Embolization by sinusoidal lining cells obstructs the microcirculation in rat sinusoidal obstruction syndrome

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DeLeve, Laurie D., Yoshiya Ito, Nancy W. Bethea, Margaret K. McCuskey, Xiangdong Wang, and Robert S. McCuskey. Embolization by sinusoidal lining cells obstructs the microcirculation in rat sinusoidal obstruction syndrome. Am J Physiol Gastrointest Liver Physiol 284: G1045–G1052, 2003. First published February 12, 2003; 10.1152/ajpgi.00526.2002.—Mechanisms leading to the obstruction of the microcirculation in sinusoidal obstruction syndrome (SOS) have been unclear. Because this occurs at the onset of disease, this is a potential key target for therapeutic intervention. Rats were treated with monocrotaline with or without continuous intraportal infusion of glutathione and were studied at 0.5, 1, 2, 4, 6, and 10 days after monocrotaline treatment with the use of in vivo microscopy and transmission electron microscopy. Sinusoidal perfusion decreased from days 1 through 10 with a nadir on day 4. At 12 h, numerous swollen sinusoidal endothelial cells (SECs) were observed. Subsequently, red blood cells penetrated into the space of Disse through gaps between and through swollen SEC and dissected the sinusoidal lining away from the parenchymal cells. Sinusoidal blood flow was obstructed by an embolism of aggregates of sinusoidal lining cells, red blood cells, and adherent monocytes. All changes were prevented by glutathione infusion, notably the initial swelling of SEC. SOS is initiated by changes in SEC. Microcirculatory obstruction is due to dissection of the sinusoidal lining, followed by embolization of the sinusoid by sinusoidal lining cells, compounded by aggregates of monocytes adherent in the sinusoids. Glutathione prevents SOS by preserving an intact sinusoidal barrier.

endothelium; hepatitis; toxic; glutathione; pyrrolizidine alkaloid

IN NORTH AMERICA AND Western Europe, sinusoidal obstruction syndrome (SOS) is mainly seen in the setting of hematopoietic stem cell transplantation. It is the main dose-limiting toxicity for the myeloablative conditioning regimens (i.e., chemotherapy) used before hematopoietic stem cell transplantation for malignancy. Incidence of SOS in adults who receive hematopoietic stem cell transplantation for malignancy varies from 1 to 54% (2). The wide range in incidence is likely due to differences in diagnostic criteria and clinical choices in patient eligibility criteria and conditioning regimen at different centers. Mortality for severe SOS exceeds 95% (9).

SOS differs from most other liver diseases in that cirrhotical changes are the cause rather than the consequence of the liver injury. Impairment of circulation leads to hepatocyte necrosis and eventual liver failure. Thus the key target for potential therapeutic intervention would be the early sequence of events that leads to the obstruction of the circulation. Of note, the early impairment of the microcirculation that initiates the disease must be distinguished from the fixed obstruction that occurs later due to fibrosis (1, 4).

Although early studies and the original name of the disease (hepatic venoocclusive disease) suggest that SOS originates in the hepatic veins, analysis of clinical material demonstrated that the critical change is obstruction at the level of the sinusoids with increased frequency of involvement of the central veins in more severe cases (11). Animal studies have confirmed that changes in the sinusoids occur earlier than changes in the central veins (4). These clinical and experimental findings prompted the change in name from hepatic venoocclusive disease to sinusoidal obstructive syndrome (5). The mechanism of the obstruction of the microcirculation has been a topic for speculation, but there has been no solid evidence to support any of the proposed mechanisms.

The present paper uses a reproducible rat model of monocrotaline-induced SOS that shares the clinical and histological features of SOS after hematopoietic stem cell transplantation (4). Monocrotaline is a pyrrolizidine alkaloid, the most extensively studied group of toxins that cause SOS. To allow the reader to put the current findings into perspective, the course of SOS in this model will be summarized. At 24–48 h after a single gavage of monocrotaline, i.e., days 1 and 2 (“preSOS”), there is ultrastructural evidence of damage to sinusoidal endothelial cells (SECs), but little histological or clinical evidence of SOS. On days 3–5 (early SOS), the clinical and histological manifestations are rated severe, i.e., severe centrilobular (CL) necrosis.
and hemorrhage, damage to central vein endothelial cells with subendothelial hemorrhage, and ultrastructural evidence of extensive destruction of the sinusoidal wall. Clinically, there is hepatomegaly, ascites formation, and hyperbilirubinemia. On days 6 and 7, severe late SOS occurs with the characteristic subendothelial and adventitial fibrosis of the central veins, absence of hepatocyte necrosis, continued sinusoidal and subendothelial hemorrhage, gradual resolution of the ultrastructural evidence of damage to the sinusoidal lining, and persistent clinical evidence of SOS. From days 8 through 10, SOS resolves completely in some animals, whereas severe, late SOS persists in others. Throughout the 10-day period after monocrotaline, there is a decrease in the number of Kupffer cells and an increase in the number of detectable monocytes in the lobule and within the lumen of the central vein.

The present study asks two questions. First, what causes the initial impairment to the microcirculation in SOS? To examine this, in vivo microscopy and scanning electron microscopy were used to characterize the changes, starting within 12 h of the treatment with monocrotaline. Second, because infusion of glutathione (GSH) or N-acetyl-L-cysteine into the portal circulation prevents SOS in vivo (15) and may be of clinical efficacy (10), the effect of GSH on the sinusoidal architecture was examined.

MATERIALS AND METHODS

Experiments described in this paper were performed in adherence with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205]. The experiments followed protocols approved by the Animal Care and Use Committees of the University of Arizona and University of Southern California.

Fig. 1. In vivo microscopy findings in rats treated with monocrotaline. Parameters were assessed in 10 periportal and 10 centrilobular regions in each animal (n = 6 per group). A: number of sinusoids containing blood flow (SCF)/microscopic field. B: number of swollen sinusoidal endothelial cells (SEC)/microscopic field. C: number of leukocytes adhering per 100-μm length of sinusoid adhering leukocyte/100 μm. D: number of macrophages per microscopic field that phagocyted latex particles injected through a mesenteric vein. Phagocytic activity was expressed as the ratio of phagocytic macrophages to the number of sinusoids containing blood flow. *P < 0.05 vs. controls (day 0).

Animals and Experimental Protocols

Experiments were performed on male Sprague-Dawley rats (~200 g body wt) maintained on a standard diet with water ad libitum. The rats received one intragastric dose of monocrotaline (Sigma, St. Louis, MO), 160 mg/kg body wt in water on day 0. Animals similarly dosed with an equivalent volume of vehicle (water) served as controls. Intraperitoneal infusions of GSH (2 μmol/h) were started 1 day before the monocrotaline gavage and were delivered by an Alzet mini-osmotic pump (model 2ML1; Alza, Palo Alto, CA) via a cannula inserted into the inferior mesenteric vein at a rate of 10.0 μl/h. The dose of GSH used was previously determined to be the minimal effective dose to completely prevent SOS (15).

In Vivo Microscopy

On 0.5, 1, 2, 4, 6, and 10 days after oral gavage with monocrotaline, the hepatic microvascular responses were examined by using established high resolution in vivo microscopic methods (8) under pentobarbital anesthesia (50 mg/kg body wt ip). The objective used to study the liver was a Leitz 80X/NA 1.0 water immersion objective. To assess regional distribution, parameters were counted per microscopic field in 10 periportal (PP) and 10 CL regions in each animal. Quantitation of sinusoidal perfusion, leukocyte adherence and phagocytes was done in the same microscopic fields. The relative adequacy of blood perfusion through the sinusoids was evaluated by counting the number of sinusoids containing blood flow per 10 microscopic fields. SEC swelling was assessed by counting the number of swollen cells whose nuclear regions protruded across one-third or more of the lumen. To examine the interaction of leukocytes with the sinusoidal wall, quantification of leukocytes adhering to the endothelial lining of sinusoids was calculated by counting the number of leukocytes per 100-μm length of sinusoid in the same microscopic fields. A leukocyte was defined as adhering to the sinusoidal wall, if it remained stationary for at least 30 s.
The phagocytic function of hepatic macrophages was assessed by measuring the phagocytosis of fluorescent 1.0-μm latex particles (Fluoresbrite-fluorescent monodispersed polystyrene microspheres; Polysciences, Warrington, PA) by individual cells. The latex particles were diluted 1:10 with sterile saline and injected into a mesenteric vein by using a 30-gauge lymphangiography needle (Becton Dickinson, Franklin Lakes, NJ). Distribution and relative number of phagocytic macrophages was measured by counting the number of cells that phagocytosed latex particles in a standardized microscopic field (4,125 μm²) 15 min after each rat received the latex particle suspension. Phagocytic cells firmly affixed to the sinusoidal wall were considered as macrophages, and this group included both recruited monocytes/macrophages and resident macrophages (Kupffer cells). Because reduced perfusion of individual sinusoids can limit the delivery of latex particles to macrophages in these vessels, the ratio of macrophages that phagocytosed latex particles to sinusoids containing flow was used as an overall index of macrophage phagocytic activity.

Results were averaged and the data were represented as the average numbers of the parameters in each animal. There were six animals in each experimental group of the monocrotaline-treated animals; one control rat infused with sterile saline was used per experimental group. Implantation of GSH-infusion pumps led to peritoneal adhesions due to surgery in the majority of animals that obscured the liver capsule. These adhesions limited the number of fields suitable for observation, but did not impair the circulation. Rats with an inadequate number of fields suitable for observation due to adhesions were excluded from further analysis. Thus three animals were included in the analysis of day 2 and day 4 groups, and two animals were included in the day 1 group of the monocrotaline/GSH infusion-treated rats.

Scanning Electron Microscopy

Tissue for scanning electron microscopy was fixed by routine perfusion through the portal vein by routine methods (4).
Statistical Analysis

Numerical data were expressed as the means ± SE. Statistical analyses were performed by using ANOVA followed by a Student-Newman-Keuls test. The 95% confidence level \((P < 0.05)\) was considered significant.

RESULTS

Monocrotaline-Treated Rats

Sinusoidal perfusion. The number of sinusoids containing blood flow after oral gavage with monocrotaline...
are shown in Fig. 1A. The numbers of perfused sinusoids in both PP and CL regions were significantly reduced by day 1, and they continued to decrease progressively toward a nadir on day 4 with ~55% reduction from the control levels (note that day 4 is the period of severe, early SOS). On days 6 and 10, the number of sinusoids containing blood flow was somewhat increased compared with day 4 ($P < 0.05$) but was still around 65–75% of control levels in both PP and CL regions.

**Rounding up of SECs.** Figure 1B quantifies the novel finding that monocrotaline causes SEC to swell or round up. Notably, this occurs within 12 h (day 0.5) of the administration of monocrotaline. The fivefold increase in the number of enlarged SEC in the CL region at 12 h approaches the maximum level observed; thus this is an early event. Rounding up of SEC varied across the lobule with more enlarged SEC between days 0.5 and 2 in the CL regions than in the PP regions. There was no significant increase in the number of swollen SEC on day 4, but this may be due to loss of most of the sinusoidal lining at that time point (4). On day 6, the number of swollen SEC was again significantly elevated, and the number returned to control levels by day 10.

**Sinusoidal adherence of leukocytes.** Figure 1C shows the numbers of leukocytes adhering to the sinusoidal wall after oral gavage with monocrotaline. Within 12 h of monocrotaline (day 0.5), leukocyte adherence was already increased threefold. On days 1 and 2, the number of adherent leukocytes increased to six- to sevenfold above control levels. On day 4, there was a marked difference in the lobular distribution of adherent leukocytes; in the PP region, the number remained similar to day 2, but in the CL region, the number of adherent leukocytes rose to a 15-fold increase above baseline. Adherence of leukocytes then decreased on days 6 and 10 when compared with day 4 ($P < 0.05$), although the levels were still slightly greater than controls.

**Phagocytic activity within the sinusoid.** Figure 1D demonstrates the phagocytic activity of hepatic macrophages after treatment with monocrotaline. On days 4, 6, and 10, the activity in both PP and CL regions was markedly increased compared with controls: 2.5- and 8.4-fold, respectively, on day 4; 1.8- and 5.0-fold, respectively, on day 6; and 2.2- and 5.6-fold, respectively, on day 10.

**Morphological changes in SOS.** Figure 2 shows the morphological events as they unfolded over the first 2 days after monocrotaline treatment (these changes are quantified in Fig. 3). During the first 2 days, there was rounding up of SEC with penetration of red blood cells beneath these enlarged SEC (Figs. 2, A and B, and 4A). Red blood cells that penetrated into the space of Disse dissect the sinusoidal lining away from the parenchymal cells (Figs. 2C and 4B). During this period, many sinusoids are engorged and the dilated sinusoids contain sluggish blood flow (Figs. 2, B and C, and 4B). What cannot be adequately conveyed with static images but can be appreciated with in vivo microscopy, is the rapid flow of blood through the space of Disse once the sinusoidal lining has been separated from the parenchymal cells (Fig. 2B); and the actual peeling away of sinusoidal lining cells (Fig. 2C).

**GSH Infusion into Monocrotaline-Treated Rats**

In vivo microscopy was used to compare sinusoidal perfusion, rounding up of SEC, and leukocyte adhesion in monocrotaline-treated rats in the presence or ab-
Fig. 5. Effect of glutathione on scanning electron microscopy after monocrotaline. Rats were treated with McT on day 0 plus either continuous infusion of sterile saline (A, C, E, and G) or GSH (B, D, and F). A: day 1, McT/NaCl: large gaps in SEC with microvilli of hepatocytes visible through gaps, loss of fenestrae and sieve plates. B: day 1, McT/GSH: fenestrae and sieve plates still present, although reduced in number; only an occasional gap was seen. C: day 2, McT/NaCl: extensive destruction of SEC with very large gaps. D: day 2, McT/GSH: SEC with intact fenestrae and sieve plates; only an occasional gap. E: day 4, McT/PBS: loss of endothelium and obstruction of sinusoid; fragment of SEC and stellate cell seen; remainder of sinusoidal lining is destroyed, exposing parenchymal cells to blood. F: day 4, McT/GSH: fenestrae and sieve plates intact. G: day 4, McT/NaCl: extensive gaps and destruction of SEC. Magnification: A, ×8,600; B, ×8,900; C, ×6,900; D, ×8,700; E, ×8,900; F, ×8,900; G, ×7,100.

sence of a continuous intraportal infusion of GSH. Figure 3, A and B, demonstrates that GSH infusion maintains normal sinusoidal perfusion in contrast to the progressive decrease in the number of perfused sinusoids seen with monocrotaline alone. Figure 3, C and D, shows that GSH completely prevents the three- to fivefold increase in the number of swollen SEC on days 1 and 2. Similarly, Fig. 3, E and F, demonstrates that GSH infusion completely abolishes the increase in leukocyte adherence on days 2 and 4 and attenuates it on day 1.

Scanning electron microscopy (Fig. 5) also demonstrated the protective effect of GSH on the sinusoidal architecture. The loss of fenestrae and sieve plates, the formation of large gaps in the SEC, and the penetration of red blood cells through the gaps seen 1 and 2 days after monocrotaline (Fig. 5, A and C) were almost entirely prevented by the administration of GSH (Fig. 5, B and D). Furthermore, the destruction of SEC with near-total loss of the sinusoidal lining on day 4 (Fig. 5, E and G) was prevented by the GSH infusion (Fig. 5F).

DISCUSSION
The major point of the present study is the elucidation of the cause of the initial circulatory obstruction in
SOS, which has been the least understood aspect of this disease. Earlier studies (4) demonstrated damage to SEC but did not determine how changes to SEC led to impairment of the microcirculation. The findings presented here suggest the following sequence of morphological events. In the first 12–48 h after monocrotaline treatment, there is minimal clinical and histological evidence of disease. In this period, the initial event is rounding up of the SEC. Red blood cells enter the space of Disse by penetrating through gaps within and between these swollen SEC. Once blood has entered below the SEC, the flow of blood begins to dissect the entire sinusoidal lining away from the underlying parenchymal cells. Dissection of the sinusoidal lining results in an embolism of sinusoidal lining cells that obstruct much of the microcirculation by day 4. Day 4 falls in the period in the middle of the 3-day period in which SOS manifests itself clinically and during which the histological manifestations of CL coagulative necrosis and hemorrhage are most pronounced. At this time point monocyte accumulation peaks in areas of necrosis. Accumulated monocytes contribute to the aggregates that narrow and obstruct the sinusoids. Taken together, these findings show that the earliest morphological event is the change in the SEC that permits blood to dissect off the sinusoidal lining. Embolism of the sinusoidal lining cells compounded by monocyte aggregates is the cause of the microcirculatory obstruction.

Kupffer cells are lost along with the sinusoidal lining destroying the physiological gradient, i.e., the presence of more Kupffer cells in the PP area compared with the centrilobular area. Kupffer cells are replaced between days 4 and 10 with an influx of phagocytic monocytes [not Kupffer cells (4)], which accumulate in the injured CL area giving the impression of a change in the physiological gradient. However, this is a response to injury. Notably, the accumulation of monocytes is abolished when the GSH infusion prevents injury.

The reactive metabolite of monocrotaline, monocrotaline pyrrole, binds covalently within the endothelial cell to actin (7). Monocrotaline induces F-actin depolymerization in SEC and this leads to increased expression and activity of matrix metalloproteinase-9 within 12 h of monocrotaline administration (unpublished observation). The present study showed that penetration of red blood cells into the space of Disse occurs under SEC that are rounded up. The actin cytoskeleton plays a major role in cell shape and depolymerization of F-actin leads to rounding up of cells. Release of matrix metalloproteinases on the contraluminal side of the SEC breaks down extracellular matrix in the space of Disse, so that increased matrix metalloproteinase activity could account for the dehiscence of the SEC (unpublished observation). Thus F-actin depolymerization and release of matrix metalloproteinases would explain how red blood cells can penetrate beneath the endothelial barrier and dissect the endothelium off of the matrix in the space of Disse.

Studies have shown that infusion of GSH prevents SOS, but that discontinuation of the infusion on day 5 after monocrotaline administration precipitates accelerated development of severe SOS within 24 h of the discontinuation of GSH (15). In the rat, 90% of monocrotaline and its metabolites is excreted within 7 h of administration (6). Thus the need for prolonged continuous GSH infusion cannot be attributed to detoxification of monocrotaline metabolites. Rather, it appears that the continuous GSH infusion suppresses a persistent change in the liver sinusoid induced by monocrotaline. This persistent change may be increased matrix metalloproteinase-9 or matrix metalloproteinase-2 activity in SEC. The ability of GSH to prevent monocrotaline-induced SOS even after monocrotaline is excreted (15), may be due to inhibition of matrix metalloproteinase activity by GSH (3, 12–14).

In summary, this study has used in vivo microscopy and electron microscopy to examine the cause of sinusoidal obstruction in the monocrotaline-induced model of SOS. The findings suggest that the circulatory obstruction in SOS is initiated by changes in SEC that allow red blood cells to penetrate into the space of Disse and to dissect off the sinusoidal lining. Subsequent embolization of the sinusoidal lining, compounded by aggregates of monocytes, is the proximate cause of the obstruction. All of these changes are prevented by infusion of GSH.

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REFERENCES


