Ischemia/Reperfusion Accelerates the Outgrowth of Hepatic Micrometastases in a Highly Standardized Murine Model

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Mortality in colorectal cancer is associated with the development of liver metastases. Surgical removal of these tumors is the only hope for cure, but recurrence is common. During liver surgery, ischemia/reperfusion (I/R) often occurs as a result of hemorrhage or vascular clamping. Although the adverse effects of I/R on postoperative liver function are well documented, the influence of I/R on the outgrowth of residual micrometastases is unknown. We used a highly standardized mouse model of partial hepatic I/R to study the effects of I/R on the outgrowth of preestablished colorectal micrometastases. Five days following intrasplenic injection of C26 colon carcinoma cells, the vascular structures of the left lobe were clamped for 45 minutes under hemodynamically stable conditions. Tissue glutathione, plasma liver enzymes, hepatocellular necrosis, and tumor growth were assessed over time. I/R caused oxidative stress and early liver tissue damage. The outgrowth of micrometastases in occluded liver lobes was accelerated five- to sixfold compared with nonoccluded lobes and was associated with areas of necrotic liver tissue surrounded by inflammatory cells and apoptotic hepatocytes. Accelerated tumor growth and tissue necrosis were completely prevented by occluding blood flow intermittently. In contrast, ischemic preconditioning or treatment with the antioxidants α-tocopherol or ascorbic acid failed to protect against late tissue necrosis and tumor growth, although early hepatocellular damage was largely prevented by these methods. In conclusion, I/R is a strong stimulus of recurrent intrahepatic tumor growth. Measures to prevent I/R-induced late tissue necrosis cross-protect against this phenomenon. (HEPATOLOGY 2005;42:165-175.)

The liver is the prime target organ for the development of metastases in colorectal cancer. Mortality is almost invariably attributable to complications associated with tumor growth in the liver.1 Although surgical removal of hepatic tumors is as yet the only hope for cure, the vast majority of patients ultimately present with recurrent disease, predominantly in the liver.2 During hepatic surgery, intraoperative hemorrhage is common, often necessitating blood transfusion, the latter being suggested as a predictor of postoperative outcome and poor prognosis.3,4 Therefore, approaches to control intraoperative bleeding are presently applied worldwide and include vascular clamping methods.5,6 However, such measures cause hepatic ischemia/reperfusion (I/R) to the liver tissue, which may contribute to postoperative liver failure. The adverse effects of I/R on hepatocellular function have been well documented.7,8 In contrast, the influence of I/R on intrahepatic tumor growth has remained underexposed. The few studies available show that I/R, when applied prior to a challenge with tumor cells, stimulates tumor cell adhesion and promotes the incidence of metastases formation.9,10 These studies are likely to be relevant for the implantation of tumor cells that are shed into the blood circulation during surgical manipulation.11 However, in the majority of patients undergoing liver
surgery, microscopic tumor cell deposits are already present at the time of surgery, and their detection in the liver parenchyma is associated with tumor recurrence and poor life expectancy. At present, it is unknown how I/R in general, and vascular clamping in particular, affects the behavior of these micrometastases. We hypothesized that I/R, such as that frequently encountered during liver surgery, accelerates the outgrowth of preexistent hepatic micrometastases, thereby worsening prognosis.

I/R-induced tissue damage may be affected by many confounding systemic parameters. Most importantly, hemodynamic stability is crucial, because hypotension and systemic hypo-oxygenation may induce temporary tissue ischemia. In addition, hypothermia affects hemodynamic stability and also reduces I/R-induced injury. Therefore, we established a highly standardized murine model of partial hepatic I/R by blood flow occlusion in which special attention was paid to anesthetic management, hemodynamic stability, and body temperature. Before studying the effects of hepatic I/R on the outgrowth of micrometastases, the model was validated by measuring parameters of oxidative stress and hepatocellular damage.

Several therapeutic strategies have been successfully developed to prevent liver tissue damage following I/R. These include alternative clamping techniques such as ischemic preconditioning and intermittent clamping as well as pharmacological intervention with antioxidants such as \( \alpha \)-tocopherol and ascorbic acid. We evaluated whether those strategies cross-protect the liver against (accelerated) outgrowth of micrometastases, thereby worsening prognosis.

**Materials and Methods**

**Animals.** All experiments were performed in accordance with the guidelines of the Animal Welfare Committee of the University Medical Center Utrecht, The Netherlands. Male Balb/C mice (10-12 weeks) were purchased from Charles River (Sulzfeld, Germany) and were housed under standard laboratory conditions.

**Murine Model of Hepatic I/R.** Partial hepatic I/R was induced by occluding the vascular inflow of the left lateral liver lobe for 45 minutes, corresponding to approximately 40% of the liver mass. All surgical procedures were performed under inhalation anesthesia with a 1.5% to 2% isoflurane/O\(_2\) mixture using a mask. Buprenorphine (3 \( \mu \)g/mouse) was administered intramuscularly before surgery to provide sufficient intraoperative and postoperative analgesia. Surgical procedures were performed under aseptic conditions, and surgical foil was placed over the laparotomy wound to avoid dehydration. Heparin was not administered. Body temperature was maintained at 36.5°C to 37.5°C by placing the animals on a heated table and covering them with aluminium foil. After all procedures, a small amount of saline was left in the abdominal cavity, and the peritoneum and skin were separately closed with 5.0 vicryl. Sham-operated mice underwent laparotomy with exposure of the liver but without interruption of hepatic flow.

Blood pressure was measured by placing a 26-gauge catheter in the carotid artery in sham-operated mice and in mice subjected to 45 minutes of ischemia followed by 40 minutes of reperfusion. Mean arterial blood pressure was continuously measured for at least 120 minutes from the onset of anesthesia. Blood perfusion of the left liver lobe during clamping was measured via laser doppler (Oxyflo; Oxford Optronix, Oxford, UK), and tissue pO\(_2\) analyses were performed using a fine needle probe (Oxylite; Oxford Optronix). To demonstrate local oxidative stress tissue levels of glutathione (GSH) and GSH disulfide (GSSG) were measured after 1 and 6 hours of reperfusion. Concurrently, the degree of hepatocellular injury was assessed according to plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Animals in the latter group were allowed to recover after surgery and were reanesthetized for blood withdrawal and liver harvesting following 6 hours of reperfusion.

**Cell Culture and Induction of Liver Micrometastases.** The murine colon carcinoma cell line C26 was cultured in Dulbecco’s modified Eagle medium supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 U/mL) and streptomycin (100 \( \mu \)g/mL) in a 5% carbon dioxide environment. Confluent cultures were harvested by brief trypsinization (0.05 trypsin in 0.02% EDTA) and after centrifugation, single cell suspensions were prepared in phosphate-buffered saline to a final concentration of 5 \( \times \) 10\(^4\) cells/100 \( \mu \)L. Cell viability was determined by trypan blue staining, and was always 98% or more. Colorectal liver metastases were induced in mice as previously described. In brief, through a left lateral flank incision, 5 \( \times \) 10\(^4\) C26 colorectal carcinoma cells were injected into the splenic parenchyma. After 10 minutes, the spleen was removed to prevent intrasplenic tumor growth. Micrometastases were allowed to develop throughout the liver for 5 days. At that time point, animals were subjected to the different I/R protocols as described and morphological assessment of tumor growth, hepatocellular necrosis, and apoptosis was performed on nonischemic and ischemic lobes harvested 4, 5, and 7 days later.

**Preconditioning and Intermittent Clamping Protocol.** Ischemic preconditioning was applied by occluding the blood supply to the left liver lobe for 10 minutes
followed by a 15-minute reperfusion period before 45 minutes of ischemia. In pilot experiments, this preconditioning protocol proved to be the most optimal schedule in preventing elevation of liver enzymes (data not shown), as has also been recognized in other studies.\textsuperscript{20,21} Intermittent clamping was performed by occluding the blood supply to the left liver lobe in three cycles of 15 minutes separated by 5 minutes of reperfusion,\textsuperscript{35} leaving the total time of ischemia unaltered (45 minutes). Tissue levels of GSH and plasma levels of ALT and AST were assessed after 6 hours of reperfusion. The percentage of tissue necrosis, the presence of apoptosis, and tumor load were evaluated 5 days after ischemia.

**Antioxidant Treatment Protocol Using \(\alpha\)-Tocopherol and Ascorbic Acid.** \(\alpha\)-Tocopherol was prepared for intraperitoneal injection by compounding (+)-\(\alpha\)-tocopherol acetate (Sigma-Aldrich Chemie, Schnelldorf, Germany) with ethyl alcohol (20%), benzyl alcohol (1%), and pure vegetable oil (79%). The solvent without \(\alpha\)-tocopherol served as a control vehicle. \(\alpha\)-Tocopherol was administered via intraperitoneal injection at a dose of 300 mg/kg/d for 3 days before surgery and a fourth injection just before I/R or sham operation. L-ascorbic acid sodium salt was dissolved in saline (control vehicle) to a final concentration of 100 mg/kg and was administered by intravenous injection 5 minutes before I/R or sham operation. In pilot experiments, the antioxidative effect of both treatments was confirmed by prevention of a rise in GSSG levels after 1 hour of reperfusion (data not shown). Tissue GSH levels and plasma liver enzymes were analyzed following 6 hours of reperfusion. Late hepatocellular necrosis, apoptosis, and tumor growth were measured 4 days after ischemia.

**GSSG and GSH Assay.** After liver harvesting, pieces of the ischemic and nonischemic lobes were snap frozen and stored at \(-80^\circ\text{C}\). Liver contents of GSSG and GSH were measured in whole tissue extracts as previously described.\textsuperscript{36} Briefly, the liver samples were homogenized in 5-sulfosalicylic acid, diluted, and added to the assay reagent containing 5,5’-dithiobis(2-nitrobenzoic acid) and nicotinamide adenine dinucleotide phosphate (both purchased from ICN Biomedicals BV, Zoetermeer, The Netherlands). For GSSG measurements, 2-vinylpyridine and triethanolamine (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) were added to undiluted extracts to conjugate GSH and neutralize the acidic constitution before adding the assay reagent. The reaction was initiated by adding GSH reductase (Sigma-Aldrich Chemie BV). GSSG and GSH were determined via kinetic measurements of the absorbance change of 5,5’-dithiobis(2-nitrobenzoic acid) at 412 nm by comparing it with known standards. Liver contents of GSSG and GSH were expressed as nanomoles per milligram of protein. The liver protein content was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

**Liver Enzymes.** Heparin plasma samples (500 \(\mu\)L) were obtained via cardiac puncture and were centrifuged at 14,000 rpm for 10 minutes. Plasma levels of ALT and AST served as indicators of liver tissue damage and were analyzed using commercially available diagnostic kits (Instruchemie BV, Delfzijl, The Netherlands).

**Tumor Analysis.** Intrahepatic tumor load was scored as the hepatic replacement area (HRA),\textsuperscript{34} the percentage of hepatic tissue that has been replaced by tumor cells. On two nonsequential hematoxylin-eosin–stained sections per liver lobe, at least 100 fields were selected using an interactive video overlay system, including an automated microscope (Leica-Q-Prodit; Leica Microsystems, Ryswyk, The Netherlands) at a magnification of \(\times40\). Using a four-points grid overlay, the ratio of tumor cells versus normal hepatocytes plus necrotic cells was determined for each field. Tumor load (HRA) was expressed as the average area ratio of all fields. Observers were blinded to treatment. Using this method, we obtained less than 5% interobserver and intraobserver variability. Finally, HRA ratios between ischemic and nonischemic lobes were calculated for each animal to express the proportional increase in HRA in the ischemic (left) lobes versus the nonischemic (right plus median) lobes.

**Quantification of Hepatocellular Necrosis.** The percentage of hepatocellular necrosis was scored simultaneously with tumor HRA analyses on nonsequential hematoxylin-eosin–stained sections. The ratio of necrotic cells versus healthy hepatocytes plus tumor cells was determined for each field. The percentage of hepatocellular necrosis was expressed as the average area ratio of all fields.

**Evaluation of Apoptosis.** Activated caspase 3 was analyzed via immunohistochemistry on tissue sections of clamped and unclamped liver lobes. Non–tumor-bearing sections served as controls. After deparaffinization and rehydration, sections were stained with an anti-active caspase-3 (C92-605, BD Biosciences PharMingen, Alphen aan den Rijn, The Netherlands) antibody followed by rabbit anti-mouse horseradish peroxidase (Pierce) antibody. The reaction was developed using diaminobenzidine/ \(\text{H}_2\text{O}_2\) as a chromogen substrate.

**Statistical Analysis.** Statistical differences between groups were analyzed using the Mann-Whitney \(U\) test for nonparametric data. Data are expressed as the mean \(\pm\) SEM.

**Results**

**Validation of a Standardized Murine Model of Partial Hepatic I/R.** In our standardized model of left
lobar I/R, blood pressure remained stable for at least 120 minutes before and during I/R (Fig. 1A) (n = 3 each group) without overt changes in blood pH, pO2, or pCO2 (data not shown). Local hypoperfusion and hypoxia of the left liver lobe during clamping were confirmed by laser doppler and tissue pO2 measurements. Following application of the clamp, the blood flow in the left lobe was obstructed by 85% to 90% (Fig. 1B), followed by a dramatic drop in pO2 to approximately 1 to 2 mm Hg (Fig. 1C). To demonstrate local oxidative stress, we measured consumption of the endogenous antioxidant GSH and production of its oxidized form, GSSG. Tissue levels of GSSG in the ischemic lobes were elevated after 1 hour of reperfusion but were not significantly different after 6 hours of reperfusion (Fig. 1D) (n = 7 each group). GSH content was not altered after 1 hour of reperfusion but had decreased by 50% after 6 hours of reperfusion in liver tissue isolated from the clamped liver lobes, but not in that from the unclamped liver lobes (Fig. 1E) (n = 7 each group). Plasma ALT and AST levels were markedly increased after 1 and 6 hours of reperfusion (Fig. 1F-G) (n = 7 each group), as shown in similar I/R models. Mortality was not observed in this model of left lobar I/R. In conclusion, this partial I/R model induces hepatic oxidative stress and tissue injury in a background of stable hemodynamic parameters.

**Equal Tumor Loads in All Liver Lobes Provide an Internal Control for Local Effects of I/R.** Metastatic tumor growth was initiated by injecting C26 colorectal carcinoma cells into the splenic parenchyma. Single tumor cells reach the liver through the portal vein, where a subset grows out to form intrahepatic micrometastases. Tumor growth was assessed in control animals by determining the percentage of liver tissue that was replaced by tumor cells (HRA) 12 days after tumor cell injection. The tumor load in the right plus median lobes was similar to that in the left liver lobe (33.3% vs. 32.8%). Consequently, the ratio between the HRA values was approximately 1. Based on this result, we conclude that the right plus median liver lobes may serve as an internal control for tumor growth after selective clamping of the left lobe.
I/R Accelerates the Local Outgrowth of Preestablished Liver Metastases. We next examined how I/R affects the growth rate of preestablished liver micrometastases. After intrasplenic tumor cell injection, micrometastases were allowed to develop throughout the liver for 5 days. Subsequently, the left liver lobes were selectively subjected to 45 minutes of ischemia, followed by reperfusion. Tumor growth was analyzed on hematoxylin-eosin–stained tissue sections. (A) Liver harvested 7 days after partial ischemia clearly shows an increase in macroscopic tumor growth in the clamped lobe (arrow). (B) Time course of tumor growth, expressed as the hepatic replacement area (HRA). In ischemic liver lobes (solid line), HRA was significantly increased compared with nonischemic liver lobes (dotted line) and with the left (dashed line) and right plus median (dashed/dotted line) lobes of sham-operated mice at 4 (n = 5), 5 (n = 6), and 7 (n = 6) days after I/R. (C) Time course of the proportional increase in HRA in the ischemic (left) lobes versus the nonischemic (right plus median) lobes, expressed as HRA ratio. Tumor growth was stimulated over four- to fivefold on the fourth and fifth day after ischemia (white bars represent sham operation; black bars represent I/R). *P < .05; **P < .01. I/R, ischemia/reperfusion; HRA, hepatic replacement area.

Fig. 2. Accelerated outgrowth of established liver metastases following I/R. Five days after the induction of liver metastases, the left liver lobe was subjected to 45 minutes of ischemia, followed by reperfusion. The livers were harvested 4, 5, and 7 days after ischemia, and tumor growth was analyzed on hematoxylin-eosin–stained tissue sections. (A) Liver harvested 7 days after partial ischemia clearly shows an increase in macroscopic tumor growth in the clamped lobe (arrow). (B) Time course of tumor growth, expressed as the hepatic replacement area (HRA). In ischemic liver lobes (solid line), HRA was significantly increased compared with nonischemic liver lobes (dotted line) and with the left (dashed line) and right plus median (dashed/dotted line) lobes of sham-operated mice at 4 (n = 5), 5 (n = 6), and 7 (n = 6) days after I/R. (C) Time course of the proportional increase in HRA in the ischemic (left) lobes versus the nonischemic (right plus median) lobes, expressed as HRA ratio. Tumor growth was stimulated over four- to fivefold on the fourth and fifth day after ischemia (white bars represent sham operation; black bars represent I/R). *P < .05; **P < .01. I/R, ischemia/reperfusion; HRA, hepatic replacement area.
within metastases in nonischemic liver lobes occurs very infrequently (<1% of cells). In perinecrotic tumor tissue, however, caspase-3–positive cells were more frequently detected. Because apoptotic hepatocytes surround necrotic areas in tumor-bearing as well as non–tumor-bearing livers (see Fig. 3A), the caspase-3–positive cells in perinecrotic tumor tissue most likely represent apoptotic hepatocytes rather than apoptotic tumor cells. These observations suggest that tumor cells preferably grow into the zones surrounding necrotic tissue areas that are characterized by infiltrating lymphocytes and apoptotic hepatocytes.

Tissue necrosis, lymphocyte infiltration, and apoptosis were only sporadically observed in the non-clamped lobes or in the liver lobes from sham-operated animals, involving less than 1% of the liver tissue. At day 5 and day 7 after I/R, the area of tissue necrosis was reduced to 17.7 ± 4.4% (n = 6) and 10.6 ± 5.4% (n = 6), respectively, of the total tissue area. This reduction was attributed to invasion of tumor cells, overgrowing the necrotic areas (Fig. 3B).

**Intermittent Clamping But Not Ischemic Preconditioning Protects the Liver Against Accelerated Tumor Growth.** Because I/R-accelerated tumor growth is associated with liver tissue necrosis, we examined whether alternative clamping methods that protect against hepatocellular damage would cross-protect the liver against accelerated tumor growth. The application of a short period of ischemia before prolonged clamping, called ischemic preconditioning, can render liver tissue less vulnerable to a sustained ischemic insult by triggering hepatocellular defense mechanisms.8,22 In addition, several clinical and experimental studies have demonstrated that intermittent clamping reduces hepatocellular injury.24-26 We measured the effects of both clamping methods on GSH tissue levels, on early and late hepatic damage, and on tumor growth. Both pre-
conditioning and intermittent clamping prevented the depletion of GSH (Fig. 4A; n = 6 each group). In addition, both clamping methods largely prevented early hepatocellular damage (by 85% and 93%, respectively) as judged by plasma ALT and AST levels (Fig. 4B-C; n = 6 each group). Late hepatocellular damage (i.e., liver tissue necrosis) was quantified via morphometric analysis of hematoxylin-eosin-stained tissue sections (Fig. 4D). Tissue necrosis was modestly (twofold) reduced by ischemic preconditioning 5 days after ischemia but was virtually abolished by intermittent clamping (Fig. 4D; n = 8 each group). Tumor growth was quantified via morphometric analysis of the hepatic replacement areas 5 days after ischemia (Fig. 4E-F; n = 8 each group). Ischemic preconditioning and intermittent clamping both (A) prevented the reduction in GSH tissue levels (white bars represent nonischemic lobes; black bars represent ischemic lobes) and (B,C) reduced early hepatocellular damage, as assessed by plasma ALT and AST levels following 6 hours of reperfusion (n = 6 each group). Late hepatocellular damage (i.e., liver tissue necrosis) was quantified via morphometric analysis of hematoxylin-eosin-stained tissue sections. (D) Tissue necrosis was modestly (twofold) reduced by ischemic preconditioning 5 days after ischemia but was virtually abolished by intermittent clamping (white bars represent nonischemic lobes; black bars represent ischemic lobes) (n = 8 each group). (E) Tumor growth was quantified via morphometric analysis of the hepatic replacement areas 5 days after ischemia. Intermittent clamping, but not ischemic preconditioning, prevented the stimulatory effect of I/R on tumor growth. (F) Hematoxylin-eosin-stained sections of nonischemic and ischemic liver lobes of each group (original magnification ×2). *P < .05; **P < .01. GSH, glutathione; IPC, ischemic preconditioning; INT, intermittent clamping; I/R, ischemia/reperfusion; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HRA, hepatic replacement area; t, C26 tumor cells; n, necrosis.

These results suggest that the extent of late local tissue necrosis with associated inflammatory cells and apoptotic hepatocytes, rather than initial hepatocellular damage, is correlated with I/R-induced acceleration of tumor growth.

α-Tocopherol and Ascorbic Acid Fail to Protect Against I/R-Stimulated Tumor Growth. Oxygen radicals are generated predominantly during the early phases of reperfusion and contribute to early hepatocellular cell damage. α-Tocopherol is the most effective lipid-solu-
ble antioxidant in biological systems and has been proven effective in reducing oxidative stress and hepatocellular damage in both clinical and preclinical trials in doses from 10 to 300 mg/kg. Ascorbic acid is a water-soluble antioxidant known to reduce oxidative stress with maximal hepatoprotective effects at a dose of 100 mg/kg. Therefore, we examined the effects of \( \alpha \)-tocopherol (300 mg/kg) and ascorbic acid (100 mg/kg) on GSH levels, early hepatocellular damage, late tissue necrosis, apoptosis, and accelerated tumor growth. Both \( \alpha \)-tocopherol and ascorbic acid pretreatment reduced consumption of GSH (Figs. 5A, 6A) (\( n = 6 \) each group) and prevented early hepatocellular damage by 50%, as indicated by plasma ALT and AST levels (6 h post-I/R) (\( n = 6 \) each group). Tissue necrosis and tumor growth were measured 4 days after ischemia. \( \alpha \)-tocopherol had no discernable effect on I/R-induced tissue necrosis and failed to reduce the stimulatory effect of I/R on intrahepatic tumor growth. Hematoxylin-eosin-stained sections of nonischemic and ischemic liver lobes of each group (original magnification \( \times 2 \)). *\( P < .05 \), **\( P < .01 \). GSH, glutathione; I/R, ischemia/reperfusion; \( \alpha \)-T, \( \alpha \)-tocopherol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HRA, hepatic replacement area; t, C26 tumor cells; n, necrosis.

**Discussion**

The outgrowth of preexisting micrometastases may be accelerated through various mechanisms. First, the outgrowth of distant micrometastases may be promoted by...
removal of the primary tumor, because the production of anti-angiogenic factors by the primary tumor suppresses distant tumor growth.39 Second, dormant tumor cells may be stimulated by pro-angiogenic factors that are released during surgery-induced tissue injury and hypoxia.40-42 Third, partial hepatectomy may augment the growth of micrometastases by paracrine proliferative stimuli.33,43 In the present study, we show that local I/R induced by vascular clamping is an additional and strong stimulus that promotes the outgrowth of micrometastases in the liver.

Our finding that tumor growth is stimulated in clamped but not nonclamped lobes suggests that the mechanism operates locally and does not involve systemically released factors. Local responses to I/R are complex7,8 but can be divided into two distinct phases. Acute hepatocellular injury is caused by reactive oxygen species and cytokines released by Kupffer and endothelial cells,38,44 and is reflected by a rise in plasma liver enzymes. The late phase is characterized by neutrophil infiltration causing further damage to the parenchyma, mainly through a protease-dependent pathway.17,45 Moreover, endothelial cell swelling, a local imbalance in vasoconstrictors and vasodilators, and activation of the coagulation system, together with the influx of neutrophils, will lead to microcirculatory disturbances, a phenomenon known as no-reflow.46 This no-reflow further aggravates the damage and is reflected by microscopic tissue necrosis. Thus, distinct mechanisms underlie I/R-induced early liver tissue damage and late tissue necrosis. I/R-accelerated tumor growth was shown to be associated with late tissue necrosis but not early hepatocellular injury. These results are based on our finding that ischemic preconditioning, α-tocopherol, and ascorbic acid reduced the I/R-stimulated consumption of GSH as well as the rise in liver enzyme levels but could not prevent late tissue necrosis nor I/R-stimulated tumor growth. Intermittent clamping prevented both early and late hepatocellular damage and I/R-accelerated tumor growth, confirming a relationship with late events in I/R-induced injury when necrotic tissue areas are apparent. Neutrophils that infiltrate the parenchyma during the late phase may contrib-
ute to tumor growth by producing proliferation- and angiogenesis-stimulating factors and cytokines. Moreover, microcirculatory disturbances following reperfusion will prolong intrahepatic hypoxia. Prolonged exposure of micrometastases in the liver to hypoxia could lead to the activation of several distinct pathways that contribute to I/R-accelerated tumor growth. These include pathways that stimulate tumor cell proliferation and tumor angiogenesis.\(^6\)\(^7\)\(^8\) Intermittent clamping appears to be superior to ischemic preconditioning in maintaining hepatic microcirculation, especially after prolonged periods of ischemia.\(^9\) This outcome might explain the remarkable difference between ischemic preconditioning and intermittent clamping in preventing hepatocellular necrosis\(^10\) and accelerated outgrowth of micrometastases (present study). Finally, a change in the structure of the liver parenchyma may facilitate the outgrowth of invading tumor cells. Infiltrating lymphocytes may induce apoptosis in surrounding hepatocytes through stimulation of Fas, which may facilitate tumor outgrowth as a result of an altered tissue structure.\(^8\)\(^7\) Our observation that the areas of enhanced tumor outgrowth were closely associated with zones of inflammatory cells and apoptotic hepatocytes surrounding the necrotic tissue areas supports this hypothesis. Obviously, the above-mentioned possibilities are not mutually exclusive, and the mechanism of I/R-accelerated tumor growth is likely to be multifactorial. Finally, we have also found increased tumor outgrowth of preestablished CC531 colon carcinoma micrometastases following partial hepatic I/R in Wag/Rij rats (data not shown). Therefore, I/R-stimulated tumor growth in the liver is not restricted to the C26 cell line and presumably represents a general phenomenon.

In conclusion, our results identify hepatic ischemia and reperfusion as a result of vascular clamping as a major cause of accelerated tumor growth in a standardized mouse model of colorectal liver metastases. Clamping-induced acceleration of tumor growth may be prevented by simply interrupting blood flow intermittently, without losing the benefit of decreasing blood loss.\(^6\) We found that intermittent clamping was superior to ischemic preconditioning, both with respect to preventing late tissue necrosis and preventing accelerated tumor growth. Therefore, we consider intermittent clamping as the clamping method of choice. Finally, these results may also be relevant for other circumstances that are associated with intrahepatic I/R, such as low-flow states following hemorrhagic or circulatory shock and resuscitation.\(^4\) In those cases, anti-inflammatory agents or pharmacological interventions that preserve microcirculation might protect against accelerated tumor growth.

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**References**

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