Essential Role of P-Selectin in the Initiation of the Inflammatory Response Induced by Hemorrhage and Reinfusion

By Rosario Scalia, Valerie E. Armstead, Alexander G. Minchenko, and Allan M. Lefer

From the Department of Physiology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Summary

Resuscitation from hemorrhage induces profound pathophysiologic alterations and activates inflammatory cascades able to initiate neutrophil accumulation in a variety of tissues. This process is accompanied by acute organ damage (e.g., lungs and liver). We have previously demonstrated that significant leukocyte–endothelium interactions occur very early in other forms of ischemia/reperfusion (i.e., splanchnic ischemia/reperfusion and traumatic shock) which are largely mediated by increased expression of the adhesion molecule, P-selectin, on the vascular endothelium. Here we postulated that increased endothelial expression of P-selectin in the microvasculature would play an essential role in initiating the inflammatory signaling of hemorrhagic shock. Using intravital microscopy, we found that hemorrhagic shock significantly increased the number of rolling and adherent leukocytes in the mouse splanchnic microcirculation. In contrast, mice genetically deficient in P-selectin, or wild-type mice given either an anti–P-selectin monoclonal antibody or a recombinant soluble P-selectin glycoprotein ligand (PSGL)-1 immunoglobulin, exhibited markedly attenuated leukocyte–endothelium interaction after hemorrhagic shock. Thus, activation of P-selectin protein on the microvascular endothelium is essential for the initial upregulation of the inflammatory response occurring in hemorrhagic shock. Moreover, endogenous levels of PSGL-1 mRNA were significantly increased in the lung, liver, and small intestine of wild-type mice subjected to hemorrhagic shock. Since PSGL-1 promotes adhesive interactions largely through P-selectin expressed on the vascular endothelium, this result further supports the crucial role played by P-selectin in the recruitment of leukocytes during hemorrhagic shock.

Key words: mRNA • leukocyte • endothelium • immunohistochemistry • intravital microscopy

Hemorrhagic shock initiates an inflammatory response characterized by the upregulation of cytokine expression (1) and emigration of neutrophils into a variety of tissues (2). The accumulation of neutrophils in splanchnic and thoracic organs is likely to contribute to end-organ damage and resultant dysfunction after this form of shock. The mechanism by which hemorrhage triggers this inflammatory response remains poorly understood. Heightened adrenergic activity (3), increased production of reactive free radicals (4), and systemic release of proinflammatory agents from the gut (5, 6) have been hypothesized to contribute to acute organ injury after hemorrhage. Nevertheless, none of these studies has clarified the specific pathway that promotes leukocyte recruitment into visceral organs during hemorrhagic shock.

Before transmigration into inflamed tissue, leukocytes must initially interact with the vascular endothelium (7), as demonstrated during ischemia/reperfusion (8) and in several experimental models of circulatory shock (9). Moreover, we have reported that under shock conditions very early endothelial dysfunction (i.e., 2.5–5 min) is associated with increased endothelial dysfunction (10, 11) and expression of the cell adhesion molecule, P-selectin (12). P-selectin is able to initiate the cascade of events that increases cell adherence and leukocyte infiltration into injured tissues by first promoting leukocyte rolling along the vascular endothelium (13, 14). Therefore, we hypothesized that increased surface expression of P-selectin in the microvascular endothelium would exert an essential role in recruitment of leukocytes in the case of hemorrhagic shock. Hemorrhage was therefore carried out in control wild-type mice, in wild-type mice treated with anti-P-select-
Materials and Methods

Hemorrhagic Shock Protocol. This study was performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University. All animal protocols have been approved by the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University.

Wild-type (C57BL/6) mice and P-selectin–deficient (C57BL/6-Sel) mice were obtained from The Jackson Laboratory. Male and female mice (8–14 wk old and 20–30 g body wt) were anesthetized with sodium pentobarbital (120 mg/kg) injected intraperitoneally. A tracheotomy was performed to maintain a patent airway throughout the experiment. The left carotid artery was cannulated for continuous blood pressure monitoring, and the right jugular vein was cannulated for blood withdrawal and fluid or antibody administration. Mice were subjected to hemorrhage by withdrawal of blood to allow mean arterial blood pressure (MABP) to be maintained at 40 mmHg for 45 min. The mean bleedout volume was 0.72 ± 0.12 ml and 0.81 ± 0.09 ml for the wild-type and P-selectin–deficient mouse, respectively. Blood was collected in a heparinized (5 U) syringe and kept at 37°C until reinfusion. Mice were then resuscitated by infusion of the shed blood and intravenous injection of 0.5 ml 0.9% NaCl alone or with either an anti–P-selectin mAb or rs.PSGL.Ig. Mice were killed by exsanguination 45 min after reinfusion. Mice were then cut into blocks and dehydrated in graded acetone washes at 4°C. Tissue blocks were embedded in paraffin (Immunobrid; Polysciences, Inc.), and 4-μm-thick sections were cut and transferred to Vectabond-coated slides (Vector Laboratories, Inc.). The slides were soaked in ethanol for 10 min to remove the plastic embedding material and to allow staining of the tissue. After the 10-min ethanol wash, the tissue sections were stained with hematoxylin and eosin and examined blindly. The number of PMNs was counted and tallied. Five fields from each of two slides were counted from each organ, and three mice were studied per group. In addition, grading of the histopathologic changes in the lung (i.e., neutrophil infiltration, interstitial edema, and intra-alveolar hemorrhage) was performed on a 0 to 3 scale (with 0 being normal and 3 being the most severe abnormality).

 Howell and Weir (20) demonstrated that P-selectin deficiency or blockade prevents the increase in leukocyte-endothelium interaction observed in the mouse microcirculation after reinfusion of hemoglobin. The data strongly indicate that increased P-selectin surface expression on the surface of the microvascular endothelium plays an essential role in the initiation of the inflammatory response observed in hemorrhage and reinfusion, and that this mechanism can be abrogated in a variety of ways.

Abbreviations used in this paper: MABP, mean arterial blood pressure; PMNs, polymorphonuclear leukocytes; PSGL-1, P-selectin glycoprotein ligand 1.
The concentration of RNA suspended in ribonuclease-free water was determined spectrophotometrically.

The plasmid containing the full-length mouse P-selectin cDNA was provided by Professor Dietmar Vestweber (Institut für Zellbiologie, ZMBE, Westfalisches Wilhelms Univesitat M unster, M unster, Germany). The plasmid for synthesis of mouse tissue P-selectin probe for ribonuclease protection assays was created by recloning of a PstI–AvrII fragment of mouse P-selectin (from 1878 to 2270; sequence data available from EMBL/GenBank/ DDBJ under accession number M87861) into a PstI–Spel site of pBluescript II SK + vector (Stratagene). This plasmid was digested with XhoI and used as a template for in vitro transcription of a 474-base radiolabeled antisense fragment containing a 393-base protected fragment using T3 RNA-polymerase (Boehringer Mannheim) in the presence of [32P]UTP (Amersham Corp.).

Mouse PSGL-1 cDNA was synthesized by reverse transcriptase PCR using mouse lung total RNA and oligo(dT), and was amplified using forward primer (5'-CCTGGGAATTCACCTGGCC-3') and reverse primer (5'-GAGAGTGGAGCTAGCAAAGG-3'). These oligonucleotides correspond to amino acid sequences 267-283 and 394-388 of mouse PSGL-1, respectively (sequence data available from EMBL/GenBank/DDBJ under accession number X91144). A 384-bp PCR fragment was cloned using PCR 2.1-TOPO Cloning Kit (a gift from Invitrogen Corp.). The Sst I–XbaI fragment of this plasmid was recloned in pBluescript II SK + vector (Stratagene). This plasmid was digested with XbaI to make, with T7 polymerase, a 570-base radiolabeled antisense probe that contained a 364-base-protected fragment. All constructs used in this investigation were verified by sequencing the insert in the plasmid. These sequences were found to be 100% identical to the published sequences. The expression of PSGL-1 mRNA was analyzed by reverse transcriptase PCR analysis. Amplified PCR products were analyzed by electrophoresis on a 1.5% agarose gel. The intensity of each P-selectin and PSGL-1 mRNA band was normalized for GAPDH (data not shown).

Mouse β-actin antisense RNA probes were used to evaluate total RNA, which in turn were used for PSGL-1 mRNA expression analysis. The mouse β-actin plasmid for the synthesis of the antisense RNA probe was received from Ambion. For the ribonuclease protection assay, 2 μg of total RNA for detection of β-actin mRNA, and 60 μg for detection of PSGL-1 mRNA in the lung, liver, and small intestine have been used accordingly to previously described procedures (20). The intensity of each P-selectin and PSGL-1 mRNA band was normalized for β-actin mRNA levels.

Statistical Analysis. All values for data listed in the text and figures are presented as means ± SEM of n independent experiments. Data were compared by analysis of variance using post hoc analysis with Fisher's correct t test. P ≤ 0.05 was considered significant in all cases.

Results

Hemodynamic Changes Induced by Hemorrhagic Shock. Fig. 1 illustrates the time course of systemic MABP in the five experimental groups of mice. All groups of mice exhibited initial MABP values in the range of 110–120 mmHg (Fig. 1). In control wild-type mice, MABP did not significantly change over the entire 90-min observation period (Fig. 1). In hemorrhaged mice, MABP was maintained at 40 mmHg for 45 min. After reinfusion of the shed blood to hemorrhaged mice, MABP increased to values not significantly different from control wild-type mice at that time (Fig. 1). In the wild-type hemorrhaged group receiving only saline, MABP progressively decreased to 90 ± 5 mmHg at the end of the experiment. In contrast, hemorrhaged P-selectin−/− mice as well as hemorrhaged wild-type mice receiving the anti–P-selectin mAb or the rsPSGL.Ig maintained significantly higher MABP at the end of the 45-min observation period in the range of 115–125 mmHg (P < 0.05, Fig. 1).

This higher MABP was not due to decreased bleedout volumes, since the volume of shed blood was not significantly different among all groups of mice. These final blood pressures were also not statistically different from the initial MABP in these same groups of mice. Thus, either gene deficiency or functional inactivation of P-selectin expressed on the vascular endothelium limits the systemic hemodynamic consequences of hemorrhagic shock.

Venular shear rates for the five experimental groups of mice are reported in Table I. No significant differences were observed in initial shear rates among the five groups of mice. After hemorrhage, shear rates in peri-intestinal venules abruptly decreased to less than half of the observed initial control values. Therefore, the present hemorrhagic shock model is characterized by a marked hypoperfusion of the splanchic microvasculature during the oligemic phase. However, upon reinfusion of shed blood, venular shear rates returned to normal values (Table I). This strongly suggests that blood flow was reestablished to control levels during the postsiliclastic phase. Since shear rates were normal after reinfusion, the adhesive interactions observed between leukocytes and the microvascular endothelium dur-

Figure 1. Time course of MABP over the course of hemorrhage and reinfusion for the five experimental groups of mice. Wild-type and P-selectin−/− mice were subjected to hemorrhagic shock. Functional blockade of P-selectin in wild-type mice was achieved by systemic administration of either anti–P-selectin mAb (1 mg/kg) or rsPSGL.Ig (1 mg/kg). Each point represents mean values ± SEM; numbers indicate surviving rats at each interval. *P < 0.05 and **P < 0.01 versus wild-type control mice. □, control wild-type (n = 6); ■, hemorrhage wild-type (n = 7); ○, hemorrhage P-selectin−/− (n = 6); ◇, hemorrhage wild-type + anti–P-selectin mAb (n = 5); ●, hemorrhage wild-type + rsPSGL.Ig (n = 5).
ing resuscitation from hemorrhage could not be attributed to alterations in physical hydrodynamic forces brought about by perturbations in local hemodynamics.

P-selectin is required for the upregulation of leukocyte–endothelium interaction in hemorrhagic shock. A low baseline number of rolling (i.e., 10–20 cells/min; Fig. 2) and adherent (i.e., 2–3 cells/100 \( \mu \text{m} \); Fig. 3) leukocytes was observed in the mesenteric microvasculature for all experimental groups of wild-type mice. Furthermore, neither rolling (Fig. 2) nor adherence (Fig. 3) of leukocytes increased in peri-intestinal venules of P-selectin–deficient mice at any time. Baseline leukocyte rolling (Fig. 2) and adherence (Fig. 3) were not significantly changed during the first 45 min of the hemorrhage period in all wild-type and P-selectin–deficient mice. However, the number of rolling and adherent leukocytes in untreated hemorrhaged wild-type mice exhibited a threefold (\( P < 0.01 \)) increase after reinfusion compared with normal control values (Figs. 2 and 3). In contrast, no significant increase in the number of rolling or adhered leukocytes was observed in the peri-intestinal venules of P-selectin–deficient mice (Figs. 2 and 3). Similarly, intravenous infusion of either 1 mg/kg of an anti-P-selectin mAb or 1 mg/kg of rs.PSGL.Ig significantly attenuated both leukocyte rolling (Fig. 3) and leukocyte adherence (Fig. 3) induced by hemorrhage and reinfusion of shed blood. In addition, no significant change in the total number of circulating leukocytes was observed in the five experimental groups of mice, so that the changes in rolling and adherence could not be attributed to leukopenia. The average number of circulating leukocytes in wild-type mice and P-selectin–deficient mice was 5.9 ± 0.6 and 7.2 ± 0.4 \( 10^3 \) cells/mm\(^3\) (mean ± SEM), respectively. These values are not significantly different from each other, nor was leukopenia observed at the end of the experimen-

### Table I. Diameters and Shear Rates for Mouse Peri-intestinal Venules

| Group                                | n  | Venular diameter (\( \mu \text{m} \)) | Venular shear rate (s\(^{-1} \)) | Baseline (0 min) | Hemorrhage (45 min) | Resuscitation (90 min) |
|--------------------------------------|----|-------------------------------------|---------------------------------|------------------|---------------------|-----------------------|
| Control wild-type                    | 6  | 35 ± 3.3                            |                                | 620 ± 19         | 612 ± 33            | 563 ± 42              |
| Hemorrhage P-selectin gene deleted   | 7  | 33 ± 2.7                            |                                | 653 ± 20         | 320 ± 41            | 625 ± 40              |
| Hemorrhage wild-type                 | 6  | 31 ± 6.3                            |                                | 616 ± 39         | 303 ± 28            | 618 ± 46              |
| Hemorrhage wild-type + anti-P-selectin mAb | 5  | 36 ± 6.5                            |                                | 614 ± 41         | 310 ± 51            | 609 ± 37              |
| Hemorrhage wild-type + rs.PSGL.Ig    | 5  | 32 ± 8.1                            |                                | 626 ± 54         | 287 ± 47            | 617 ± 24              |

All values are means ± SEM. n = numbers of mice studied.

![Figure 2](image.png) **Figure 2.** Leukocyte rolling observed in peri-intestinal venules of wild-type mice, P-selectin–deficient (P-selectin\(^{-/-}\)) mice, and wild-type mice given either anti-P-selectin mAb or rs.PSGL.Ig, and subjected to hemorrhagic shock. Bar heights represent means and brackets indicate ± SEM. *P < 0.05 and **P < 0.01 from control wild-type mice. White bars, control wild-type (n = 6); black bars, hemorrhage wild-type (n = 7); gray bars, hemorrhage P-selectin\(^{-/-}\) (n = 6); cross-hatched bars, hemorrhage wild-type + anti-P-selectin mAb (n = 5); hatched bars, hemorrhage wild-type + rs.PSGL.Ig (n = 5).

![Figure 3](image.png) **Figure 3.** Leukocyte adherence observed in peri-intestinal venules of wild-type mice, P-selectin–deficient (P-selectin\(^{-/-}\)) mice, and wild-type mice given either anti-P-selectin mAb or rs.PSGL.Ig, and subjected to hemorrhagic shock. Bar heights represent means and brackets indicate ± SEM. *P < 0.05 and **P < 0.01 from control wild-type mice. White bars, control wild-type (n = 6); black bars, hemorrhage wild-type (n = 7); gray bars, hemorrhage P-selectin\(^{-/-}\) (n = 6); cross-hatched bars, hemorrhage wild-type + anti-P-selectin mAb (n = 5); hatched bars, hemorrhage wild-type + rs.PSGL.Ig (n = 5).
tal protocol or after systemic administration of either anti-P-selectin mAb or rsPSGL.1g. Therefore, functional expression of P-selectin protein on the mouse splanchnic microvascular endothelium exerts a crucial role in triggering inflammatory events after hemorrhage and fluid resuscitation.

Determination of P-selectin Surface Expression In Situ by Immunohistochemical Localization. Immunolocalization of P-selectin was studied in the venular endothelium of the mouse ileum immediately after completion of intravital microscopic measurements. The percentage of venules staining positively for P-selectin in ileal sections from control wild-type mice was consistently low (21 ± 4% positive venules; Fig. 4). Moreover, virtually no surface P-selectin expression was detected by immunohistochemistry at any time in P-selectin gene-deficient mice (Fig. 4). However, hemorrhage plus reperfusion resulted in a significant increase in P-selectin expression in wild-type mice (Fig. 4). Intravenous infusion of either anti-P-selectin mAb or rsPSGL.1g did not attenuate the number of venules staining positively for P-selectin after hemorrhage and reinfusion (74 ± 4% and 70 ± 6 positive venules respectively; NS versus hemorrhage wild-type mice). This clearly indicates that inhibition of leukocyte-endothelium interaction induced by anti-P-selectin mAb or rsPSGL.1g is due to functional neutralization of P-selectin on the endothelial cell surface rather than to significant attenuation of P-selectin expression on the microvascular endothelium.

Inhibition of Neutrophil Infiltration into Lung, Liver, and Intestine Is Associated with Decreased Organ Injury in P-selectin–deficient Mice. As an additional verification of organ injury, we performed histological analysis of lung, liver, and intestine in control and hemorrhaged mice. After hemorrhage and reinfusion, P-selectin-deficient mice had fewer infiltrated neutrophils in lung, liver, and intestine (Fig. 5) than did wild-type mice. Moreover, P-selectin-deficient mice subjected to hemorrhage and reinfusion developed less interstitial lung edema and intra-alveolar hemorrhage compared with hemorrhaged wild-type mice, as assessed by histological analysis (histopathological score 0.45 ± 0.08 and 2.7 ± 0.15, respectively; P < 0.01). This decreased inflammation and injury in lungs and splanchnic organs of P-selectin–deficient mice strongly demonstrates the crucial role exerted by selectin-mediated leukocyte recruitment during the early pathophysiologic events of hemorrhagic shock.

Hemorrhagic Shock Increases PSGL-1 mRNA Expression in Several Organs in the Wild-type Mouse. Levels of mRNA codifying for endogenous PSGL-1 were assessed in hemorrhaged wild-type mice, using a ribonuclease protection assay. As shown in Fig. 6, the intensity of each PSGL-1 mRNA band was normalized to that of β-actin. After the 45-min resuscitation period, PSGL-1 transcripts were significantly increased in the lungs of mice subjected to hemorrhagic shock (Fig. 6). Similar results were also observed in the liver and small intestine of hemorrhaged wild-type mice. After hemorrhage and reinfusion PSGL-1 transcripts in both liver and intestine increased 34 ± 8.4% and 32 ± 2%, respectively (P < 0.001 versus control mouse tissue). In contrast, no significant changes were observed for the
intestine of hemorrhaged wild-type mice. These data pro-
nounce PSGL-1 occurs in the lung as well as in the liver and
an increased expression of mRNA codifying for endoge-
We also found that upon resuscitation from hemorrhage,
cruitment of leukocytes observed in hemorrhagic shock.
In this regard, several investigators have demonstrated
that inhibition of the rolling phase of leukocytes plays a key
role in attenuating the acute inflammatory response (28,
29). Consistent with such findings, we now demonstrate
that soon after resuscitation from hemorrhage, leukocyte-
endothelium interactions are significantly upregulated in
the microcirculation, an event that is associated with in-
creased expression of P-selectin on the microvascular en-
dothelium. Moreover, as confirmed in P-selectin-deficient
mice, in the absence of P-selectin protein virtually no leu-
kocyte–endothelium interaction occurs after hemorrhage and
reinfusion of shed blood. This finding agrees with pre-
vious observations showing severe attenuation of leukocyte
rolling and extravasation in P-selectin-deficient mice (14).
In addition, we observed de novo synthesis of PSGL-1 in
the lungs and splanchnic organs of hemorrhaged wild-type
mice, as confirmed by quantification of PSGL-1 mRNA N. This increase in PSGL-1 mRNA levels may contribute to
widespread increases in cell-to-cell interaction during acute
inflammatory conditions such as hemorrhagic shock.

The inhibitory effect on leukocyte–endothelium interac-
tion exerted by blockade of P-selectin may contribute to
normalization of the pathophysiologic events in hemor-
ragic shock. One possible explanation is that inhibition of
the initial P-selectin-mediated tethering of leukocytes to
endothelium may diminish the localized production of
proinflammatory cytokines, which subsequently induce
expression of endothelial cell adhesion molecules. In this
regard, other investigators have demonstrated that after
hemorrhagic shock increased infiltration of blood cells into
vital organs increases intraparenchymal cytokine expres-
sion, starting 2 h after reinfusion and reaching a peak value
after 3 d (30, 31). Moreover, inhibition of leukocyte ex-
travasation exerts a key role during inflammation because
activated neutrophils, which have adhered to the endo-

Figure 6. Representative autoradiograph of a polyacrylamide gel used
in typical ribonuclease protection assay comparing PSGL-1 and P-selectin
mRNA expression in lungs of sham-operated control and hemorrhaged
wild-type mice. Compared with lanes containing PSGL-1, mRNA levels
from sham-operated wild-type mice (lane 1) there is a marked increase in
PSGL-1 mRNA in lung isolated from wild-type mice subjected to hem-
orrhagic shock (lane 2). No significant difference was observed in P-selectin
mRNA levels between the two groups of mice. Denitometric quanti-
fication of the effect of hemorrhage on lung PSGL-1 mRNA expression
is summarized in the right panel of the figure. Bar heights represent means
and brackets indicate ± SEM. Numbers at the bottom of each bar repres-
ent the number of mice studied.

Discussion
This study was undertaken to determine the role of
P-selectin in the early inflammatory response occurring af-
after resuscitation from hemorrhagic shock. Using either
mice genetically deficient in P-selectin protein or func-
tionally blocking P-selectin in wild-type mice, by either an
anti-P-selectin mAb or rsPSGL-Ig, we first demonstrate
that P-selectin plays an essential role in the pathological re-
cruitment of leukocytes observed in hemorrhagic shock.
We also found that upon resuscitation from hemorrhage,
a increased expression of mRNA codifying for endoge-
uous PSGL-1 occurs in the lungs as well as in the liver and
intestine of hemorrhaged wild-type mice. These data pro-
vide compelling evidence that P-selectin plays a key role in
the activation of the inflammatory cascades occurring in
hemorrhagic shock, thus designating P-selectin as a possible
strategic target in the therapy of hemorrhagic shock. Our
findings are also consistent with an earlier report that an
mAb against P-selectin markedly reduced the volume of
fluid necessary for resuscitation of hemorrhaged rabbits
(21), although no analysis of leukocyte–endothelium inter-
actions was attempted.

A multistep series of adhesive and signaling events regu-
lates inflammatory responses to infection or injury (22, 23).
To initiate these responses, circulating leukocytes must first
roll along the endothelium, and then adhere to the vascular
wall under shear forces. Selectins mediate the first adhesive
step, which is characterized by tethering and rolling of leu-
kocytes on endothelial cells, platelets, or other leukocytes
(24). In particular, P-selectin, expressed on activated plate-
lets and endothelial cells, binds to ligands on most leuko-
cytes (25). The regulated expression of the selectins and
their high affinity ligand (i.e., PSGL-1), helps modulate the
inflammatory response. However, inappropriate expression
of these molecules contributes to leukocyte-mediated tissue
damage in a variety of acute inflammatory disorders (26,
27). In this regard, several investigators have demonstrated
that inhibition of the rolling phase of leukocytes plays a key
role in attenuating the acute inflammatory response (28,
29). Consistent with such findings, we now demonstrate
that soon after resuscitation from hemorrhage, leukocyte-
endothelium interactions are significantly upregulated in
the microcirculation, an event that is associated with in-
creased expression of P-selectin on the microvascular en-
dothelium. Moreover, as confirmed in P-selectin-deficient
mice, in the absence of P-selectin protein virtually no leu-
kocyte–endothelium interaction occurs after hemorrhage and
reinfusion of shed blood. This finding agrees with pre-
vious observations showing severe attenuation of leukocyte
rolling and extravasation in P-selectin-deficient mice (14).
In addition, we observed de novo synthesis of PSGL-1 in
the lungs and splanchnic organs of hemorrhaged wild-type
mice, as confirmed by quantification of PSGL-1 mRNA N. This increase in PSGL-1 mRNA levels may contribute to
widespread increases in cell-to-cell interaction during acute
inflammatory conditions such as hemorrhagic shock.

The inhibitory effect on leukocyte–endothelium interac-
tion exerted by blockade of P-selectin may contribute to
normalization of the pathophysiologic events in hemor-
ragic shock. One possible explanation is that inhibition of
the initial P-selectin-mediated tethering of leukocytes to
endothelium may diminish the localized production of
proinflammatory cytokines, which subsequently induce
expression of endothelial cell adhesion molecules. In this
regard, other investigators have demonstrated that after
hemorrhagic shock increased infiltration of blood cells into
vital organs increases intraparenchymal cytokine expres-
sion, starting 2 h after reinfusion and reaching a peak value
after 3 d (30, 31). Moreover, inhibition of leukocyte ex-
travasation exerts a key role during inflammation because
activated neutrophils, which have adhered to the endo-
thelium, release cytotoxic mediators including proteases, eicosanoids, cytokines, and oxygen-derived free radicals (1, 32), each of which can promote tissue injury and exacerbate endothelial dysfunction in hemorrhagic shock.

One may speculate on the mechanism triggering the upregulation of P-selectin to the vascular endothelial cell surface during hemorrhagic shock. Hemorrhagic shock represents a severe form of whole body ischemia/reperfusion. Several investigators have demonstrated that a common pathophysiologic event occurring during ischemia/reperfusion is the early occurrence of acute endothelial dysfunction characterized by impaired endothelial release of nitric oxide (8, 9, 11, 28, 33). In this connection, acute endothelial dysfunction associated with severe organ injury has been reported in myocardial ischemia/reperfusion (11), splanchnic ischemia/reperfusion (9), traumatic shock (10), and hemorrhagic shock (33). Moreover, a functional relationship between loss of endothelium-derived nitric oxide and the upregulation of P-selectin on the venular endothelium has been reported previously (12). Therefore, hemorrhage-induced loss of endothelium-derived nitric oxide release is probably responsible for increased expression of cell adhesion molecules in the microvascular endothelium. This conclusion is supported by the observation that endothelial cell dysfunction occurs very early after hemorrhage and persists despite fluid resuscitation (33), and that an mAb against P-selectin attenuates fluid leakage in hemorrhagic shock (21).

This study provides the first clear in vivo evidence of a purely P-selectin-dependent leukocyte-endothelium interaction in hemorrhagic shock. This work also suggests that neutralization of P-selectin in the early phase of hemorrhagic shock may limit infiltration of leukocytes into inflamed organs.

We thank Ms. Irina Opentanova for her expert technical assistance in the molecular biological measurements used in this study.

This work was supported in part by Research Grant No. GM-45434 from the National Institutes of General Medical Science of the National Institutes of Health.

Address correspondence to Allan M. Lefer, Department of Physiology, Jefferson Medical College, Thomas Jefferson University, 1020 Locust St., Philadelphia, PA 19107-6799. Phone: 215-503-7760; Fax: 215-503-2073; E-mail: allan.m. lefer@mail.tju.edu

Received for publication 13 October 1998 and in revised form 21 December 1998.

References

1. Barroso-Aranda, J., B.W. Zweifach, J.C. Mathison, and G.W. Schmid-Schönbein. 1995. Neutrophil activation, tumor necrosis factor, and survival after endotoxic and hemorrhagic shock. J. Cardiovasc. Pharma col. 25(Suppl 2):S23–S29.

2. Harbrecht, B.G., B. Wu, S.C. Watkins, T.R. Billiar, and A.B. Peitzman. 1997. Inhibition of nitric oxide synthesis during severe shock but not after resuscitation increases hepatic injury and neutrophil accumulation in hemor rhaged rats. Shock. 8:415–421.

3. Letulzo, Y., R. Shenkar, D. Kaneko, P. Moine, G. Fantuzzi, C.A. Dinarello, and E. Abraham. 1997. Hemorrhage increases cytokine expression in lung mononuclear cells in mice: involvement of catecholamines in nuclear factor-κB regulation and cytokine expression. J. Clin. Invest. 99:1516–1524.

4. Lander, H.M. 1997. An essential role for free radicals and derived species in signal transduction. FASEB J. 11:118–124.

5. Delitch, E.A., W. Bridges, R. Berg, R.D. Specian, and D.N. Granger. 1990. Hemorrhagic shock-induced bacterial translocation: the role of neutrophils and hydroxyl radicals. J. Trauma. 30:942–951.

6. Petzman, A.B., A.O. Udekwu, J. Ochoa, and S. Smith. 1991. Bacterial translocation in trauma patients. J. Trauma. 31:1083–1086.

7. McEver, R.P. 1992. Leukocyte-endothelial cell interactions. Curr. Opin. Cell Biol. 4:840–849.

8. Lefer, A.M., X.L. Ma, A.W. Weyrich, and D.J. Lefer. 1993. Endothelial dysfunction and neutrophil adherence as critical events in the development of reperfusion injury. Agents Actions. 41:127–135.

9. Lefer, A.M., and D.J. Lefer. 1993. Pharmacology of the endothelium in ischemia-reperfusion and circulatory shock. Annu. Rev. Pharmacol. Toxicol. 33:71–90.

10. Scalia, R., S. Pearlman, B. Campbell, and A.M. Lefer. 1996. Time course of endothelial dysfunction and neutrophil adherence and infiltration during murine traumatic shock. Shock. 6:177–182.

11. Tsoo, P.S., N. Aoki, D.J. Lefer, G. Johnson, and A.M. Lefer. 1990. Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. Circulation. 82:1402–1412.

12. Gauthier, T.W., K.L. Davenport, and A.M. Lefer. 1994. Nitric oxide attenuates leukocyte-endothelium interaction via P-selectin in splanchnic ischemia-reperfusion. Am. J. Physiol. 267:G562–G568.

13. Lorant, D.E., M.K. Topham, R.E. Whatley, R.P. McEver, T.M. McIntyre, S.M. Prescott, and G.A. Zimmerman. 1993. Inflammatory roles of P-selectin. J. Clin. Invest. 92:559–570.

14. Mayadas, T.N., R.C. Johnson, H. Rayburn, R.O. Hynes, and D.D. Wagner. 1993. Leukocyte rolling and extravasation are severely compromised in P-selectin-deficient mice. Curr. Biol. 74:541–554.

15. Scalia, R., J. Gefen, N.A. Petasis, C.N. Serhan, and A.M. Lefer. 1997. Lipoxin A₄ stable analogs inhibit leukocyte roll-
ing and adherence in the rat mesenteric microvasculature: role of P-selectin. Proc. Natl. Acad. Sci. USA. 94:9967–9972.
16. Borders, J.L., and H.J. Granger. 1984. An optical doppler intravital velocimeter. Microvasc. Res. 27:117–127.
17. Granger, D.N., J.N. Benoit, M. Suzuki, and M.B. Grisham. 1989. Leukocyte adherence to venular endothelium during ischemia-reperfusion. A m. j. Physiol. 257:G683–G688.
18. Weyrich, A.S., M. Buerke, K.H. Albertine, and A.M. Lefer. 1995. Time course of coronary vascular endothelial adhesion molecule expression during reperfusion of the ischemic feline myocardium. J. Leukocyte Biol. 57:45–55.
19. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. A nal. Biodem. 162:156–159.
20. Minchenko, A., T. Bauer, S. Salceda, and J. Caro. 1994. Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. Lab. Invest. 71:374–379.
21. Winn, R.K., J.C. Paulson, and J.M. Harlan. 1994. A monoclonal antibody to P-selectin ameliorates injury associated with hemorrhagic shock in rabbits. A m. j. Physiol. 267: H2391–H2397.
22. Zimmerman, G.A., T.M. McIntyre, and S.M. Prescott. 1996. Adhesion and signaling in vascular cell-cell interactions. J. Clin. Invest. 98:1699–1702.
23. Springer, T.A. 1995. Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. A nnu. R ev. Physiol. 57:827–872.
24. McEver, R.P., K.L. Moore, and R.D. Cummings. 1995. Leukocyte trafficking mediated by selectin-carbohydrate interactions. J. Biol. Chem. 270:11025–11028.
25. Norman, K.E., K.L. Moore, R.P. McEver, and K. Ley. 1995. Leukocyte rolling in vivo is mediated by P-selectin glycoprotein ligand-1. Blood. 86:4417–4421.
26. Lefer, A.M., A.S. Weyrich, and M. Buerke. 1994. Role of selectins, a new family of adhesion molecules, in ischaemia-reperfusion injury. Cardiovasc. Res. 28:289–294.
27. Lowe, J.B., and P.A. Ward. 1997. Therapeutic inhibition of carbohydrate-protein interactions in vivo. J. Clin. Invest. 100: S47–S51.
28. Weyrich, A.S., X.Y. Mao, D.J. Lefer, K.H. Albertine, and A.M. Lefer. 1993. In vivo neutralization of P-selectin protects feline heart and endothelium in myocardial ischemia and reperfusion injury. J. Clin. Invest. 91:2620–2629.
29. Kubes, P., M. Jutila, and D. Payne. 1995. Therapeutic potential of inhibiting leukocyte rolling in ischemic reperfusion. J. Clin. Invest. 95:2510–2519.
30. Shenkar, R., W.F. Coulson, and E. Abraham. 1994. Hemorrhage and resuscitation induce alterations in cytokine expression and the development of acute lung injury. A m. J. Respir. Cell Mol. Biol. 10:290–297.
31. Abraham, E., S. Bursten, R. Shenkar, J. Allbee, R. Tuder, P. Woodson, D.M. Guidot, G. Rice, J.W. Singer, and J. Ripine. 1995. Phosphatidic acid signaling mediates lung cytokine expression and lung inflammatory injury after hemorrhage in mice. J. Exp. Med. 181:569–575.
32. Suzuki, M., W. Inauen, P.R. Kvietys, M.B. Grisham, C. Meininger, M.E. Schelling, H.J. Granger, and D.N. Granger. 1989. Superoxide mediates reperfusion-induced leukocyte-endothelial cell interactions. A m. J. Physiol. 257:H1740–H1745.
33. Wang, P., Z.F. Ba, and I.H. Chaudry. 1993. Endothelial cell dysfunction occurs very early following trauma-hemorrhage and persists despite fluid resuscitation. A m. J. Physiol. 265: H973–H979.