of ischemia followed by 6 hours of reperfusion. Neutrophils stain dark brown.

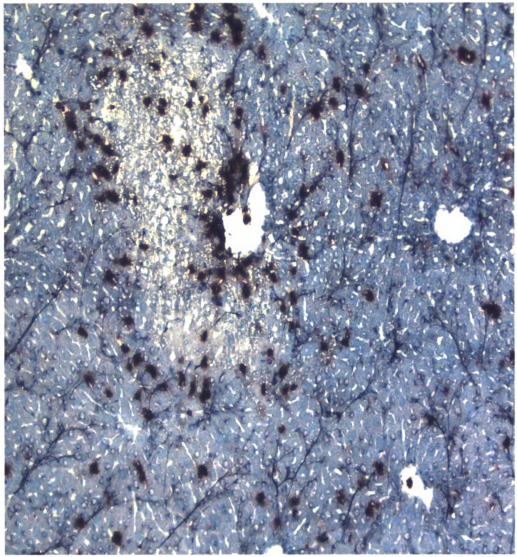

Figure 11C. Immunohistochemical Staining For Neutrophils In P/I Null Mice After 6 Hours of Reperfusion

This hepatic tissue section represents the typical extensive neutrophil infiltration seen in P/I null mice after 90 minutes of ischemia followed by 6 hours of reperfusion. Neutrophils stain dark brown.

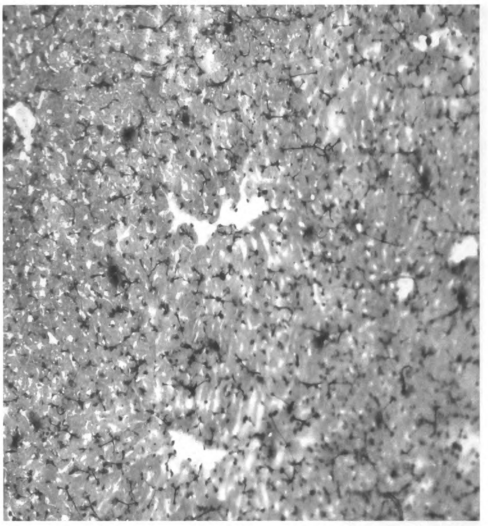

Figure 12A. Immunohistochemical Staining For Neutrophils In Wild-Type Mice After 15 Hours of Reperfusion

This hepatic tissue section represents the typical neutrophil infiltration seen in wild-type mice after 90 minutes of ischemia followed by 15 hours of reperfusion. Neutrophil infiltration at this time point is greater than in shams. Neutrophils stain dark brown.

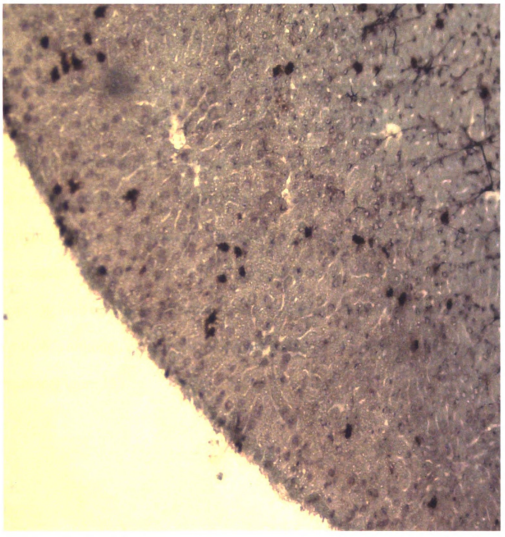

Figure 12B. Immunohistochemical Staining For Neutrophils In P/I Null Mice After 15 Hours of Reperfusion

This hepatic tissue section represents the typical neutrophil infiltration seen in wild-type mice after 90 minutes of ischemia followed by 15 hours of reperfusion. Neutrophil infiltration at this time point is greater than in shams. Neutrophils stain dark brown.

Survival Study

Ten wild-type and ten P/I null mice were subjected to 90 minutes of partial hepatic ischemia after which the vascular clamp was removed and the abdomen closed in the manner described in the methods section. The mice were observed post-operatively and their approximate times of death were recorded. At the end of three weeks, all surviving mice were euthanized. This study found that all P/I null mice survived the full three weeks while only 7 out of 10 of the wild-type mice survived that long. The Kaplan-Meier log rank test showed no statistically significant difference between the two groups ($p = 0.067$) although a trend towards improved survival in the P/I null group was apparent (Figure 13).

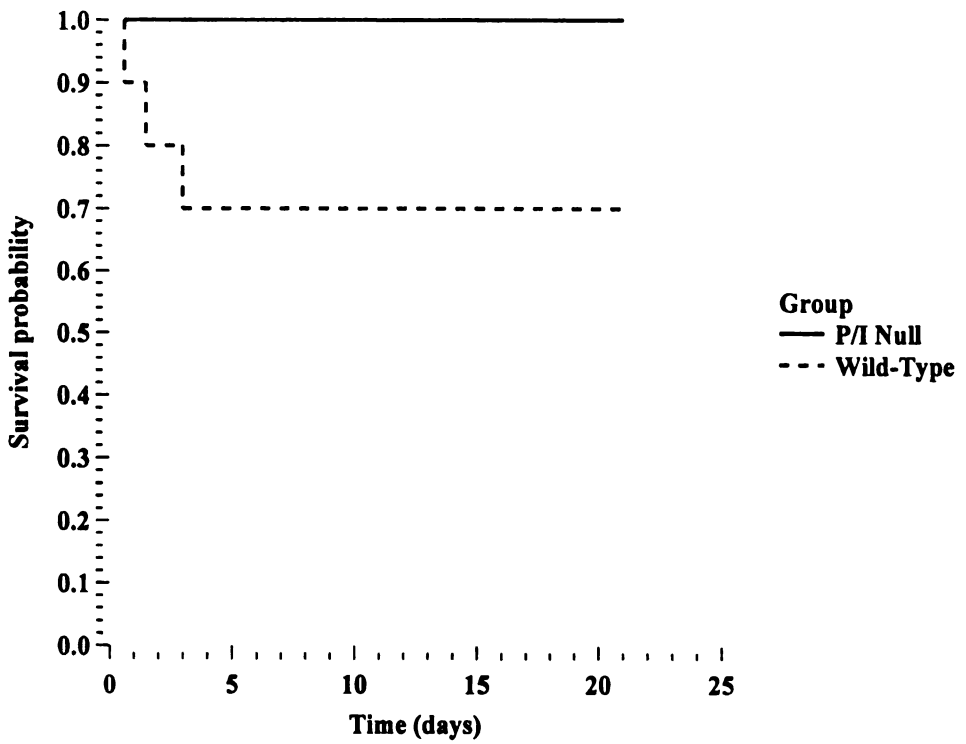

Figure 13. Kaplan-Meier Survival Curve

The plot shows four curves. The top curve (horizontal line) is the survival curve for the P/I null mice (10/10 mice survived). The third curve from the top is the survival curve for the wild-type mice (7/10 mice survived). The second and last curves from the top represent the 95% confidence intervals for the wild-type mice. Wild-type survival was not significantly different from P/I null survival ($p = 0.067$) although a trend is apparent.

DISCUSSION

Previous studies of hepatic ischemia and reperfusion have suggested a role for adhesion molecules in neutrophil-mediated injury. Specifically, studies using monoclonal antibodies in murine models of hepatic I/R have implicated ICAM-1 and P-selectin as important adhesion molecules in this capacity. In this study it was hypothesized that the cellular adhesion molecules P-selectin and ICAM-1 are necessary for neutrophil-mediated hepatic I/R injury. Transgenic mice with deficiencies in the adhesion molecules P-selectin and ICAM-1 were employed in a murine model of partial hepatic I/R injury to examine the effects of this injury on these mice compared to wild-type mice with normal P-selectin and ICAM-1 expression. Sham-operated mice that underwent laparotomy and dissection but no ischemia were used as a negative control. The outcome variables assessed were neutrophil infiltration into the liver, hepatocyte injury, and overall survival. Neutrophil infiltration was assessed using hepatic MPO levels and immunohistochemical staining specific for mouse neutrophils. Hepatocyte damage was assessed using plasma ALT levels and histopathologic examination. Survival rates were assessed in separate groups of mice undergoing the same ischemia and reperfusion procedure. Hepatic I/R injury was shown to occur in both P/I null and wild-type I/R groups in comparison to sham-operated negative controls. However, by all quantitative assessments made of all three outcome variables, there were no statistically significant differences between P/I null and wild-type mice subjected to partial hepatic I/R. These findings strongly suggest that P-selectin and ICAM-1 are in fact not necessary for neutrophil-mediated hepatic I/R injury to occur and that hepatic I/R injury occurs to a similar extent in the absence of these adhesion molecules.

Results from a previous study performed at our laboratory are in accordance with the present findings. In this study shorter periods of reperfusion were examined (0, 1.5 and 3 hours) in the same model of partial hepatic I/R comparing P/I null mice with wild-type mice. This study determined that there were no statistically significant differences between the two groups with respect to neutrophil sequestration and hepatocyte injury.[122] However, since the peak time period for neutrophil-mediated hepatocyte injury is after at least 6 hours,[27] we felt it prudent to design the present study to focus attention on these later time points.

Among all of the outcome variables that were assessed the only one that detected a difference among I/R groups was the qualitative assessment of hepatocellular injury by histopathologic examination. By this assessment, the wild-type group after 6 hours of reperfusion generally had larger areas of necrosis compared to P/I null mice at the same time point (Figures 7A and B). The 6 hour wild-type group generally had areas of necrosis involving more than 75% of the liver section while the 6 hour P/I null group had hepatocellular necrosis involving approximately 20 to 50% of the hepatic parenchyma. However, this finding is inconsistent with the rest of our data that suggests no difference exists. This data includes ALT measurements comparing wild-type and P/I null mice after 6 hours of reperfusion. ALT measurement is a more quantitative assessment of the extent of hepatocellular injury that occurred throughout the entire liver. Histopathologic assessment, in contrast, was qualitative and more prone to sampling error since the pathologist was provided with only 1-2 small tissue sections per animal. Individual tissue sections represent only the pathologic process that is occurring in that small two-dimensional area of the liver and may not be an accurate representation of the entire post-

ischemic liver lobe. Additionally, this finding of greater than 75% necrosis in the 6 hour wild-type group was inconsistent with observations in wild-type mice after 15 hours of reperfusion. Mice in this latter group contained only 20 to 50% areas of hepatocyte necrosis which was comparable with both the P/I null mice at that time point and after 6 hours of reperfusion. Clearly it is not possible for mice to truly have 75% hepatocyte necrosis and then only 20 to 50% necrosis 9 hours later. For these reasons, we considered ALT levels a more accurate measure for quantifying hepatocellular injury. Histopathologic analysis, however, was still quite useful for observing the qualitative changes that occurred in the liver after hepatic I/R including focal areas of hepatocellular degeneration and coagulative necrosis with associated sinusoidal congestion, hemorrhage and neutrophil accumulation.

There are a number of possible explanations for why P-selectin and ICAM-1 did not seem to be required for hepatic I/R injury to occur. In order to examine these explanations, let us consider the step-wise model of neutrophil-mediated hepatic injury that has been proposed for hepatic I/R injury.[reviewed in 71] The first step in this model is neutrophil sequestration within the liver. In the present study, we found neutrophil sequestration in the liver to be similar among P/I null mice and wild-type mice as determined by hepatic MPO levels and immunohistochemical staining of neutrophils. Although adhesion molecule dependent neutrophil sequestration has been demonstrated in other organs such as skeletal muscle,[46-48] mesentery,[49;50] heart,[51-53] lung,[54] and brain,[55] in the liver this process is likely a result of the mechanical consequences of inflammation rather than chemical ones mediated by adhesion molecule interactions. Hepatic inflammatory stimuli induce a number of physical changes that contribute to this

mechanical sequestration. First, swelling of the endothelial lining cells occurs in response to various inflammatory mediators.[(reviewed in 130)] Second, increasing concentrations of endothelin-1 relative to nitric oxide cause vasoconstriction of the sinusoids resulting in further narrowing the sinusoidal lumen.[131;132] Third, peptide chemoattractants released during hepatic inflammation cause stiffening and decreased deformability of neutrophils.[133] These three factors contribute to the mechanical trapping of neutrophils in the hepatic sinusoids independent of adhesion molecule interactions. In support of this concept are a several studies that show neutrophil accumulation in the sinusoids is not attenuated by blocking a number of different adhesion molecules, including P-selectin and ICAM-1, with monoclonal antibodies.[40;41;76;91;134;135]

The next step in neutrophil-mediated hepatic I/R injury, after neutrophil sequestration, is neutrophil transmigration across the endothelial barrier into the hepatic parenchyma. In the case of endotoxin-induced hepatic injury, little endothelial cell damage occurs and transendothelial migration of neutrophils is required to cause hepatocyte injury.[75] However, in I/R injury, initial activation of Kupffer cells results in extensive vascular injury. In this scenario, transendothelial migration of neutrophils may be less critical for the induction of hepatocyte damage since the endothelial barrier is compromised and even absent in some areas. Still, various studies using blocking antibodies against either ICAM-1 or Mac-1 have shown decreased hepatocellular injury after I/R.[40;87;90;91] It is unlikely that these results are secondary to neutrophil sequestration effects since neutrophils seem to be sequestered in the liver in similar numbers with or without these molecules.[40] Although ICAM-1 and Mac-1 dependent transendothelial migration of neutrophils is important for hepatic injury after various inflammatory insults

such as endotoxemia, its role after hepatic I/R is likely to be less important due to Kupffer cell-mediated injury and disruption of the endothelial barrier. Antibodies that block ICAM-1 or Mac-1 may attenuate hepatic injury by blocking some transendothelial migration of neutrophils, preventing neutrophil binding to hepatocytes, or via non-specific antibody interactions unrelated to these adhesion molecules. It is also possible that attenuation of injury in these experiments results from a combination of these factors.

Additionally, it is unlikely that P-selectin plays an important role in hepatic I/R injury through either neutrophil sequestration or transendothelial migration, as it appears to do in other organs. P-selectin is stored in endothelial cells in Weibel Palade bodies which fuse with the cell membrane in response to inflammatory mediators.[74] This results in rapidly increased expression of P-selectin on the surface of endothelial cells. P-selectin is thought to be involved in neutrophil sequestration through multiple weak interactions with its ligand which is expressed on the surface of neutrophils. These interactions result in rolling of the neutrophils along the endothelial surface. Such rolling slows neutrophil passage through the liver microvasculature and puts the neutrophils in close contact with the endothelial surface. Neutrophil rolling allows for further adhesion molecule interactions, such as those between ICAM-1 and the $\beta 2$ integrins. Such P-selectin-mediated induction of neutrophil rolling has been demonstrated in the post-sinusoidal venules after inflammatory stimuli including hepatic I/R.[76;107] However, sinusoidal endothelial cells do not contain Weibel Palade bodies[78;136] and transcriptional activation of P-selectin occurs in large vessel endothelial cells but not in endothelial cells of the sinusoids.[76] Selectins are not expressed on the sinusoidal endothelia, even in the face of severe inflammation.[77] Furthermore, the extravasation of neutrophils in response

to inflammatory stimuli was found to occur only at the sinusoids and not the post-sinusoidal venules.[60] There is no evidence that neutrophils tethered in the post-sinusoidal venules actually transmigrate and cause injury to hepatocytes. At least in the case of endotoxin-induced hepatic injury, it is the transmigrated neutrophils and not the intravascular neutrophils that significantly contribute to the hepatic injury.[60;137] Therefore, it is unlikely that P-selectin plays an important role in hepatic injury through neutrophil sequestration or facilitation of transendothelial migration.

After neutrophil sequestration and migration into the hepatic parenchyma, activated neutrophils adhere to hepatocytes via adhesion molecule interactions.[(reviewed in 71)] This adhesion increases and prolongs neutrophil release of cytotoxic molecules which facilitates neutrophil-induced hepatocyte necrosis.[66;67] Activated neutrophils adhere to hepatocytes via two independent groups of adhesion molecule interactions with β2-integrins. These include a lymphocyte function-associated antigen (LFA-1; CD11a/CD18) interaction with ICAM-1 and a Mac-1 dependent adhesion that is independent of ICAM-1.[138] Therefore, there is evidence that although ICAM-1 does play a role in neutrophil adherence-mediated hepatocyte injury, there are also other pathways that can accomplish the same end. As we have seen for both neutrophil sequestration and transmigration, the adhesion molecules P-selectin and ICAM-1 may play a role in hepatic I/R injury, but they are not necessary for its occurrence. The results of the present study are consistent with this concept. Hepatocyte injury, as determined by plasma ALT levels and histopathology, was similar between P/I null mice with wild-type mice undergoing hepatic I/R.

Just as neutrophil adherence to hepatocytes has been shown to occur via two separate and independent groups of adhesion molecules,[138] there is evidence that such functional redundancy of adhesion molecules exists at multiple steps in the I/R pathway. A study of the inflammatory mediators leukotriene B4 and PAF on the behavior of leukocytes in mesenteric venules demonstrated such functional redundancy of adhesion molecules.[139] This study showed that leukocyte rolling was attenuated by mAbs against either P-selectin or E-selectin. Additionally, mAbs against either ICAM-1 or E-selectin reduced leukocyte adherence and transmigration.[139] In a murine model of endotoxin shock, mAb against VCAM-1 had no effect on sequestration of neutrophils within the liver microvasculature. However, this mAb against VCAM-1 was successful in attenuating neutrophil transendothelial migration by 84% and hepatocyte necrosis by 60%.[134] Therefore, VCAM-1 may share the function of mediating neutrophil transendothelial migration with ICAM-1. Yet another study demonstrated in vitro that neutrophil adherence to endothelial cells was only partially dependent on ICAM-1 interactions with β2-integrins and that Mac-1 appeared to recognize and bind an ICAM-1 independent ligand.[138]

In addition to overlap in function of specific adhesion molecules, there is also evidence that complement factors mediate neutrophil adherence to endothelial cells. As previously discussed, the complement cascade is activated by hepatic I/R. In response to complement activation neutrophils rapidly adhere to endothelial cells via complement receptor 3 interactions with endothelial bound activated C3.[140] Therefore, neutrophil adherence to endothelial cells, transendothelial migration, and subsequent adherence to hepatocytes may be mediated, at least to some extent, by complement factors and

adhesion molecules other than P-selectin and ICAM-1. Functional redundancy of adhesion molecules and the complement system may be sufficient to compensate for the absence of P-selectin and ICAM-1 in mediating neutrophil-induced hepatic I/R injury in P/I null mice.

Despite these arguments, several past studies have implicated a major role for P-selectin and ICAM-1 in hepatic inflammatory injury. Earlier studies employed blocking antibodies against either P-selectin or ICAM-1. Studies that blocked P-selectin with specific mAb in animals undergoing hepatic I/R reported a reduction in neutrophil adherence and hepatocellular injury.[105;106] Similar studies that used mAb to block ICAM-1 reported decreased hepatocellular injury.[90;91;111-113]Neutrophil sequestration in the liver, however, was not affected by administration of the antibody.[90;91;115] Although these studies appear to demonstrate an important role for P-selectin and ICAM-1 in hepatic I/R injury, it should be considered that blocking antibodies as a means of eliminating adhesion molecules is an imperfect model. Such antibodies may not have the exclusive effect of reducing neutrophil-mediated injury by specifically blocking P-selectin or ICAM-1. These mAbs may exhibit non-specific binding and cause unanticipated stimulatory or inhibitory effects on multiple arms of the inflammatory cascade. All of the selectins are structurally similar to one another. It is entirely possible that an antibody to P-selectin may bind other selectins as well. Evidence exists that suggests antibodies directed against adhesion molecules for the purpose of preventing neutrophil binding may have unanticipated effects. A mAb against P-selectin was shown to inhibit the deposition of complement membrane attack complexes (C5b-9) in a rat model of intestinal I/R injury.[85] Similarly, in a murine model of skeletal muscle I/R, soluble P-selectin blunted

the inflammatory response by inhibiting the classical complement pathway.[121] In microvessels, anti-ICAM-1 antibodies were found to reduce leukocyte rolling, an action mediated by selectins.[91] These effects may have been a result of decreased inflammation resulting from specific blocking of the adhesion molecules or they may have occurred because of non-specific antibody blocking of other molecules involved in the inflammatory process.

Due to these uncertainties, a more desirable model would eliminate the adhesion molecules in question without employing antibodies. Hence, genetic knockout mice were designed with deletions encoding for specific adhesion molecules. Several studies have used such mice to investigate the roles of various adhesion molecules in inflammation. Sligh et al, used ICAM-1 deficient mice in a model of chemical peritonitis and skin contact hypersensitivity. They found peritoneal neutrophil emigration to be impaired and contact hypersensitivity to be decreased in ICAM-1 deficient mice.[141] Sarman et al, in a model of bacterial peritonitis and skin infection found that ICAM-1 deficient mice had higher rates of bacteremia after peritoneal injection with *Pseudomonas aeruginosa* and larger skin lesions after intradermal injection with *Staphylococcus aureus*. Interestingly, ICAM-1 deficient mice were similar to wild-type controls in response to peritoneal injection of *S. aureus* and *Escherichia coli* and in response to intradermal injection of *P. aeruginosa* and *E. coli*. This study demonstrated that the role of ICAM-1 in response to inflammation is both site and stimulus dependent.[142]

Controversy in the literature has been generated through the use of P-selectin deficient mice in models of hepatic inflammation. Martinez-Mier et al used P-selectin deficient mice in a model of hepatic I/R injury.[109] Examining these mice after 90 minutes

of ischemia followed by 3 hours of reperfusion they reported reduced hepatic neutrophil infiltration and decreased hepatocellular injury in the P-selectin deficient mice compared to wild-type ischemic controls.[109] Yadav et al also found decreased hepatocellular injury and neutrophil adherence in the P-selectin deficient mice in most, but not all, of the time points they examined.[110] These studies seem to directly contrast others which have demonstrated that neutrophil-mediated hepatic injury was not attenuated in P-selectin deficient mice subjected to inflammatory stimuli. Essani et al, in a model of endotoxin shock, found no differences in neutrophil sequestration nor in hepatocellular injury in P-selectin deficient mice when compared to wild-type controls.[76] Young et al, in a model of hepatic I/R injury, used P/I null mice undergoing reperfusion times shorter than in the present study to show there was no difference in neutrophil sequestration or hepatic injury between P/I null mice and wild-type ischemic controls.[122] The present study is consistent with these latter studies which suggest P-selectin is not critical for neutrophil-mediated hepatic I/R injury.

Although genetic knockout models seem preferable to blocking antibody models for examining the roles of adhesion molecules in inflammation, genetic knockout models are also not without flaws. Eppihimer et al demonstrated that genetic knockout mice with deficiencies in CD11/CD18, P-selectin or ICAM-1 have altered expression of other adhesion molecules including P-selectin and E-selectin.[96] The chronic deficiency of P-selectin or ICAM-1 resulted in both altered basal and induced surface expression of other adhesion molecules on the surface of endothelial cells in some vascular beds. Additionally, it has been demonstrated that genetic knockout mice with deficiencies in P-selectin, ICAM-1, or both have significantly higher numbers of circulating neutrophils

relative to wild-type mice of similar genetic background.[120;141;143] These elements of the mutant mice are potential confounding variables.

The survival study resulted in the survival of all P/I null mice while 3 of the 10 wild-type mice died within the first 72 hours of reperfusion. This survival difference between the two groups did not quite reach statistical significance ($p = 0.67$) but a trend towards decreased survival was apparent. Future study with a larger sample size may detect a significant difference between the two groups. Lack of differences between wild-type and P/I null mice in neutrophil infiltration, hepatic MPO, plasma ALT, and hepatic histopathology suggest that the potential survival advantage of P/I null mice was not a result of decreased hepatic injury. Since local organ injury appeared to be similar between both groups, it is likely that the P/I null mice were less susceptible to the systemic manifestations of hepatic I/R injury such as ARDS and MODS.[92-94] Large quantities of inflammatory mediators such as TNF-α and IL-1 released during hepatic I/R travel systemically through the circulation resulting in the widespread activation of vascular endothelium in multiple organs. This endothelial cell activation results in the upregulation of P-selectin and ICAM-1 on the surface of endothelial cells.[95] Concurrently, the ligands for P-selectin and ICAM-1 are upregulated on the surface of leukocytes that are also activated by the systemic inflammatory mediators.[98] Previous studies have documented an important role for P-selectin and/or ICAM-1 in mediating parenchymal cell injury to organs other than the liver, including the heart,[52;118] intestine,[56] lung,[57] and skeletal muscle.[47] Because of this, the mice with deficiencies in P-selectin and ICAM-1 may have a survival advantage over their wild-type counterparts.

In conclusion, this study suggests that the adhesion molecules P-selectin and ICAM-1 did not play a significant role in mediating hepatic I/R injury. Neutrophil infiltration and hepatocellular injury occur to a similar extent in the absence of these adhesion molecules. These adhesion molecules, although not necessary for injury to occur in the liver itself during hepatic I/R injury, may influence overall survival by playing a role in the systemic organ injury that often ensues following ischemia and reperfusion of the liver. Continued research in this area is likely to yield important therapeutic strategies for the prevention and control of hepatic I/R injury and its disastrous systemic consequences in the critically ill patient.

REFERENCES

1. Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. *J.Pathol.* 2000; **190**: 255-266.

2. Maier RV. Pathogenesis of Multiple Organ Dysfunction Syndrome - Endotoxin, Inflammatory Cells, and Their Mediators: Cytokines and Reactive Oxygen Species. *Surgical Infections* 2000; **1**: 197-204.

3. Kvietys PR, Granger DN. Endothelial cell monolayers as a tool for studying microvascular pathophysiology. *Am.J.Physiol* 1997; **273**: G1189-G1199.

4. Rymsa B, Wang JF, de Groot H. O2-. release by activated Kupffer cells upon hypoxia-reoxygenation. *Am.J.Physiol* 1991; **261**: G602-G607.

5. Bouwens L. Structural and functional aspects of Kupffer cells. *Revis.Biol.Celular.* 1988; **16**: 69-94.

6. Wardle EN. Kupffer cells and their function. *Liver* 1987; **7**: 63-75.

7. Thurman RG, Marzi I, Seitz G, Thies J, Lemasters JJ, Zimmerman F. Hepatic reperfusion injury following orthotopic liver transplantation in the rat. *Transplantation* 1988; **46**: 502-506.

8. Caldwell-Kenkel JC, Thurman RG, Lemasters JJ. Selective loss of nonparenchymal cell viability after cold ischemic storage of rat livers. *Transplantation* 1988; **45**: 834-837.

9. Hearse DJ, Humphrey SM, Nayler WG, Slade A, Border D. Ultrastructural damage associated with reoxygenation of the anoxic myocardium. *J.Mol.Cell Cardiol.* 1975; **7**: 315-324.

10. Parks DA, Granger DN. Contributions of ischemia and reperfusion to mucosal lesion formation. *Am.J.Physiol* 1986; **250**: G749-G753.

11. Green CJ, Healing G, Lunec J, Fuller BJ, Simpkin S. Evidence of free-radical-induced damage in rabbit kidneys after simple hypothermic preservation and autotransplantation. *Transplantation* 1986; **41**: 161-165.

12. Jaeschke H. Reactive oxygen and ischemia/reperfusion injury of the liver. *Chem.Biol.Interact.* 1991; **79**: 115-136.

13. McCord JM. Oxygen-derived free radicals in postischemic tissue injury. *N.Engl.J.Med.* 1985; **312**: 159-163.

14. Granger DN. Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. *Am.J.Physiol* 1988; **255**: H1269-H1275.

15. Jaeschke H, Smith CV, Mitchell JR. Reactive oxygen species during ischemia-reflow injury in isolated perfused rat liver. *J.Clin.Invest* 1988; **81**: 1240-1246.

16. Jaeschke H, Benzick AE. Pathophysiological consequences of enhanced intracellular superoxide formation in isolated perfused rat liver. *Chem.Biol.Interact.* 1992; **84**: 55-68.

17. Mathews WR, Guido DM, Fisher MA, Jaeschke H. Lipid peroxidation as molecular mechanism of liver cell injury during reperfusion after ischemia. *Free Radic.Biol.Med.* 1994; **16**: 763-770.

18. Yokoyama Y, Beckman JS, Beckman TK *et al.* Circulating xanthine oxidase: potential mediator of ischemic injury. *Am.J.Physiol* 1990; **258**: G564-G570.

19. Shiratori Y, Kiriyama H, Fukushi Y *et al.* Modulation of ischemia-reperfusion-induced hepatic injury by Kupffer cells. *Dig.Dis.Sci.* 1994; **39**: 1265-1272.

20. Lindert KA, Caldwell-Kenkel JC, Nukina S, Lemasters JJ, Thurman RG. Activation of Kupffer cells on reperfusion following hypoxia: particle phagocytosis in a low-flow, reflow model. *Am.J.Physiol* 1992; **262**: G345-G350.

21. Rao PN, Liu T, Synder JT, Platt JL, Starzl TE. Reperfusion injury following cold ischemia activates rat liver Kupffer cells. *Transplant.Proc.* 1991; **23**: 666-669.

22. Jaeschke H, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia- reperfusion injury in rat liver. *Am.J.Physiol* 1991; **260**: G355-G362.

23. Mosher B, Dean R, Harkema J, Remick D, Palma J, Crockett E. Inhibition of Kupffer cells reduced CXC chemokine production and liver injury. *J.Surg.Res.* 2001; **99**: 201-210.

24. Suzuki S, Nakamura S, Sakaguchi T *et al.* Alteration of reticuloendothelial phagocytic function and tumor necrosis factor-alpha production after total hepatic ischemia. *Transplantation* 1997; **64**: 821-827.

25. Kukan M, Vajdova K, Horecky J, Nagyova A, Mehendale HM, Trnovec T. Effects of blockade of Kupffer cells by gadolinium chloride on hepatobiliary function in cold ischemia-reperfusion injury of rat liver. *Hepatology* 1997; **26**: 1250-1257.

26. Bremer C, Bradford BU, Hunt KJ *et al.* Role of Kupffer cells in the pathogenesis of hepatic reperfusion injury. *Am.J.Physiol* 1994; **267**: G630-G636.

27. Jaeschke H, Farhood A, Smith CW. Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. *FASEB J.* 1990; **4**: 3355-3359.

28. Shito M, Wakabayashi G, Ueda M *et al.* Interleukin 1 receptor blockade reduces tumor necrosis factor production, tissue injury, and mortality after hepatic ischemia- reperfusion in the rat. *Transplantation* 1997; **63**: 143-148.

29. Warren JS, Yabroff KR, Remick DG *et al.* Tumor necrosis factor participates in the pathogenesis of acute immune complex alveolitis in the rat. *J.Clin.Invest* 1989; **84**: 1873-1882.

30. Warren JS. Intrapulmonary interleukin 1 mediates acute immune complex alveolitis in the rat. *Biochem.Biophys.Res.Commun.* 1991; **175**: 604-610.

31. Lentsch AB, Ward PA. Regulation of inflammatory vascular damage. *J.Pathol.* 2000; **190**: 343-348.

32. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. *Annu.Rev.Immunol.* 1994; **12**: 141-179.

33. Jaeschke H, Farhood A, Bautista AP, Spolarics Z, Spitzer JJ. Complement activates Kupffer cells and neutrophils during reperfusion after hepatic ischemia. *Am.J.Physiol* 1993; **264**: G801-G809.

34. Tanhehco EJ, Yasojima K, McGeer PL, Washington RA, Lucchesi BR. Free radicals upregulate complement expression in rabbit isolated heart. *Am.J.Physiol Heart Circ.Physiol* 2000; **279**: H195-H201.

35. Mollnes TE, Fosse E. The complement system in trauma-related and ischemic tissue damage: a brief review. *Shock* 1994; **2**: 301-310.

36. Hammerschmidt DE, Harris PD, Wayland JH, Craddock PR, Jacob HS. Complement-induced granulocyte aggregation in vivo. *Am.J.Pathol.* 1981; **102**: 146-150.

37. Jagels MA, Daffern PJ, Hugli TE. C3a and C5a enhance granulocyte adhesion to endothelial and epithelial cell monolayers: epithelial and endothelial priming is required for C3a- induced eosinophil adhesion. *Immunopharmacology* 2000; **46**: 209-222.

38. Tonnesen MG, Smedly LA, Henson PM. Neutrophil-endothelial cell interactions. Modulation of neutrophil adhesiveness induced by complement fragments C5a and C5a des arg and formyl-methionyl-leucyl-phenylalanine in vitro. *J.Clin.Invest* 1984; **74**: 1581-1592.

39. Grattagliano I, Vendemiale G, Lauterburg BH. Reperfusion injury of the liver: role of mitochondria and protection by glutathione ester. *J.Surg.Res.* 1999; **86**: 2-8.

40. Jaeschke H, Farhood A, Fisher MA, Smith CW. Sequestration of neutrophils in the hepatic vasculature during endotoxemia is independent of beta 2 integrins and intercellular adhesion molecule-1. *Shock* 1996; **6**: 351-356.

41. Essani NA, Fisher MA, Farhood A, Manning AM, Smith CW, Jaeschke H. Cytokine-induced upregulation of hepatic intercellular adhesion molecule-1 messenger RNA expression and its role in the pathophysiology of murine endotoxin shock and acute liver failure. *Hepatology* 1995; **21**: 1632-1639.

42. Bautista AP, Spitzer JJ. Platelet activating factor stimulates and primes the liver, Kupffer cells and neutrophils to release superoxide anion. *Free Radic.Res.Commun.* 1992; **17**: 195-209.

43. Atherton A, Born GV. Relationship between the velocity of rolling granulocytes and that of the blood flow in venules. *J.Physiol* 1973; **233**: 157-165.

44. Ley K, Gaehtgens P. Endothelial, not hemodynamic, differences are responsible for preferential leukocyte rolling in rat mesenteric venules. *Circ.Res.* 1991; **69**: 1034-1041.

45. McEver RP, Moore KL, Cummings RD. Leukocyte trafficking mediated by selectin-carbohydrate interactions. *J.Biol.Chem.* 1995; **270**: 11025-11028.

46. Forbes TL, Harris KA, Jamieson WG, DeRose G, Carson M, Potter RF. Leukocyte activity and tissue injury following ischemia-reperfusion in skeletal muscle. *Microvasc.Res.* 1996; **51**: 275-287.

47. Jerome SN, Dore M, Paulson JC, Smith CW, Korthuis RJ. P-selectin and ICAM-1-dependent adherence reactions: role in the genesis of postischemic no-reflow. *Am.J.Physiol* 1994; **266**: H1316-H1321.

48. Kanwar S, Smith CW, Kubes P. An absolute requirement for P-selectin in ischemia/reperfusion-induced leukocyte recruitment in cremaster muscle. *Microcirculation.* 1998; **5**: 281-287.

49. Hernandez LA, Grisham MB, Twohig B, Arfors KE, Harlan JM, Granger DN. Role of neutrophils in ischemia-reperfusion-induced microvascular injury. *Am.J.Physiol* 1987; **253**: H699-H703.

50. Kurose I, Anderson DC, Miyasaka M *et al.* Molecular determinants of reperfusion-induced leukocyte adhesion and vascular protein leakage. *Circ.Res.* 1994; **74**: 336-343.

51. Ma XL, Tsao PS, Lefer AM. Antibody to CD-18 exerts endothelial and cardiac protective effects in myocardial ischemia and reperfusion. *J.Clin.Invest* 1991; **88**: 1237-1243.

52. Ma XL, Lefer DJ, Lefer AM, Rothlein R. Coronary endothelial and cardiac protective effects of a monoclonal antibody to intercellular adhesion molecule-1 in myocardial ischemia and reperfusion. *Circulation* 1992; **86**: 937-946.

53. Weyrich AS, Ma XY, Lefer DJ, Albertine KH, Lefer AM. In vivo neutralization of P-selectin protects feline heart and endothelium in myocardial ischemia and reperfusion injury. *J.Clin.Invest* 1993; **91**: 2620-2629.

54. Adkins WK, Taylor AE. Role of xanthine oxidase and neutrophils in ischemia-reperfusion injury in rabbit lung. *J.Appl.Physiol* 1990; **69**: 2012-2018.

55. Mori E, del Zoppo GJ, Chambers JD, Copeland BR, Arfors KE. Inhibition of polymorphonuclear leukocyte adherence suppresses no- reflow after focal cerebral ischemia in baboons. *Stroke* 1992; **23**: 712-718.

56. Carden DL, Young JA, Granger DN. Pulmonary microvascular injury after intestinal ischemia-reperfusion: role of P-selectin. *J.Appl.Physiol* 1993; **75**: 2529-2534.

57. Goldman G, Welbourn R, Rothlein R *et al.* Adherent neutrophils mediate permeability after atelectasis. *Ann.Surg.* 1992; **216**: 372-378.

58. Jerome SN, Smith CW, Korthuis RJ. CD18-dependent adherence reactions play an important role in the development of the no-reflow phenomenon. *Am.J.Physiol* 1993; **264**: H479-H483.

59. Granger DN, Kubes P. The microcirculation and inflammation: modulation of leukocyte- endothelial cell adhesion. *J.Leukoc.Biol.* 1994; **55**: 662-675.

60. Chosay JG, Essani NA, Dunn CJ, Jaeschke H. Neutrophil margination and extravasation in sinusoids and venules of liver during endotoxin-induced injury. *Am.J.Physiol* 1997; **272**: G1195-G1200.

61. Asako H, Kubes P, Baethge BA, Wolf RE, Granger DN. Colchicine and methotrexate reduce leukocyte adherence and emigration in rat mesenteric venules. *Inflammation* 1992; **16**: 45-56.

62. Asako H, Wolf RE, Granger DN, Korthuis RJ. Phalloidin prevents leukocyte emigration induced by proinflammatory stimuli in rat mesentery. *Am.J.Physiol* 1992; **263**: H1637-H1642.

63. Kubes P, Grisham MB, Barrowman JA, Gaginella T, Granger DN. Leukocyte-induced vascular protein leakage in cat mesentery. *Am.J.Physiol* 1991; **261**: H1872-H1879.

64. Caldwell-Kenkel JC, Currin RT, Tanaka Y, Thurman RG, Lemasters JJ. Reperfusion injury to endothelial cells following cold ischemic storage of rat livers. *Hepatology* 1989; **10**: 292-299.

65. McKeown CM, Edwards V, Phillips MJ, Harvey PR, Petrunka CN, Strasberg SM. Sinusoidal lining cell damage: the critical injury in cold preservation of liver allografts in the rat. *Transplantation* 1988; **46**: 178-191.

66. Nathan C, Srimal S, Farber C *et al.* Cytokine-induced respiratory burst of human neutrophils: dependence on extracellular matrix proteins and CD11/CD18 integrins. *J.Cell Biol.* 1989; **109**: 1341-1349.

67. Shappell SB, Toman C, Anderson DC, Taylor AA, Entman ML, Smith CW. Mac-1 (CD11b/CD18) mediates adherence-dependent hydrogen peroxide production by human and canine neutrophils. *J.Immunol.* 1990; **144**: 2702-2711.

68. Marubayashi S, Dohi K, Ochi K, Kawasaki T. Role of free radicals in ischemic rat liver cell injury: prevention of damage by alpha-tocopherol administration. *Surgery* 1986; **99**: 184-192.

69. Lentsch AB, Yoshidome H, Warner RL, Ward PA, Edwards MJ. Secretory leukocyte protease inhibitor in mice regulates local and remote organ inflammatory injury induced by hepatic ischemia/reperfusion. *Gastroenterology* 1999; **117**: 953-961.

70. Li XK, Matin AF, Suzuki H, Uno T, Yamaguchi T, Harada Y. Effect of protease inhibitor on ischemia/reperfusion injury of the rat liver. *Transplantation* 1993; **56**: 1331-1336.

71. Jaeschke H. Cellular adhesion molecules: regulation and functional significance in the pathogenesis of liver diseases. *Am.J.Physiol* 1997; **273**: G602-G611.

72. Crockett-Torabi E, Fantone JC. Signal Transduction and Adhesion Molecules. In: Ward PA, Fantone JC, eds. Adhesion Molecules and the Lung. Marcel Dekker, Inc., New York, 1996; 63-98.

73. Crockett-Torabi E. Selectins and mechanisms of signal transduction. *J.Leukoc.Biol.* 1998; **63**: 1-14.

74. McEver RP, Beckstead JH, Moore KL, Marshall-Carlson L, Bainton DF. GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J.Clin.Invest* 1989; **84**: 92-99.

75. Essani NA, McGuire GM, Manning AM, Jaeschke H. Differential induction of mRNA for ICAM-1 and selectins in hepatocytes, Kupffer cells and endothelial cells during endotoxemia. *Biochem.Biophys.Res.Commun.* 1995; **211**: 74-82.

76. Essani NA, Fisher MA, Simmons CA, Hoover JL, Farhood A, Jaeschke H. Increased P-selectin gene expression in the liver vasculature and its role in the pathophysiology of neutrophil-induced liver injury in murine endotoxin shock. *J.Leukoc.Biol.* 1998; **63**: 288-296.

77. Steinhoff G, Behrend M, Schrader B, Duijvestijn AM, Wonigeit K. Expression patterns of leukocyte adhesion ligand molecules on human liver endothelia. Lack of ELAM-1 and CD62 inducibility on sinusoidal endothelia and distinct distribution of VCAM-1, ICAM-1, ICAM-2, and LFA- 3. *Am.J.Pathol.* 1993; **142**: 481-488.

78. Irving MG, Roll FJ, Huang S, Bissell DM. Characterization and culture of sinusoidal endothelium from normal rat liver: lipoprotein uptake and collagen phenotype. *Gastroenterology* 1984; **87**: 1233-1247.

79. Corral L, Singer MS, Macher BA, Rosen SD. Requirement for sialic acid on neutrophils in a GMP-140 (PADGEM) mediated adhesive interaction with activated platelets. *Biochem.Biophys.Res.Commun.* 1990; **172**: 1349-1356.

80. Walcheck B, Moore KL, McEver RP, Kishimoto TK. Neutrophil-neutrophil interactions under hydrodynamic shear stress involve L-selectin and PSGL-1. A mechanism that amplifies initial leukocyte accumulation of P-selectin in vitro. *J.Clin.Invest* 1996; **98**: 1081-1087.

81. Dore M, Korthuis RJ, Granger DN, Entman ML, Smith CW. P-selectin mediates spontaneous leukocyte rolling in vivo. *Blood* 1993; **82**: 1308-1316.

82. Lawrence MB, Springer TA. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* 1991; **65**: 859-873.

83. Ley K, Gaehtgens P, Fennie C, Singer MS, Lasky LA, Rosen SD. Lectin-like cell adhesion molecule 1 mediates leukocyte rolling in mesenteric venules in vivo. *Blood* 1991; **77**: 2553-2555.

84. Lindbom L, Xie X, Raud J, Hedqvist P. Chemoattractant-induced firm adhesion of leukocytes to vascular endothelium in vivo is critically dependent on initial leukocyte rolling. *Acta Physiol Scand.* 1992; **146**: 415-421.

85. Gibbs SA, Weiser MR, Kobzik L, Valeri CR, Shepro D, Hechtman HB. P-selectin mediates intestinal ischemic injury by enhancing complement deposition. *Surgery* 1996; **119**: 652-656.

86. Witthaut R, Farhood A, Smith CW, Jaeschke H. Complement and tumor necrosis factor-alpha contribute to Mac-1 (CD11b/CD18) up-regulation and systemic neutrophil activation during endotoxemia in vivo. *J.Leukoc.Biol.* 1994; **55**: 105-111.

87. Jaeschke H, Farhood A, Bautista AP, Spolarics Z, Spitzer JJ, Smith CW. Functional inactivation of neutrophils with a Mac-1 (CD11b/CD18) monoclonal antibody protects against ischemia-reperfusion injury in rat liver. *Hepatology* 1993; **17**: 915-923.

88. Nolte D, Hecht R, Schmid P *et al.* Role of Mac-1 and ICAM-1 in ischemia-reperfusion injury in a microcirculation model of BALB/C mice. *Am.J.Physiol* 1994; **267**: H1320-H1328.

89. Perry MA, Granger DN. Role of CD11/CD18 in shear rate-dependent leukocyte-endothelial cell interactions in cat mesenteric venules. *J.Clin.Invest* 1991; **87**: 1798-1804.

90. Farhood A, McGuire GM, Manning AM, Miyasaka M, Smith CW, Jaeschke H. Intercellular adhesion molecule 1 (ICAM-1) expression and its role in neutrophil-induced ischemia-reperfusion injury in rat liver. *J.Leukoc.Biol.* 1995; **57**: 368-374.

91. Vollmar B, Glasz J, Menger MD, Messmer K. Leukocytes contribute to hepatic ischemia/reperfusion injury via intercellular adhesion molecule-1-mediated venular adherence. *Surgery* 1995; **117**: 195-200.

92. Liu DL, Jeppsson B, Hakansson CH, Odselius R. Multiple-system organ damage resulting from prolonged hepatic inflow interruption. *Arch.Surg.* 1996; **131**: 442-447.

93. Liu P, Yin K, Yue G, Wong PY. Role of nitric oxide in hepatic ischemia-reperfusion with endotoxemia. *J.Inflamm.* 1995; **46**: 144-154.

94. Weinbroum A, Nielsen VG, Tan S *et al.* Liver ischemia-reperfusion increases pulmonary permeability in rat: role of circulating xanthine oxidase. *Am.J.Physiol* 1995; **268**: G988-G996.

95. Eppihimer MJ, Wolitzky B, Anderson DC, Labow MA, Granger DN. Heterogeneity of expression of E- and P-selectins in vivo. *Circ.Res.* 1996; **79**: 560-569.

96. Eppihimer MJ, Russell J, Anderson DC, Wolitzky BA, Granger DN. Endothelial cell adhesion molecule expression in gene-targeted mice. *Am.J.Physiol* 1997; **273**: H1903-H1908.

97. Meyer K, Brown MF, Zibari G *et al.* ICAM-1 upregulation in distant tissues after hepatic ischemia/reperfusion: a clue to the mechanism of multiple organ failure. *J.Pediatr.Surg.* 1998; **33**: 350-353.

98. Foulds S, Mireskandari M, Kalu P *et al.* Visceral ischemia and neutrophil activation in sepsis and organ dysfunction. *J.Surg.Res.* 1998; **75**: 170-176.

99. Upperman JS, Deitch EA, Guo W, Lu Q, Xu D. Post-hemorrhagic shock mesenteric lymph is cytotoxic to endothelial cells and activates neutrophils. *Shock* 1998; **10**: 407-414.

100. Adams CA, Jr., Magnotti LJ, Xu DZ, Lu Q, Deitch EA. Acute lung injury after hemorrhagic shock is dependent on gut injury and sex. *Am.Surg.* 2000; **66**: 905-912.

101. Deitch EA, Xu D, Franko L, Ayala A, Chaudry IH. Evidence favoring the role of the gut as a cytokine-generating organ in rats subjected to hemorrhagic shock. *Shock* 1994; **1**: 141-145.

102. Magnotti LJ, Upperman JS, Xu DZ, Lu Q, Deitch EA. Gut-derived mesenteric lymph but not portal blood increases endothelial cell permeability and promotes lung injury after hemorrhagic shock. *Ann.Surg.* 1998; **228**: 518-527.

103. Gallay P, Jongeneel CV, Barras C *et al.* Short time exposure to lipopolysaccharide is sufficient to activate human monocytes. *J.Immunol.* 1993; **150**: 5086-5093.

104. Crouser ED, Dorinsky PM. Gastrointestinal tract dysfunction in critical illness: pathophysiology and interaction with acute lung injury in adult respiratory distress syndrome/multiple organ dysfunction syndrome. *New Horiz.* 1994; **2**: 476-487.

105. Garcia-Criado FJ, Toledo-Pereyra LH, Lopez-Neblina F, Phillips ML, Paez-Rollys A, Misawa K. Role of P-selectin in total hepatic ischemia and reperfusion. *J.Am.Coll.Surg.* 1995; **181**: 327-334.

106. Singh I, Zibari GB, Zizzi H *et al.* Anti-P-selectin antibody protects against hepatic ichemia-reperfusion injury. *Transplant.Proc.* 1998; **30**: 2324-2326.

107. Sawaya DE, Jr., Zibari GB, Minardi A *et al.* P-selectin contributes to the initial recruitment of rolling and adherent leukocytes in hepatic venules after ischemia/reperfusion. *Shock* 1999; **12**: 227-232.

108. Dulkanchainun TS, Goss JA, Imagawa DK *et al.* Reduction of hepatic ischemia/reperfusion injury by a soluble P- selectin glycoprotein ligand-1. *Ann.Surg.* 1998; **227**: 832-840.

109. Martinez-Mier G, Toledo-Pereyra LH, McDuffie JE, Warner RL, Ward PA. P-selectin and chemokine response after liver ischemia and reperfusion. *J.Am.Coll.Surg.* 2000; **191**: 395-402.

110. Yadav SS, Howell DN, Steeber DA, Harland RC, Tedder TF, Clavien PA. P-Selectin mediates reperfusion injury through neutrophil and platelet sequestration in the warm ischemic mouse liver. *Hepatology* 1999; **29**: 1494-1502.

111. Kuzume M, Nakano H, Yamaguchi M *et al.* A monoclonal antibody against ICAM-1 suppresses hepatic ischemia- reperfusion injury in rats. *Eur.Surg.Res.* 1997; **29**: 93-100.

112. Marubayashi S, Oshiro Y, Maeda T *et al.* Protective effect of monoclonal antibodies to adhesion molecules on rat liver ischemia-reperfusion injury. *Surgery* 1997; **122**: 45-52.

113. Nakano H, Kuzume M, Namatame K, Yamaguchi M, Kumada K. Efficacy of intraportal injection of anti-ICAM-1 monoclonal antibody against liver cell injury following warm ischemia in the rat. *Am.J.Surg.* 1995; **170**: 64-66.

114. Vollmar B, Glasz J, Post S, Menger MD. Role of microcirculatory derangements in manifestation of portal triad cross-clamping-induced hepatic reperfusion injury. *J.Surg.Res.* 1996; **60**: 49-54.

115. Rentsch M, Post S, Palma P, Lang G, Menger MD, Messmer K. Anti-ICAM-1 blockade reduces postsinusoidal WBC adherence following cold ischemia and reperfusion, but does not improve early graft function in rat liver transplantation. *J.Hepatol.* 2000; **32**: 821-828.

116. Yadav SS, Howell DN, Gao W, Steeber DA, Harland RC, Clavien PA. L-selectin and ICAM-1 mediate reperfusion injury and neutrophil adhesion in the warm ischemic mouse liver. *Am.J.Physiol* 1998; **275**: G1341-G1352.

117. Kamochi M, Kamochi F, Kim YB *et al.* P-selectin and ICAM-1 mediate endotoxin-induced neutrophil recruitment and injury to the lung and liver. *Am.J.Physiol* 1999; **277**: L310-L319.

118. Lefer DJ, Flynn DM, Anderson DC, Buda AJ. Combined inhibition of P-selectin and ICAM-1 reduces myocardial injury following ischemia and reperfusion. *Am.J.Physiol* 1996; **271**: H2421-H2429.

119. Briaud SA, Ding ZM, Michael LH, Entman ML, Daniel S, Ballantyne CM. Leukocyte trafficking and myocardial reperfusion injury in ICAM-1/P- selectin-knockout mice. *Am.J.Physiol Heart Circ.Physiol* 2001; **280**: H60-H67.

120. Bullard DC, Qin L, Lorenzo I *et al.* P-selectin/ICAM-1 double mutant mice: acute emigration of neutrophils into the peritoneum is completely absent but is normal into pulmonary alveoli [see comments]. *J.Clin.Invest* 1995; **95**: 1782-1788.

121. Kyriakides C, Woodcock SA, Wang Y *et al.* Soluble P-selectin moderates complement-dependent reperfusion injury of ischemic skeletal muscle. *Am.J.Physiol Cell Physiol* 2000; **279**: C520-C528.

122. Young CS, Palma JM, Mosher BD *et al.* Hepatic ischemia/reperfusion injury in P-selectin and intercellular adhesion molecule-1 double-mutant mice. *Am.Surg.* 2001; **67**: 737-744.

123. Colletti LM, Burtch GD, Remick DG *et al.* The production of tumor necrosis factor alpha and the development of a pulmonary capillary injury following hepatic ischemia/reperfusion. *Transplantation* 1990; **49**: 268-272.

124. Kawano K, Kim YI, Kaketani K, Kobayashi M. The beneficial effect of cyclosporine on liver ischemia in rats. *Transplantation* 1989; **48**: 759-764.

125. Lichtman SN, Lemasters JJ. Role of cytokines and cytokine-producing cells in reperfusion injury to the liver. *Semin.Liver Dis.* 1999; **19**: 171-187.

126. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am.J.Clin.Pathol* 1957; **28**: 56-63.

127. Krawisz JE, Sharon P, Stenson WF. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. *Gastroenterology* 1984; **87**: 1344-1350.

128. Schierwagen C, Bylund-Fellenius AC, Lundberg C. Improved method for quantification of tissue PMN accumulation measured by myeloperoxidase activity. *J.Pharmacol.Methods* 1990; **23**: 179-186.

129. Suzuki S, Toledo-Pereyra LH, Rodriguez FJ. Role of neutrophils during the first 24 hours after liver ischemia and reperfusion injury. *Transplant.Proc.* 1994; **26**: 3695-3700.

130. McCuskey RS, Urbaschek R, Urbaschek B. The microcirculation during endotoxemia. *Cardiovasc.Res.* 1996; **32**: 752-763.

131. Nishida J, McCuskey RS, McDonnell D, Fox ES. Protective role of NO in hepatic microcirculatory dysfunction during endotoxemia. *Am.J.Physiol* 1994; **267**: G1135-G1141.

132. Zhang JX, Pegoli W, Jr., Clemens MG. Endothelin-1 induces direct constriction of hepatic sinusoids. *Am.J.Physiol* 1994; **266**: G624-G632.

133. Worthen GS, Schwab B, III, Elson EL, Downey GP. Mechanics of stimulated neutrophils: cell stiffening induces retention in capillaries. *Science* 1989; **245**: 183-186.

134. Essani NA, Bajt ML, Farhood A, Vonderfecht SL, Jaeschke H. Transcriptional activation of vascular cell adhesion molecule-1 gene in vivo and its role in the pathophysiology of neutrophil-induced liver injury in murine endotoxin shock. *J.Immunol.* 1997; **158**: 5941-5948.

135. Hill J, Lindsay T, Rusche J, Valeri CR, Shepro D, Hechtman HB. A Mac-1 antibody reduces liver and lung injury but not neutrophil sequestration after intestinal ischemia-reperfusion. *Surgery* 1992; **112**: 166-172.

136. Wisse E. An ultrastructural characterization of the endothelial cell in the rat liver sinusoid under normal and various experimental conditions, as a contribution to

the distinction between endothelial and Kupffer cells. *J.Ultrastruct.Res.* 1972; **38**: 528-562.

137. Mochida S, Ohno A, Arai M, Tamatani T, Miyasaka M, Fujiwara K. Role of adhesion molecules in the development of massive hepatic necrosis in rats. *Hepatology* 1996; **23**: 320-328.

138. Nagendra AR, Mickelson JK, Smith CW. CD18 integrin and CD54-dependent neutrophil adhesion to cytokine- stimulated human hepatocytes. *Am.J.Physiol* 1997; **272**: G408-G416.

139. Zimmerman BJ, Holt JW, Paulson JC *et al.* Molecular determinants of lipid mediator-induced leukocyte adherence and emigration in rat mesenteric venules. *Am.J.Physiol* 1994; **266**: H847-H853.

140. Marks RM, Todd RF, III, Ward PA. Rapid induction of neutrophil-endothelial adhesion by endothelial complement fixation. *Nature* 1989; **339**: 314-317.

141. Sligh JE, Jr., Ballantyne CM, Rich SS *et al.* Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. *Proc.Natl.Acad.Sci.U.S.A* 1993; **90**: 8529-8533.

142. Sarman G, Shappell SB, Mason EO, Jr., Smith CW, Kaplan SL. Susceptibility to local and systemic bacterial infections in intercellular adhesion molecule 1-deficient transgenic mice. *J.Infect.Dis.* 1995; **172**: 1001-1006.

143. Mayadas TN, Johnson RC, Rayburn H, Hynes RO, Wagner DD. Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. *Cell* 1993; **74**: 541-554.