Reactive Nitrogen and Oxygen Radicals Formed during Hepatic Ischemia-Reperfusion Kill Weakly Metastatic Colorectal Cancer Cells

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Abstract

Microscopic infarcts develop within the livers of athymic nude mice during the first 24 h after human colorectal carcinoma (CRC) cells arrest within hepatic sinusoids. Because these regions are reperfused, essentially all weakly metastatic clone A and MIP-101 CRC cells die, whereas many highly metastatic CX-1 CRC cells survive. Because hepatic sinusoidal endothelial cells kill tumor cells in vitro by producing nitric oxide, superoxide anion, and other reactive oxygen and nitrogen species, our purpose was to determine whether reoxygenation of ischemic hepatic cultures in vitro forms toxic oxygen and nitrogen radicals that kill weakly but not highly metastatic CRC cells. CRC cells (10^5) were labeled with rhodamine-dextran and calcine AM, cultured with cells from one mouse liver in a rotating suspension culture system for up to 24 h, and the metabolic activity of the CRC cells was determined. Liver fragments oxygenated normally before harvest were not toxic to either CRC cell line, but coculture with liver made ischemic by a 3-min ligation of the portal vein and hepatic artery in vivo before harvest and then cultured in oxygenated medium killed 50–70% of weakly metastatic clone A and MIP-101 cells at 24 h but <15% of highly metastatic CX-1 cells. Inhibition of nitric oxide synthase, addition of exogenous superoxide dismutase, but not catalase or hypoxia, during coculture blocked the killing of weakly metastatic CRC cells. Thus, reoxygenation of hepatic parenchymal and nonparenchymal cells after ischemia may form toxic species that eliminate weakly metastatic CRCs within 24 h of their arrest in the liver.

Introduction

Metastasis is a process that requires several steps to be completed in sequence: (a) intravasation from the primary into the circulation; (b) arrest within the microcirculation of a potential site for metastasis; (c) extravasation; and (d) proliferation within the parenchyma of the metastatic site (1–4). The process is relatively inefficient (5, 6), and Luzzi et al. (7) have observed that the majority of cells arrest in the liver by size restriction. We have found that metastatic potential (i.e., the ability of a tumor cell to colonize the liver) is proportional to the number of tumor cells that survive the first 24 h of arrest with weakly metastatic CRC (8) having very few surviving cells 24 h after arrest within the sinusoid (8, 9). However, the mechanisms that kill CRC cells during this early period after implantation are not well defined.

Our purpose was to determine whether the changes in the hepatic parenchyma that occur after tumor cell arrest produce toxic molecules that may kill metastatic precursor cells. As tumor cells arrest in the hepatic sinusoid, blood flow stops in that sinusoid for 12–24 h, on which flow resumes (9). Our approach was first to demonstrate morphologically that tumor cells induce ischemia in microscopic regions of the murine host liver during the first 24 h after arrest in the hepatic sinusoids and terminal portal venules. Then, we sought to determine whether ischemic fragments of mouse liver can alone kill human CRC cells in an in vitro culture system. Toxic oxygen and nitrogen radicals are produced during liver ischemia and reperfusion by both parenchymal (10) and nonparenchymal (11, 12) cells. Furthermore, we (13) have shown that NO, O_2·−, and, possibly, ONOO− produced by primary cultures of oxygenated, but otherwise unstimulated, murine hepatic sinusoidal endothelial cells kill weakly but not highly metastatic human CRC cells.

Our postulate was that hypoxia induced during tumor cell arrest in hepatic sinusoids leads to oxygen and nitrogen radical formation during reperfusion that are toxic to weakly, but not highly metastatic, tumor cells. Our cultures used a rotating suspension system that maintains the architecture and viability of mouse liver fragments in vitro for at least 24 h (9). Hepatic ischemia was induced by clamping the hepatic arterial and portal venous inflow for 3 min. Afterward, the liver was harvested, and 1–3-mm fragments were cultured with tumor cells for up to 24 h. This system may be controlled in vitro so that the contribution of various molecules may be ascertained. Our results suggest that NO, O_2·−, and, possibly, ONOO− formed during the reperfusion of ischemic liver kill carcinoma cells with low but not high metastatic potential. Furthermore, the data suggest that the effect of CRC lysis added to the inhibition of metabolic activity accounts for the killing observed in vivo in our xenogenetic model during the first 24 h after tumor cell arrest.

Materials and Methods

Animals. Male athymic nude mice, 6–8 weeks of age, weighing 25–30 g were obtained from Harlan Sprague Dawley Inc. (Frederick, MD) and kept under specific pathogen-free conditions. The standard diet and water were given ad libitum. Liver harvest was performed under general anesthesia with inhalational administration of methoxyfluorane (Mallinckrodt Veterinary, Inc., Mundelein, IL). All animal experiments were approved by the Animal Care and Use Committee of the University of Pittsburgh School of Medicine and were conducted in accordance with the guidelines issued by the NIH for the care of laboratory animals.

Human CRC Cell Lines. Clone A and MIP-101 are poorly differentiated human CRC lines that are weakly metastatic, whereas CX-1 is a moderately differentiated, highly metastatic human CRC line (8, 9). All CRC lines were maintained in RPMI 1640 with 10% fetal bovine serum and antibiotics (complete medium) and tested for Mycoplasma, as described previously (13, 14).

Metabolic Activity Assays. CRC cells were labeled with two fluorescent reagents, Rd-Dx and calcine AM, by electroporation, as described previously (13). The liver-tumor cell cocultures were analyzed by fluorescence microscopy for 24 h after the addition of CRC. The percentage of metabolic activity was calculated to be equal to the number of calcine-positive and Rd-Dx-positive cells divided by the total number of Rd-Dx-positive cells expressed as a percentage. Each assay was performed at least twice.
Liver–CRC Cocultures. Athymic nude mice were placed under general inhalant anesthesia, and a midline laparotomy incision was made under aseptic conditions to expose the liver. The portal vein and hepatic artery were clamped with a microvascular clamp for 3 min for the experiments with ischemic liver. The liver was excised and immediately placed in chilled RPMI 1640 in a Petri dish on ice and dissected into 1–3-mm fragments with sterile scalpel blades. The fragments and cells were aspirated into a 50-ml centrifuge tube and washed once by centrifugation at 400 × g. The fragments from one mouse liver were then resuspended in 5 ml of the medium used for the liver fragment-CRC cocultures [a 1:1 mixture of RPMI 1640 and HepatoZYME SFM (Life Technologies, Inc., Grand Island, NY) with 10% FBS and penicillin-streptomycin] and loaded into a 55-ml RWV (Rotary Cell Culture System; Synthecon, Inc., Houston, TX; Ref. 9). CRC cells (1 × 10^7) that had been prelabeled with Rd-Dx and calcine AM were then added to the RWV, and the 55-ml RWV chamber was completely filled with the liver fragment coculture medium to eliminate bubbles. Operation of the RWV and description of its function are as described previously (9). In some experiments the air pump of the RWV was disconnected to test the role of reoxygenation after liver ischemia. The dissolved PO2 in the medium with active CRC cultures after 24 h in an incubator at 37°C with 95% air and 5% CO2 is approximately 120–140 mm Hg with the air pump functioning, whereas it drops to <40 mm Hg when the air pump is disconnected (data not shown). In other experiments, mice were pretreated with an i.v. injection of 20 mg of NMMA, a NO synthase inhibitor, 5–10 min before liver harvest with 1 mM NMMA added to the medium when CRCs were cocultured with liver fragments and cells to determine the role of NO. In other experiments, the role of O2 and peroxides was also analyzed by adding SOD or CAT (Sigma Chemical Co., St. Louis, MO) at final concentrations of 286 units/ml to the liver fragment-CRC cocultures. Cocultures were incubated and analyzed for up to 24 h.

Confocal and Light Microscopy. Athymic nude mice were given injections of 2 × 10^7 CRC intrasplenically under general anesthesia, as described previously (8, 9). For analysis of regional ischemia by light microscopy, mice were allowed to recover, and their livers were harvested, as described above, 24 h after intrasplenic injection. The livers were fixed in formalin embedded in paraffin and then processed in 5-μm thick sections for routine HE staining. Routine sections were examined under a Leica upright microscope with standard ×10 and ×40 plan apo objectives.

For confocal microscopy, 1 × 10^3 clone A cells were injected intrasplenic-ally under general anesthesia. Ten minutes later, the portal vein was exposed, and 1 ml of a 1:10 dilution of 0.02 μm Green FluoSphere beads (Molecular Probes, Eugene, OR) in 2% paraformaldehyde in PBS was injected over 5 min. Livers were harvested and incubated in 2% paraformaldehyde for 1 h at 22°C and then stored in PBS at 4°C. Livers were then mounted directly on coverslips on a Leica TCS-NT confocal microscope. Selected fields were scanned with a ×60 plan apo oil immersion objective using a 488-nm line to image the FluoSpheres and a 563-nm line to image the Rd-Dx-labeled cells. Serial confocal sections were taken over the first 50 microns at 1 micron intervals from the exposed surface of the liver. Subsequently, image stacks were imported into ImageSpace (Molecular Dynamics, Sunnyvale, CA) for reconstruction into multicolor look-through projections.

Statistics. Results are presented as the mean ± SE. Comparisons among means were performed by one-way ANOVA using StatView 4.5 (Abacus Concepts, Berkeley, CA) on an Apple Macintosh computer (Macintosh, Cupertino, CA). Level of significance was ≤5%.

Results

Tumor Cell Arrest within Hepatic Sinusoids Induces Ischemia. When tumor cells arrest within the hepatic microcirculation after intrasplenic injection, they traverse the spleen to enter the hepatic microcirculation through the portal vein, and a fraction of the cells either bind to periportal venules (Fig. 1A) or are wedged into hepatic sinusoids occluding blood flow (Ref. 9 and Fig. 1B). Obstruction of blood flow causes microscopic infarcts within the liver parenchyma that are located primarily near the capsule of the liver (Fig. 1C). When examined more closely 24 h after tumor cell implantation, neutrophils and other inflammatory cells infiltrate the edge of the infarcts with coagulative necrosis of the hepatocytes, but intact nuclei within cells lining the hepatic sinusoids (Fig. 1D). These results suggest that hepatocytes within the infarct are undergoing hypoxic death while leaving endothelial, and possibly other nonparenchymal, cells intact. The infarcts are well organized by 72 h after tumor cell implantation with mononuclear cells dominating the infiltrate at the margin of the infarct and viable highly metastatic CX-1 cells (but not weakly metastatic clone A cells) within the infarct (data not shown).

The hepatic artery and portal vein were clamped, and the livers were harvested 3 min later to model these ischemic changes in the liver parenchyma without either tumor cells (so that CRC products did not affect the analysis) or the infiltrating acute and chronic inflammatory cells. Nude mouse livers were then dissected into 1–3-mm fragments at 4°C and cultured in a rotating suspension culture system. The parenchyma from normal liver maintains hepatocyte nuclear architecture for 24 h, although cytoplasmic detail is partially lost (Fig. 1E and Ref. 9). The endothelial cells in the portal venules and hepatic sinusoids appear to be intact (Fig. 1E). In contrast, fragments of ischemic liver have a more pronounced loss of cell detail: the nuclei of hepatocytes are largely pyknotic with a greater degree of loss of cytoplasmic detail than in the fragments of normal liver, although cells appear intact within the portal tracts and the sinusoids. Viability of the ischemic liver fragments and cells is decreased such that there is a 35% decrease in the ability of ischemic liver to metabolize calcine AM at 24 h compared with oxygenated liver from normal nude mice (data not shown). Nonetheless, fragments and cells from ischemic livers did contain regions of viable hepatocytes and intrasinusoidal cells after 24 h of culture (Fig. 1E).

Coculture of Human Carcinoma Cells with Ischemic Liver Fragments. CX-1 is a highly metastatic cell line because it produces colonies in 90% of mice who receive injections of 2 × 10^6 viable cells, whereas clone A and MIP-101 are weakly metastatic cell lines that form colonies in <20% of mice (8, 9). Analysis of liver by intravital videomicroscopy indicates that a high fraction (~15%) of injected CX-1 cells survive within the hepatic microcirculation compared with a low fraction (<1%) of clone A cells 24 h after tumor cell arrest in the liver (9). A major contributor to cell loss during implantation is lysis because ~50% of human CRC cells lyse when they impact into 8-μm diameter openings (9). Consequently, if lysis from impact does not occur and ischemic liver is capable of killing CRC cells, then ≥65% of the highly metastatic CX-1 cells should survive compared with ≤50% of the weakly metastatic CRC cells when cocultured with ischemic liver fragments. When clone A cells were cocultured with ischemic liver cells, only 30–50% of the cells survived at 24 h, with a significant decrease in metabolic activity (Fig. 2A). In contrast, when clone A cells were cocultured with normally oxygenated liver fragments, ≥90% of clone A cells survived for 24 h when either cultured alone or with normal liver (Fig. 2A). Similar results were obtained with MIP-101 cells cocultured with normoxic and ischemic liver fragments because 87 ± 6% of MIP-101 cells incubated with normoxic liver were metabolically active compared with 47 ± 6% of MIP-101 cells when incubated with ischemic liver fragments for 24 h (P < 0.0001). In contrast, the viability of CX-1 cells was not significantly affected by coculture with either normoxic or ischemic liver fragments (Fig. 2B). Thus, ischemic, reoxygenated liver is quite toxic to weakly but not to highly metastatic human CRC cells when the medium is oxygenated in vitro.
Fig. 1. Liver morphology after tumor cell arrest and ischemia. A, clone A (2 × 10⁶) cells were injected intrasplenically, and the liver was harvested 10 min later. Sections were processed for routine histology and analyzed with a ×40 objective. The clone A cell (arrow) is attached to a periportal venule and identified by morphology. Also note the normal architecture of the liver at this time after splenic injection. B, clone A cells that had been prelabeled with Rd-Dx were injected intrasplenically, and the liver was exposed 10 min later. The portal vein was injected with green fluorescent microbeads, as described in “Materials and Methods,” and the liver was then examined with a ×60 oil immersion objective under a confocal microscope. The green fluorescent beads outline the periportal venules and hepatic sinusoids. Red clone A cells (arrows) at the end of green vessels appear to be obstructing the flow of blood. In contrast, other tumor cells appear to be yellow (+). These yellow cells result from the color of the red of the Rd-Dx-labeled cells mixing with the green of the microbeads where blood has flowed past the CRC cell like the clone A cell shown in A. B suggests that a fraction of clone A cells obstruct blood flow. C, liver has been harvested from mice given injections of clone A cells 24 h earlier, processed for routine histology, and examined under a ×10 objective. Numerous small infarcts (I) are evident near the capsule of the liver where there is coagulative necrosis of the hepatocytes with loss of nuclear detail within the area of the infarct. D, the margin of an infarct (I) is shown at higher magnification under a ×40 objective and demonstrates that neutrophils and other acute inflammatory cells are present in the margin of the infarct. Nuclei within the vessels of the infarct are still present. E, normal liver has been harvested and cultured in vitro in the RWV for 24 h. Although there is some loss of cytoplasmic detail and nuclear structure, the hepatocytes and sinusoidal cells appear to be viable. F, ischemic liver fragments were cultured for 24 h and processed for routine histology. Both the hepatocytes and the sinusoidal cells are partially viable, but there is more loss of hepatocytes than in E. Bars, 30 μm.
HEPATIC ISCHEMIA-REPERFUSION KILLS TUMOR CELLS

Fig. 2. Loss of metabolic activity of clone A and CX-1 CRC cells by coculture with ischemic and normal liver. Clone A (A) and CX-1 (B) cells were cultured either alone (●), with liver fragments from normal nude mice (▲), or with liver fragments from mice whose livers were made ischemic shortly before harvest (■), as described in the text. Cultures were performed for 24 h at 37°C in a rotating cell suspension culture system, and the metabolic activity of the cells was determined as described in the text. The results suggest that ischemic liver fragments in the presence of oxygenated medium decreases the metabolic activity of clone A, but not CX-1 cells. Results are from one of six independent experiments.

data not shown and Ref. 13). The next experiments sought to determine whether the same species might also be involved in the killing observed in the present culture experiments.

When donor mice were pretreated with NMMA, a NO synthase inhibitor, a significant increase was observed in the number of clone A cells that survived coculture with ischemic liver fragments for 24 h (Table 1). Similarly, the addition of exogenous SOD to the culture medium also significantly blocked the killing of clone A cells (Table 1). However, the addition of CAT did not decrease the killing of clone A cells by ischemic liver fragments (Table 1). When the medium was hypoxic during the coculture, clone A cells survived even when cocultured with ischemic liver (Table 1). Thus, liver made ischemic in vitro requires a fresh source of oxygen to kill weakly metastatic CRC cells. In addition, inhibition of NO and O2,−, but not peroxides, blocks the killing of weakly metastatic cells.

Discussion

The present results demonstrate that ischemic liver may be toxic to weakly metastatic CRC cells on reoxygenation and that this toxicity involves reactive oxygen and nitrogen species and extend our previous findings that normal mouse liver was not inherently toxic to human CRC (9, 13). Neither oxygenated nor hypoxic normal liver parenchyma is toxic to human CRC cells of either high or low metastatic potential during the first 24 h of coculture. Thus, the ischemia produced by some tumor cells as they arrest in the hepatic microcirculation may be a major barrier to their ability to colonize liver. Weiss et al. (6) observed that certain tumor cells entering a capillary network will arrest as they are carried by the circulating blood into a sinusoid, the diameter of which is smaller than that of the cells. Luzzi et al. (7) and Chambers et al. (15) have used intravital videomicroscopy to determine that the majority of tumor cells entering the hepatic microcirculation are arrested by size restriction rather than adhesion to large diameter periportal venules. Although they did not observe intravascular cell lysis during arrest, they documented the process of “clasmatosis,” in which cells relieve the stress of shape change by shedding membrane fragments (16). We (9) and others (5) have observed that nearly 50% of human CRC cells die when they enter an 8-μm diameter pore, which is the diameter of the hepatic sinusoid under portal venous pressure. However, the cells that survive this initial arrest and deformation may obstruct blood flow and cause hepatic ischemia.

To our knowledge, other investigators have not observed that tumor cell arrest in hepatic sinusoids leads to regional ischemia within the liver. Luzzi et al. (7) estimate that only 5% of the hepatic sinusoidal volume is occluded by 3 × 105 syngeneic melanoma cells entering the portal circulation of C57Bl/6 mice and did not report any ischemia. In our experiments, nearly a log more cells are injected into the spleen, and this larger cell dose may account for the greater occlusion of the hepatic microcirculation observed in our experiments. However, this dose is used because lower doses of CRC cells may not produce reliable numbers of liver colonies, especially by weakly metastatic CRC (8). Furthermore, we use the intrasplenic injection route because our earlier data indicated that liver colonization by human CRC after intrasplenic injection is associated with the ability of patients to develop metastatic disease (17). Another possible cause for hepatic ischemia is that the human CRC used in the present experiments behave differently from the syngeneic tumor cells used by other investigators. In support of this, we have observed that xenogeneic CRC cells tend to remain within the sinusoid, do not appear to extravasate, but reestablish blood flow around the surviving cells without invasion into the hepatic parenchyma (9). In contrast, the ability of syngeneic tumor cells to extravasate and migrate through hepatic parenchyma has been demonstrated by Chambers et al. (re-reviewed in Ref. 15). The difference in invasiveness between syngeneic and xenogeneic tumor cells may be due to an inability of the xenogeneic tumor cell to respond to murine motility or invasion signals. Alternatively, xenogeneic tumor cells within the hepatic sinusoid may stimulate a greater degree of regional ischemia than do syngeneic tumor cells, which appear to be more motile in mouse liver.

Inflammatory cells may infiltrate areas of hypoxic liver and produce other mediators than reactive oxygen and nitrogen species that are toxic to tumor cells (18). However, the present experiments suggest that such mediators are not needed from infiltrating cells because the liver is harvested and removed from the circulation without any further exposure to the blood. In fact, by blocking blood flow to microscopic regions of the liver, tumor cell emboli may prevent hemoglobin from gaining access to the hypoxic regions because they are reperfused. Because hemoglobin and other compounds within erythrocytes are important scavengers of reactive oxygen and nitrogen species (19, 20), obstruction of blood flow may accentuate the toxicity of the ischemia-reperfusion injury caused by tumor cell emboli. In addition, incubation of clone A and CX-1 cells with SIN-1 recapitulates the sensitivity or resistance, respectively, to killing with similar kinetics, as observed in these experiments (13). Other investigators have confirmed the importance of SOD in metastasis by

Table 1 Inhibition of ischemic liver toxicity to weakly metastatic clone A human CRC cells after 24 h of coculture with liver fragments

<table>
<thead>
<tr>
<th>Liver fragments</th>
<th>Oxigenation</th>
<th>Treatment</th>
<th>% viable</th>
<th>% of control</th>
<th>P vs control</th>
</tr>
</thead>
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<tr>
<td>No</td>
<td>Yes</td>
<td>82 ± 5</td>
<td>100%</td>
<td>NSa</td>
<td></td>
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<tr>
<td>No</td>
<td>No</td>
<td>88 ± 6</td>
<td>100%</td>
<td>NS</td>
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<tr>
<td>Ischemic</td>
<td>Yes</td>
<td>46 ± 8</td>
<td>62%</td>
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<tr>
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<td>82 ± 11</td>
<td>100%</td>
<td>NS</td>
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<tr>
<td>Ischemic</td>
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<td>75 ± 13</td>
<td>92%</td>
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<td>100%</td>
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<tr>
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</tr>
<tr>
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<td>28%</td>
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</table>

Ps are derived from the Fisher’s PLSD assessment of differences between means after one-way ANOVA demonstrated that means were significantly different from each other.

a NS, not significant.
demonstrating that it, but not CAT, injected systemically into mice enhances the metastatic potential of a syngeneic mouse tumor (21). However, the role of SOD in the growth of malignant cells is controversial because several investigators have shown that overexpression of SOD decreases anchorage-independent growth (22) and tumorigenicity (23). Nonetheless, the present experiments suggest that reactive oxygen and nitrogen species may be toxic to weakly metastatic human CRC cells because they arrest within the liver sinusoid.

Oxygendeen murine sinusoidal endothelial cells kill weakly metastatic CRC cells in vitro (9), whereas ischemic, reoxygenated liver fragments also kill similar CRC cells. It is not clear what the effector cell is in the present system. Hepatic sinusoidal endothelial cells may be the toxic effector cell. In contrast, both hepatocytes (10) and Kupffer cells (11, 12) produce reactive oxygen and nitrogen species during hepatic ischemia and reperfusion. It is also possible that the CRC cells themselves may produce the toxic species. Nonetheless, when the 50% lysis that occurs during impaction is added to the present observed rates of loss of metabolic activity, the combined loss of viable cells is similar to the loss of metabolic activity in vitro during the first 24 h after the arrest of CRC within the hepatic microcirculation. Thus, the major determinants of survival during the first 24 h after the arrest in the liver sinusoid may be: (a) the lysis that occurs during the shape change that is associated with tumor cells impacting within small sinusoids; and (b) death from oxidative stress caused during ischemia-reperfusion by toxic oxygen and nitrogen radicals.

In summary, microscopic liver infarcts are formed during the first 24 h after the arrest of CRC within the hepatic microcirculation. Our data with an in vitro suspension culture system suggest that NO, O$_2^-$ and, possibly, ONOO$^-$ are formed during reoxygenation of ischemic liver and that these substances are more toxic to weakly than highly metastatic CRC. Weakly metastatic clone A cells have lower MnSOD activity than the highly metastatic CX-1 cells (13). Furthermore, ONOO$^-$ in low concentration induces apoptosis in human CRC and necrosis at high concentration (data not shown). Because MnSOD scavenges O$_2^-$ and prevents ONOO$^-$ production (24), the level of MnSOD may be an essential molecule for survival during the first 24 h after arrest within the hepatic microcirculation. The ability to manipulate the levels of such antioxidant molecules as MnSOD, intracellular glutathione, and Bcl-2 may offer important new approaches to preventing the establishment of metastases.

References

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