

Warm Hepatic Ischemia-Reperfusion Promotes Growth of Colorectal Carcinoma Micrometastases in Mouse Liver via Matrix Metalloproteinase-9 Induction

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Abstract

Surgical resection remains the best treatment for colorectal metastases isolated to the liver; however, 5-year survival rates following liver resection are only 40% to 50%, with liver recurrence being a significant reason for treatment failure. The ischemia-reperfusion (I/R) injury incurred during liver surgery can lead to cellular dysfunction and elevations in proinflammatory cytokines and matrix metalloproteinases (MMP). In rodents, I/R injury to the liver has been shown to accelerate the outgrowth of implanted tumors. The mechanism for increased tumor growth in the setting of liver I/R injury is unknown. To investigate the effect of I/R on tumor growth, an experimental model was used whereby small hepatic metastases form after 28 days. Mice subjected to 30 min of 70% liver ischemia at the time of tumor inoculation had significantly larger tumor number and volume, and had elevated MMP9 serum and liver tissue MMP9 as evidenced by zymography and quantitative real-time PCR. Mice treated with doxycycline, a broad-spectrum MMP inhibitor, had reduced MMP9 levels and significantly smaller tumor number and volume in the liver. MMP9-null mice were used to determine if the effects of doxycycline were due to the absence of stromal-derived MMP9. The MMP9-null mice, with or without doxycycline treatment, had reduced tumor number and volume that was equivalent to wild-type mice treated with doxycycline. These findings indicate that hepatic I/R-induced elevations in MMP9 contribute to the growth of metastatic colorectal carcinoma in the liver and that postresection MMP9 inhibition may be clinically beneficial in preventing recurrence following hepatic surgery. [Cancer Res 2007;67(6):2720–8]

Introduction

Novel chemotherapeutic regimens are effective in limiting disease progression in colorectal carcinoma, but cure with chemotherapy alone is rare in stage IV disease. Currently, liver resection is the only treatment modality that offers a significant chance for cure in colorectal carcinoma metastatic to the liver (1–3). Unfortunately, of the 15% to 30% of patients amenable to curative liver resection, only 40% to 50% will survive 5 years (2). This low rate of success is most often the result of recurrent malignant disease within the liver.

Although the high rate of recurrence within the liver may be a result of residual or dormant malignant cells, there may be a link between the conduct of the operation and recurrence in the liver as recent data suggest that hepatic resection itself increases the chances of liver recurrence (4, 5). The two lines of evidence linking the surgical procedure to recurrence are as follows: (a) surgical manipulation of the liver is known to also result in the dissemination of colon cancer cells into the peripheral blood (6–8); these circulating cells, or residual micrometastases not detected at the time of resection, may be source of the recurrent disease; and (b) following partial hepatectomy, there are elevated levels of cytokines, growth factors, and adhesion molecules following surgery, all of which are known to promote metastases and tumor growth (1, 3, 9).

Another important association between liver resection and postoperative outcome is blood loss incurred during the operation. Increased blood loss is associated with increased postoperative complications and a higher incidence of hepatic recurrence (10, 11); thus, surgeons have learned to limit the blood loss during the course of the operation by occluding the inflow of blood to the liver during the parenchymal transection (12). Although critical to minimizing blood loss, extended periods of ischemia and reperfusion (I/R) are detrimental to cellular function in the postsurgical remnant liver. I/R causes cellular damage by inducing free-radical formation, up-regulating inflammatory cytokines (13–15), dysregulating mitochondrial calcium handling (16–18), and up-regulating matrix metalloproteinases (MMP; refs. 19, 20). In particular, hepatic expression of MMP2 and MMP9 has been linked to hepatic I/R injury through recruitment of neutrophils and T cells (21). Sustained ischemia and reperfusion in the liver has been shown to accelerate growth of established hepatic metastases (4), and minimization of I/R injury through intermittent ischemia can attenuate metastasis of colorectal cancer to the liver following surgical stress (22).

Although vascular inflow occlusion is an important adjunct to safe surgery and has translated into lower postoperative morbidity, it may have negative implications for recurrent disease as I/R associated with liver resection may be a mechanism governing postoperative hepatic recurrence. We therefore hypothesized that in a model of circulating colorectal carcinoma, hepatic I/R would enhance the formation of hepatic metastases through up-regulation of MMP9 in the liver and that specific inhibition of I/R injury or MMP9 would attenuate tumor formation. We show that mice subjected to hepatic ischemia at the time of tumor inoculation have significantly larger tumor number and volume, which is associated with elevated serum and liver tissue MMP9. Mice treated with doxycycline, a broad-spectrum MMP inhibitor, have decreased I/R-induced MMP9 as evidenced by zymography

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and by quantitative real-time PCR that correlates with decreased tumor number and volume. MMP9-null mice, irrespective of doxycycline treatment, have tumor number and volume equivalent to wild-type (WT) mice treated with doxycycline. These findings indicate that hepatic I/R-induced elevations in MMP9 contribute to the growth of micrometastatic colorectal carcinoma in the liver.

Materials and Methods

Cell culture. The MC38 cell line is an adenocarcinoma of tumor grade 3 that was derived in C57BL/6 mice (23). Luciferase-expressing MC38 cells (MC38/Luc) were created by transfection with pEF-LUC-Neo plasmid DNA (0.4 $\mu\text{g}/\mu\text{L}$ stock) using the Fugene 6 reagent (Roche, Nutley, NJ) and selection with G418 (250 $\mu\text{g}/\text{mL}$) in the laboratory of Dr. Robert Coffey (Vanderbilt University Medical Center, Nashville, TN). MC38/Luc cells were

Table 1. Description of experiment groups

Experiment	Study rationale	Group	Group name	Procedure	<i>n</i>
1	To establish the effect of I/R on liver tumor growth in WT mice	1	Tumor control	Tumor injection with splenectomy	10
		2	Tumor I/R group	Tumor injection with 60 min 70% hepatic ischemia, 15 min reperfusion, and splenectomy	10
		2a	Group 2—shunted portion of liver	Within-group control: nonischemic (shunted) portion of liver	10
2	To establish the effect of I/R on serum and liver tissue MMP9 in WT mice	1	Sham	Laparotomy, liver manipulation, and closure	3
		2	30m Isc	Samples collected immediately after 30 min of 70% hepatic ischemia, while lobes were occluded	3
		3	15m Rep	Samples collected after 30 min ischemia and 15 min reperfusion	3
		4	3h Rep	Samples collected after 30 min ischemia and 3 h reperfusion	3
		5	24h Rep	Samples collected after 30 min ischemia and 24 h reperfusion	3
		6	3h Hep	Positive control for MMP9 expression, regenerating liver tissue collected 3 h after hepatectomy	3
3a	To determine the effect of doxycycline treatment in WT mice on liver tissue MMP	1	Treated	40 mg/kg doxycycline: 30 min of 70% hepatic ischemia, 15 min reperfusion, and 7 d of doxycycline	6
		2	Untreated	30 min of 70% hepatic ischemia, 15-min reperfusion, no doxycycline	6
3b	To determine the effect of doxycycline treatment on liver tumor growth in WT mice	1	Untreated	30 min of 70% hepatic ischemia, 15 min reperfusion and no doxycycline	10
		2	Early	30 min of 70% hepatic ischemia, 15 min reperfusion, and 40 mg/kg doxycycline daily for the first 14 of 28 postoperative days	10
		3	Late	30 min of 70% hepatic ischemia, 15 min reperfusion, and 40 mg/kg doxycycline daily postoperative days 14 to 28	10
		4	Continuous	30 min of 70% hepatic ischemia, 15 min reperfusion, and 40 mg/kg doxycycline daily after surgery for 28 d	10
4	To evaluate the contribution of stromal MMP9 vs MC38/Luc contributed MMP9 and inferring the specificity of doxycycline	1	Tumor Control	30 min of 70% hepatic ischemia, 15 min reperfusion in WT mice	4
		2	Doxycycline Control	30 min of 70% hepatic ischemia, 15 min reperfusion with continuous doxycycline 40 mg/kg for 28 d after surgery in WT mice	4
		3	Tumor Null	30 min of 70% hepatic ischemia, 15 min reperfusion in MMP9-null mice	4
		4	Doxy Null	30 min of 70% hepatic ischemia, 15 min reperfusion with continuous doxycycline 40 mg/kg for 28 d after surgery in MMP9-null mice	4

maintained in culture in DMEM (Cellgro Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (NovaTech, Grand Island, NY), 1% penicillin-streptomycin, 1% nonessential amino acids, and 0.5% neomycin (G418; Cellgro Mediatech) at 37°C and 5% CO₂.

Animal models. All experiments were done in accordance with NIH Guide for the Care and Use of Laboratory Animals, approved by and carried out under the guidelines of the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center (Nashville, TN). All animals were housed in a climate-controlled institutional animal facility on a 12:12 h light/dark cycle where food and water was provided *ad libitum*.

Experimental metastasis model. Male MMP9-null mice (24) were backcrossed into a C57BL/6 background (25). Ten-week old male C57BL/6j mice (The Jackson Laboratory, Bar Harbor, ME) were used as WT controls and for all other studies. Under isoflurane anesthesia, mice underwent a 1-cm midline laparotomy and medial retraction of the anterior spleen. Fifty-thousand MC38/Luc cells were injected into the pulp of the anterior pole of the spleen using a 27 ga. needle in a total volume of 0.1 mL sterile HBS. Successful injection was confirmed by splenic blush and visualization of injection flow through the anterior splenic vein. Tumor cells were allowed to circulate for 10 min. Mice not receiving I/R then underwent an additional 15 min of circulation followed by splenectomy and closure. Those mice that received I/R were subjected to 30 min of 70% hepatic ischemia with 15 min reperfusion before splenectomy. Splenectomy was done to prevent the formation of splenic tumors, such that metastatic lesions developed in the liver only.

Rodent model of hepatic I/R. Hepatic I/R was done by placement of an atraumatic vascular clamp (S&T Microclamp B-2; Fine Science Tools, Foster City, CA) across the portal vein, hepatic artery, and bile duct branches to the left and median liver lobes, rendering ~70% of the liver ischemic (26). This method has been previously reported (27–29) and prevents mesenteric congestion by allowing portal venous shunting through the right lobe and caudate lobe; these latter lobes are called the “shunted” liver. Sham-operated animals underwent laparotomy, mild liver manipulation, and equivalent anesthesia. Surgical procedures were done under aseptic conditions, and warm sterile moistened gauze was placed over the laparotomy to avoid dehydration. Body temperature was maintained at 36.5°C to 37.5°C by placing the animals on a heated table and covering them with sterile towels. The abdominal incision was closed in two layers with 6-0 PDS II (Ethicon, Inc., Cincinnati, OH). Prewarmed saline (0.5 mL) was introduced *i.p.* before closure. Buprenorphine hydrochloride (0.05 mg/kg, Buprenex Injectable, Reckitt and Colman Pharm., Inc., Richmond, VA) was used for postoperative analgesia. Animals were monitored for tumor growth every 7 days for 28 days using bioluminescent imaging (described below), after which time they were sacrificed for sample collection and quantitation of tumor burden. Individual tumors were counted and each tumor was measured in three perpendicular dimensions to estimate tumor volume based on the standard equation for volume of an ellipse: $4/3\pi \times (\text{width axis radius}) \times (\text{length axis radius}) \times (\text{height axis radius})$.

Doxycycline treatment. Doxycycline (40 mg/kg) was administered daily in drinking water as described by Pozzi et al. (30). Doxycycline has modest efficacy as a broad-spectrum inhibitor of MMP activity and reduces MMP9 levels without affecting MMP2 (30, 31).

Experimental groups. All experimental groups are outlined in Table 1. To evaluate the effects of I/R on MMP9 and metastatic tumor growth, we did three lines of experimentation in WT C57BL/6 mice and a fourth line of experimentation in MMP9-null mice. The first experiment in WT mice was designed to evaluate whether I/R increased tumor growth in our model of circulating micrometastases. To establish baseline tumor burden, seven mice received tumor injection and splenectomy only (tumor control group). Another seven mice received tumor injection followed by 30 min of 70% hepatic ischemia, 15 min of reperfusion, and splenectomy (tumor I/R group). In addition, within the I/R-treated group, the nonischemic (shunted) portion of the liver was also examined as an internal control for the effects of I/R.

The second experiment in WT mice was designed to examine the effect of I/R on serum and liver tissue MMP9 levels. Specimens were collected from three mice in each of the following groups: (a) laparotomy,

liver manipulation, and closure (sham) to serve as a negative control; (b) immediately after 30 min of 70% hepatic ischemia while occluded; (c–e) following 30 min ischemia and either 15 min, 3 h, or 24 h reperfusion; (f) regenerating liver, 3 h after hepatectomy served as a positive control because expression and activation of MMP9 is induced within 3 to 6 h following hepatectomy (20, 32).

The third experiment in WT mice was designed to determine the effects of doxycycline treatment on serum and liver tissue MMP9 levels following I/R. Twelve mice were subjected to 30 min of hepatic ischemia, and after closure, half ($n = 6$) received doxycycline for 7 days (40 mg/kg doxycycline) and the other half received normal water (untreated). Serum and liver tissue was then collected for zymography and real-time PCR. Serum and liver tissue from sham mice from experiment 2 above were used to establish baseline MMP9 levels (sham). Cytotoxicity of doxycycline on MC38/Luc cells in culture was assessed using the Cell Titer Blue viability assay (Promega, Madison, WI).

To determine the effect of doxycycline treatment on tumor growth, four groups of 10 mice were studied. All mice received tumor injection and I/R according to the method outlined above. Group 1 received no doxycycline (untreated); group 2 was treated from the day of surgery through day 14 (early); group 3 began treatment on postoperative day 14 and were treated an additional 14 days (late); and group 4 was treated from the day of surgery throughout the 28-day follow-up (continuous).

Finally, to evaluate whether decreased tumor growth using doxycycline was a result of MMP9 inhibition, we studied 16 MMP9-null mice in experiment 4. An equal number of WT C57BL/6 mice were used as controls. All mice received tumor injection according to the method above. Eight mice of each genotype were also subjected to I/R as previously described, after which, half received continuous doxycycline, whereas the other half were untreated. This resulted in four mice for each of the following four conditions: (a) tumor injection only plus doxycycline; (b) tumor injection only without doxycycline; (c) tumor injection with I/R plus doxycycline; and (d) tumor injection with I/R and no doxycycline.

Bioluminescent imaging. Nonlethal monitoring of tumor burden was accomplished by detecting luciferase-expressing MC38/Luc cells at 7-day intervals after intrasplenic injection in mice, as previously described (25). Briefly, beetle luciferin (Promega) was injected *i.p.* at a concentration of 150 mg/kg in 0.1 mL PBS 15 min before imaging. Bioluminescent image acquisition was done using a CCD camera (IVIS system, Xenogen, Alameda, CA) with 1 min exposure time and medium binning. Contoured regions of interest were selected automatically by the system software (Living Image v2.50.1, Xenogen) and quantified as total photon counts.

Serum and tissue MMP9 gelatin zymography. Serum and liver tissue MMP9 gelatin zymography was done with modifications of previously described techniques (31, 33). Under isoflurane anesthesia, blood was collected at the time of sacrifice by cardiac puncture using a 1 mL syringe and a 26 ga. needle. Approximately 0.7 mL of whole blood was collected, allowed to clot at room temperature for 30 min, and centrifuged at $1,500 \times g$ for 15 min. Serum was carefully extracted and transferred to cryotubes. Tissue specimens were snap frozen under liquid nitrogen. Pieces of frozen tissues (1 mm³) were ground under liquid nitrogen in a ceramic mortar and pestle. Pulverized tissue was transferred to sample tubes and resuspended in 250 μ L of simple lysis buffer [2:1 ratio of 10% SDS to 1 mol/L Tris (pH 7.6)]. Serum and tissue protein concentration was measured using the DC Lowry Assay (Bio-Rad, Hercules, CA). Zymography was done by electrophoresis of 10 μ g total serum or tissue protein on 1-mm-thick 1% gelatin, 10% acrylamide gels at 200 V for 60 min at 4°C under nonreducing conditions. Mouse recombinant MMP9 (R&D Systems, Inc., Minneapolis, MN) was loaded as a positive control. Gels were transferred to renaturation buffer [50 mmol/L Tris-HCl (pH 7.5), 0.1 mol/L NaCl, 2.5% Triton X-100] for 1 h with one exchange of solution after 30 min. After rinsing with water, gels were placed in developing buffer [50 mmol/L Tris-HCl (pH 7.5), 10 mmol/L CaCl₂, 0.02% NaN₃] with 1:250 mammalian tissue protease inhibitor cocktail without EDTA (2.49% AEBF [4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride], 0.05% aprotinin, 0.12% bestatin hydrochloride, 0.09% E-64 [*N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide], 0.1% leupeptin hemisulfate, and 0.1%

pepstatin A; Sigma, St. Louis, MO) and incubated at 37°C overnight. To be certain we were visualizing MMPs, initial gels were run in duplicate and 0.5 μmol/L EDTA was added to the developing solution of one of the duplicate gels. Chelation of metal ions by EDTA inhibited the gelatinolytic activity of electrophoresed proteins on that gel; however, digested bands appeared on the other gel. MMP9 and MMP2 bands were visualized using standard Coomassie blue staining.

Quantitative real-time PCR. RNA was prepared from snap-frozen liver tissue harvested from both the right lobe and caudate lobe for each animal using RNeasy Lysis Buffer (Qiagen, Crawfordsville, IN). Briefly, tissue was homogenized in RNeasy Lysis Buffer, precipitated using chloroform and isopropanol, washed and dried with ethanol, and resuspended in DEPC water. Primers for MMP9 were designed based on the full-length mouse MMP9 mRNA sequence (Genbank accession number NM_013599) and were selected after ruling out highly folded regions as indicated using the mFold software service.³ Forward 5'-CAATCCTTGAATGTGGATG-3' and reverse 5'-TAAG-GAAGGGCCCTGTAAT-3' primers yield an amplicon of 128 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were assessed using the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions to establish baseline mRNA content. A standard curve was generated from cDNA from regenerating mouse liver 6 h after hepatectomy. Real-time PCR reactions were carried out using the Bio-Rad iCycler and iQ Supermix buffer containing DNA polymerase and SYBR Green (Bio-Rad Laboratories) as described elsewhere (34). Cycling conditions were as follows: 95°C for 10 min and then 45 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 15 s. Data were analyzed using Bio-Rad iCycler PCR detection and analysis software version 3.0 (Bio-Rad Laboratories). DNA was quantitated using the standard curve method with the background subtracted. A melting curve was determined for each sample to detect primer dimers. Results are expressed as values for MMP9 cDNA divided by those for GAPDH cDNA. MMP9 values were standardized by taking the ratio of MMP9/GAPDH for each sample.

Statistical analysis. Data analysis was carried out using SPSS version 13.0 (SPSS, Inc., Chicago, IL). All values are presented as mean or mean difference ±SE unless otherwise noted. The mean difference between groups was analyzed using a one-way ANOVA with the least significant difference procedure for pairwise comparisons with $P < 0.05$ considered significant. Pairwise comparisons were considered valid only when the overall model P value was ≤ 0.05 , indicating significant difference between at least two groups.

Results

Warm hepatic I/R increases tumor in a model of hepatic micrometastases. We sought to mimic the surgical setting in which, at the time of surgical resection, there are disseminated tumor cells in the circulation that may form the basis of recurrent cancer (6–8, 35); therefore, a low inoculum of tumor cells was necessary. To determine an appropriate inoculum, we first injected mice with MC38/Luc cells at concentrations ranging from 1×10^3 to 1×10^6 cells to determine the concentration that would produce tumors in approximately two thirds of animals after 4 weeks (data not shown). Based on the results of these studies, intrasplenic injection of 5×10^4 viable cells and circulation for 10 min before I/R was selected for our model. In those mice subjected to 30 min of 70% hepatic ischemia and 15 min reperfusion, there is a 5-fold increase in average tumor number and 10-fold increase in total tumor volume when compared with mice receiving tumor injection and splenectomy alone (Fig. 1A and B). Additionally, within each I/R mouse, the shunted (nonischemic) lobe of the liver served as an internal control. We also observed significantly more tumor in the

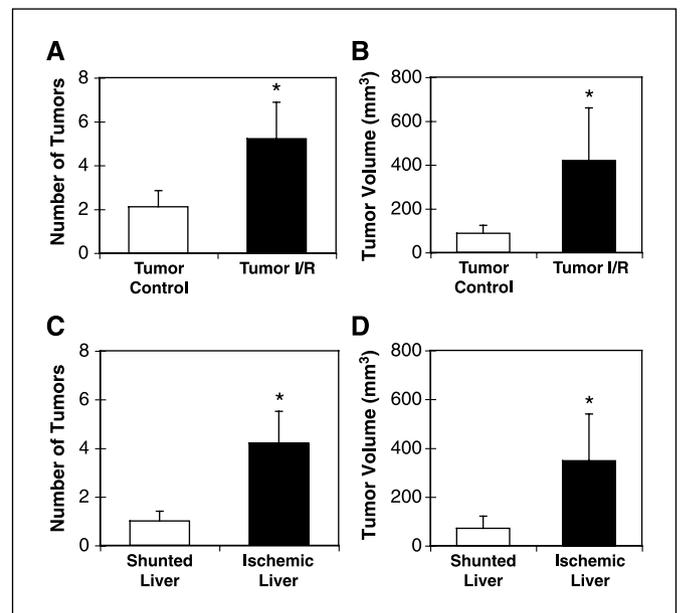


Figure 1. I/R-induced tumor growth. Twenty-eight days after surgery, animals were sacrificed and the number and volume of tumor was assessed. The number (A) and volume (B) of tumors in mice subjected to tumor injection with splenectomy (*Tumor Control*) is less than in those animals that were subjected to 30 min hepatic ischemia with reperfusion (*Tumor I/R*). Within the I/R group, the number (C) and volume (D) of tumor in shunted (right and caudate) lobes was less compared with ischemic (median and left) lobes ($*P < 0.05$).

ischemic lobes (median and left lobes) compared with shunted lobes (right and caudate; Fig. 1C and D).

Bioluminescent imaging weakly correlated with gross findings at necropsy. Comparison of photon count within regions of interest to liver weight, tumor number, or tumor volume on postoperative day 28 resulted in correlation coefficients ranging from $R^2 = 0.1778$ to 0.6023 (data not shown). Although graphical representation of photon count over time seem similar between tumor control and tumor I/R groups, more rapid elevations in photon count were observed in tumor I/R mice. Additionally, within the tumor control group, three mice (nos. 2, 412, and 414) had no tumor at necropsy, whereas all tumor I/R mice had tumor that significantly replaced the hepatic parenchyma without i.p. metastases. In a few circumstances, tumors had formed adhesions with the mesentery, bowel, or abdomen at the incision. Tumor control mice without I/R developed hepatic lesions in only 71% of cases, and none had the extensive degree of parenchymal replacement (Fig. 2).

I/R increases MMP9 mRNA and protein expression. Liver tissue samples collected from I/R and sham-operated mice 7 days after surgery were analyzed using quantitative real-time PCR and revealed a significant elevation ($P = 0.031$) in MMP9/GAPDH mRNA following warm hepatic I/R (Fig. 3A).

To further validate this observation, gelatin zymography and real-time PCR were done on tissue harvested from mouse livers during ischemia or after early reperfusion time points as outlined in experimental groups. Zymography on 10 μg of liver tissue protein showed an increase in MMP9 that was dependent on postreperfusion time. Sham-operated liver tissue and ischemic liver tissue harvested before reperfusion showed no MMP9; however, reperfusion for 15 or 30 min resulted in observable MMP9 in the liver that increased significantly after 24 h of reperfusion. Regenerating liver tissue harvested 3 h after hepatectomy was

³ <http://molbio.info.nih.gov/molbio-nih/mfold.html>

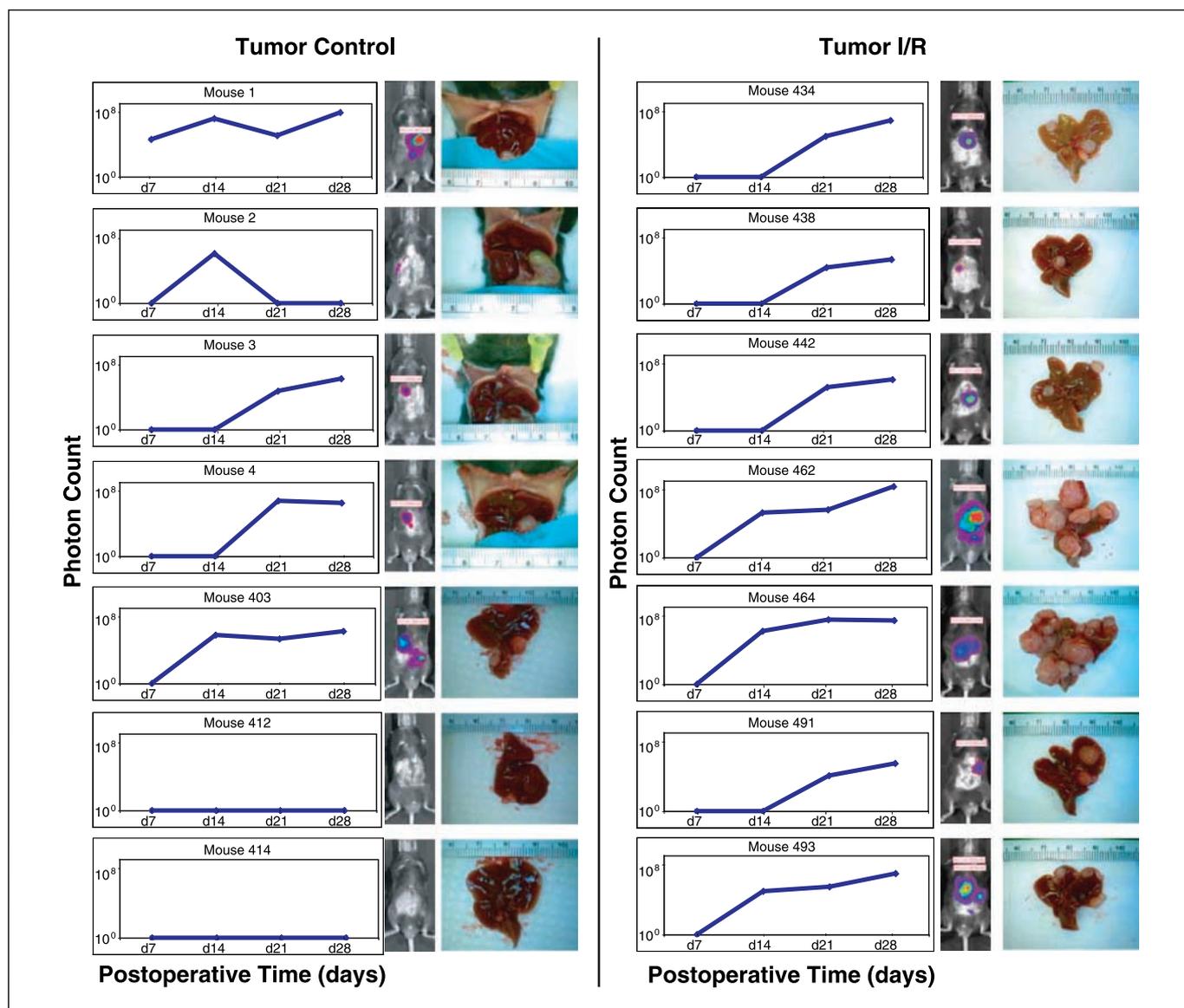


Figure 2. Nonlethal tumor tracking. Animals were monitored by bioluminescent imaging every 7 d to track tumor formation. Individual graphs of photon counts over time are displayed for tumor control mice (*left column*), of which 71% (five of seven) had tumor. Tumor I/R mice (*right column*) all developed tumor. To the right of each line graph, the bioluminescent image of individual mice and a photograph of the liver at sacrifice on postoperative day 28 are presented. Gross histologic appearance of tumor control livers at day 28 show limited tumor compared with tumor I/R livers, which consistently had significant parenchymal replacement; photon count correlated poorly with tumor burden at day 28.

used as a positive control for MMP9 expression based on data presented by Kim et al. (32) that show rapid induction in expression and activity of MMP9 between 3 and 6 h posthepatectomy. By zymography, MMP9 levels were similarly elevated in the 24 h reperfusion and the 3 h posthepatectomy samples (Fig. 3B). Figure 3C represents the average mRNA content of tissue samples used for the zymography in Fig. 3B assessed quantitatively by real-time PCR. MMP9 mRNA was highest in liver tissue subjected to 30 min ischemia and 24-h reperfusion ($P = 0.046$ compared with all other conditions). The representative zymogram in Fig. 3B indicated similar MMP9 levels in 24 h reperfusion and the 3 h posthepatectomy samples, when mRNA was evaluated and the levels for all samples were averaged, only the 24 h reperfusion condition was statistically higher than any other group when analyzed by ANOVA. Although the mRNA levels do not directly

correlate with the protein expression as presented, it is possible that early postreperfusion MMP9 mRNA levels are actually lower than in the posthepatectomy samples had more than three samples in each group been analyzed to reduce the SE.

Doxycycline treatment inhibits I/R-induced MMP9 and decreases hepatic metastases. After observing the increase in hepatic metastases after I/R associated with elevated MMP9, we investigated whether doxycycline could prevent hepatic metastases. This tetracycline derivative has been shown to reduce plasma MMP9 protein without affecting MMP2 levels (30).

A potential confounding factor regarding the effect of doxycycline treatment on tumor formation could be a direct cytotoxic effect of doxycycline on MC38/Luc cells. To rule out this possibility, we did viability assays on MC38/Luc cells seeded on 96-well plates. Doxycycline in complete DMEM was added at 0.01,

0.1, 1, and 10 mmol/L concentrations and allowed to incubate for 30, 60, or 120 min. All three incubation periods resulted in the same dose-dependent curve, with maintained viability up to 1 mmol/L, cytotoxicity at 10 mmol/L, and an estimated LD₅₀ of 5 mmol/L (Fig. 4A). The circulating plasma concentration of doxycycline in mice treated daily with 40 mg/kg has been reported as 2.3 ± 1.02 µg/mL using high-performance liquid chromatography (30). This concentration has a molar equivalent of 4.6 ± 2.04 µmol/L, which is ~1/1,000 the estimated LD₅₀. Based on these results, inhibition of tumor growth in doxycycline-treated mice was highly unlikely to be due to direct cytotoxic effects on injected tumor cells.

Early doxycycline and continuous doxycycline treatment (groups 2 and 4) resulted in 75% reduction in tumor volume compared with untreated mice (group 1), *P* = 0.05 for early (1 versus 2) and *P* = 0.04 for continuous (1 versus 4). Late treatment (group 3) resulted in only a 29% reduction in tumor volume (*P* = 0.67; Fig. 4B). This reduction in tumor volume was paralleled by reduction in tumor

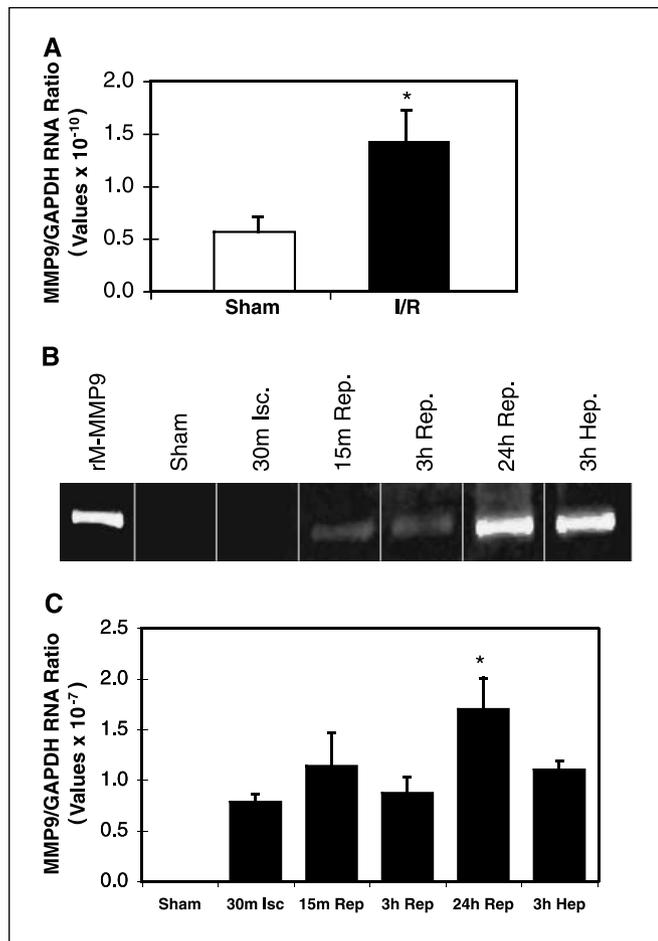


Figure 3. I/R-induced tissue MMP9. Sham-operated or I/R livers harvested 7 d after surgery were first examined by quantitative real-time PCR. **A**, MMP9 mRNA levels were significantly higher (*, *P* < 0.05) in the I/R group. **B**, gelatin zymography showed MMP9 protein levels from liver tissue in representative samples collected after sham operation (*Sham*); during 30 min ischemia (*30m Isc*); after ischemia; 15 min, 3 h, or 24 h reperfusion (*15m Rep.*, *3h Rep.*, and *24h Rep.*); and 3 h after hepatectomy (*3h Hep.*, positive control; *n* = 3 for each condition). Recombinant mouse MMP9 (*rM-MMP9*) was loaded as an additional positive control. Quantitative real-time PCR done **C** on samples used previously for zymography (**B**) shows a significant elevation of MMP9 levels 24 h following I/R (*, *P* < 0.05 compared with all other samples).

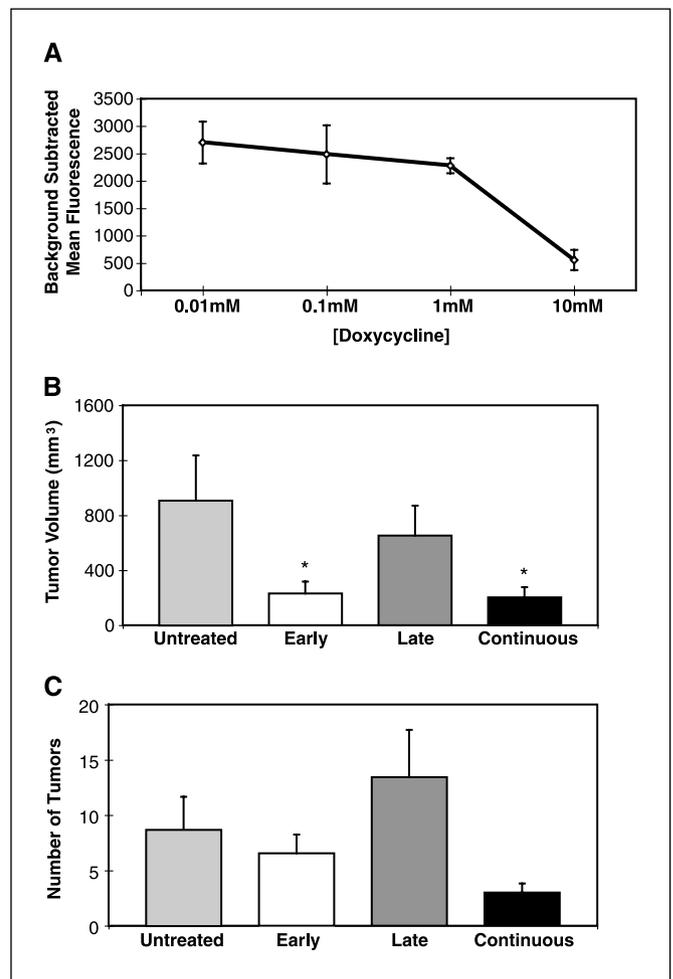


Figure 4. Doxycycline treatment prevents I/R-induced MMP9 and metastatic tumor growth in mouse liver. **A**, doxycycline was added to complete culture medium at 0.01, 0.1, 1.0, and 10 mmol/L that was used to assess cytotoxicity on MC38/Luc cells in culture. The observed LD₅₀ was estimated at 5 mmol/L based on the dose-dependent viability of cells. The mean total volume (**B**) and number (**C**) of tumors was evaluated in four groups of 10 mice that were subjected to tumor injection and 30 min warm hepatic ischemia with varied administration of 40 mg/kg doxycycline: no doxycycline (*Untreated*), treatment from the day of surgery through postoperative day 14 (*Early*), treatment from postoperative day 14 through day 28 (*Late*), or continuous treatment from the day of surgery until sacrifice at day 28 (*Continuous*). Early or continuous treatment resulted in significant reduction in tumor volume compared with untreated mice (*, *P* = 0.05 and 0.04, respectively). These same groups trended toward a significant reduction in tumor number as well (*, *P* = 0.06 and 0.07, respectively).

number; however, although all treatment groups had statistically similar total numbers of tumor, early and continuous treatment trended toward significance compared with untreated (*P* = 0.06 and 0.07, respectively; Fig. 4C).

Genetic deletion of MMP9 prevents hepatic metastases. Due to the nonspecific mechanism of doxycycline inhibition of MMPs and its potential alternative side effects, we evaluated tumor growth following I/R in C57BL/6 mice genetically deficient in MMP9 to determine if the decreased effect on tumor burden was due to MMP9 inhibition. Eight-week-old male MMP9-null mice were subdivided into the following four groups: (a) tumor injection without doxycycline, (b) tumor injection with doxycycline, (c) tumor injection and I/R without doxycycline, and (d) tumor injection and I/R with doxycycline. An equal number of age-matched WT mice were placed in the same four categories. These

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conditions were established to investigate two hypotheses. The first hypothesis was that MMP9-null mice would have less metastatic tumor after I/R. The second was that doxycycline would provide no additional protection against tumor growth in the absence of MMP9 following I/R.

We first examined the effects of doxycycline treatment on MMP9 levels after I/R. Real-time PCR revealed a trend toward significance regarding the observed decrease in MMP9 mRNA in tissue from doxycycline-treated mice compared with untreated mice after I/R ($P = 0.056$; Fig. 5A). Gelatin zymography confirmed the results of the PCR, illustrating a reduction in MMP9 in serum from doxycycline-treated mice subjected to I/R compared with untreated (Fig. 5B).

For animals receiving tumor injection without I/R (groups 1 and 2), MMP9 deficiency resulted in fewer and smaller tumor lesions (data not shown). For those mice receiving tumor injection and undergoing I/R (groups 3 and 4), we found that MMP9-null mice had significantly smaller tumor number and volume after I/R when compared with WT mice without doxycycline ($P < 0.02$ for each; Fig. 5C and D). Second, there was no statistically significant difference between WT doxycycline-treated mice and MMP9-null mice, regardless of their doxycycline treatment ($P > 0.2$ for all comparisons; Fig. 5C and D).

Discussion

The mortality associated with colorectal carcinoma is primarily due to metastatic spread of disease. In 15% to 20% of patients, when metastatic disease is isolated to the liver, cure is possible with complete surgical resection of the liver metastases. In clinical care, liver resection has become an accepted standard of care in the management of colorectal carcinoma metastatic to the liver; however, even with improved patient selection and improved surgical and anesthetic care, cure rates remain at 40% to 50%. Lower cure rates are seen in those with increased tumor burden at the time of resection as those with less tumor have higher 5-year survivals. Recent reports have shown intravascular dissemination of carcinoma cells as a result of surgical manipulation correlate with recurrent or metastatic disease (6, 7). Furthermore, several reports in rodent models have examined the outgrowth of hepatic metastases in response to hepatectomy or I/R (4, 5, 36, 37). These observations, in aggregate, suggest that liver resection itself may render the remnant liver more permissive to micrometastatic deposition and growth by up-regulating growth factors and recruiting inflammatory cells that release cytokines and adhesion molecules.

Two previous studies in rats reported that I/R before i.v. introduction of colon carcinoma cells increases metastatic liver tumor number and size that correlate with the duration of I/R (4, 5). An additional study in mice by van der Bilt et al. (37), which explored the effects of I/R on preexisting hepatic metastases of CT26 murine colorectal carcinoma tumors, found that I/R accelerated the outgrowth of those metastases. They identified that intermittent clamping, but not ischemic preconditioning, was able to prevent the outgrowth of existing metastases. They hypothesized that early reperfusion injury contributed to the release of cytotoxic cytokines and reactive oxygen species release, which promoted tumor growth; however, treatment with antioxidants α -tocopherol or ascorbic acid did not prevent outgrowth of hepatic lesions. They concluded that the late phase of I/R injury comprising neutrophil infiltration contributed to

increased tumor growth likely through a protease-dependent pathway.

Our experimental design incorporates features from both of these previously reported techniques using the splenic injection model of experimental metastases, but differs from these previous studies in the following ways: (a) a smaller inoculum of MC38 rather than the commonly used CT26 cells, (b) initiation of ischemia immediately after tumor cell injection to show the effect

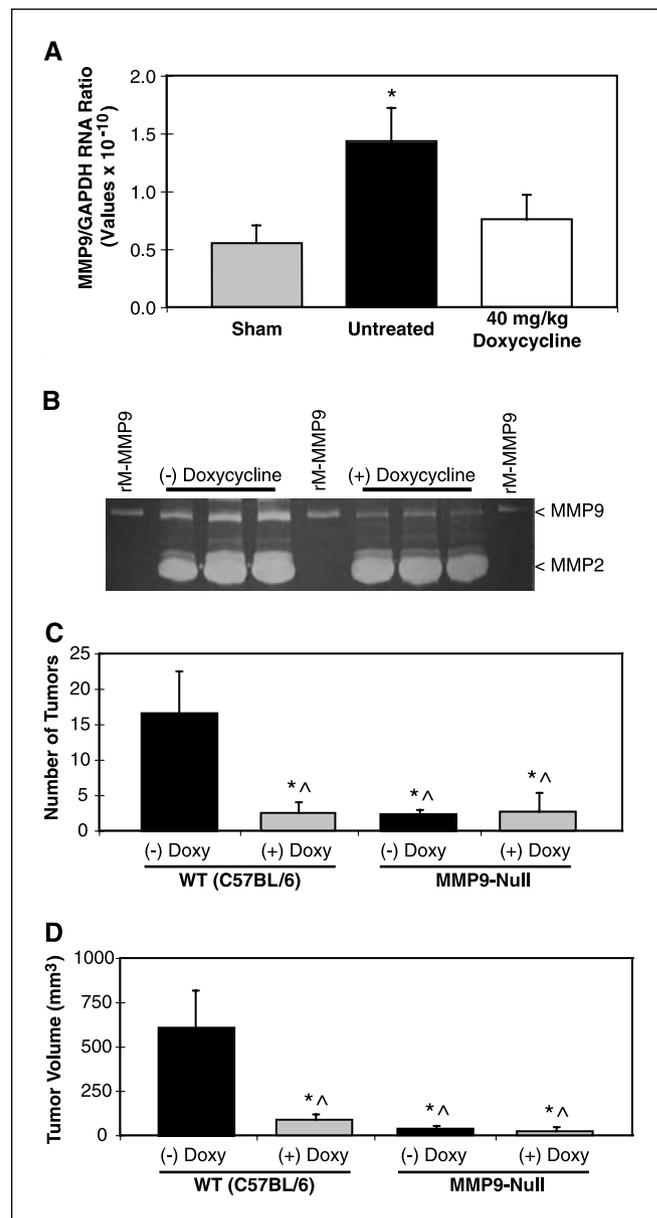


Figure 5. Genetic deletion of MMP9 prevents liver metastases. A, tissue MMP9 mRNA quantitation by real-time PCR in livers from WT sham-operated, 30 min hepatic ischemia and reperfusion, or I/R mice treated with doxycycline (40 mg/kg). Doxycycline produced a decrease in tissue MMP9 mRNA that exhibited a trend toward significance (*, $P = 0.056$). B, serum MMP9 was diminished by doxycycline treatment as evaluated by zymography. Tumor number (C) and volume (D) in WT (WT C57BL/6) and MMP9 null (MMP9 KO) with or without continuous doxycycline treatment after tumor injection and 30 min hepatic ischemia and reperfusion. MMP9 KO mice, regardless of doxycycline treatment, had significantly (*, $P < 0.02$) smaller total tumor volume and number, which was similar to WT mice treated with doxycycline.

of I/R on growth of liver metastases arising from circulating tumor cells, and (c) nonlethal monitoring of tumor growth using bioluminescent imaging. Like van der Bilt et al. (37), our finding that tumor growth was stimulated to a greater extent in clamped versus unclamped lobes suggest locally released, rather than systemically released, factors underlying the mechanism promoting tumor growth. We cannot determine whether the elevated expression of MMP9 in liver tissue after I/R observed in our study precedes neutrophil accumulation or is the result of late I/R injury; however, pharmacologic inhibition or genetic deletion of MMP9 was able to minimize tumor growth in our model.

Bioluminescent imaging has been previously validated to be a reliable predictor of hepatic tumor burden in mice (38, 39). We found a significant, but less consistent, correlation between photon count and liver weight, tumor number, or tumor volume. Unlike previous work that evaluated pairwise *in vivo* or *ex vivo* photon count compared with necropsy at exact times, we were only able to evaluate correlation coefficients at necropsy on postoperative day 28. Additionally, they used a different cell type in a BALB/c mouse, which may account for the difference in correlation values. We have found bioluminescence to correlate with tumor growth overall but cannot account for all growth as it measures only cells expressing the reporter gene and does not measure associated stromal cell infiltration and other components of a solid tumor. Another possible explanation for the lower R^2 values observed in our study is that mice with significant tumor loads had hemorrhagic ascites, which can diminish bioluminescent detection due to absorption of light by hemoglobin as discussed by Sarraff-Yazdi et al. (38). Overall, we recommend that bioluminescent imaging should be interpreted with caution before quantitative conclusions can be drawn without corresponding pathology.

The MMPs promote tumor growth and metastasis by a variety of mechanisms (40), including proteolysis of the extracellular matrix that allows the initial migration and seeding of tumor cells. The gelatinases, MMP2 and MMP9, are widely implicated in the metastasis of colorectal carcinoma (41–46). The metastatic capacity of tumor cells and the expression of MMP2 and MMP9 in tumors is not only a consequence of increased expression by the tumor cells themselves (47), but rather MMP9 in metastatic colorectal tumors is primarily produced by tissue macrophages and other immune cells in response to the tumor cells. A principal function of MMP9 in these cell types is for cell migration; however, colon cancer cells can modulate host cell expression of MMP9 to provide an invasion and

growth advantage (48). The increased expression and activity of MMP9 in colorectal tumors has made it an attractive target for therapeutic intervention. Our report shows the effectiveness of MMP9 inhibition in decreasing the number and volume of hepatic metastases following surgical manipulation of the liver in a murine model. Our findings further indicate that MMP inhibition in this setting is only effective when initiated immediately after surgery because only early or continuous doxycycline therapy was effective at minimizing tumor growth.

Circulating and tissue MMP9 levels have been investigated as a potential biomarker for predicting and detecting metastatic colorectal carcinoma; however, a consistent positive correlation has not been reported (41, 42, 44, 45, 49). Assessment of circulating MMP9 levels does not necessarily predict the activity at the tissue level. Based on observations made in our experiments, we show that elevated MMP9 expression in liver tissue is associated with the presence of tumor; however, because MMP9 was up-regulated by I/R and I/R in turn was associated with increased tumor burden, the association is not clear.

Broad-spectrum MMP inhibitors have proven ineffective in clinical trials for anticancer treatment. The ineffectiveness of these studies may be due to the fact that the majority were done on patients with advanced-stage cancer (50) or that ineffective doses were used. The benefits of early MMP inhibition established by our observations support the recommendations made by Zucker et al. (50), who suggest that future clinical trials using MMP inhibitors should focus on patients with early-stage disease and evaluation of patient MMP levels to increase the likelihood of a positive response. Further evaluation of doxycycline should be considered to determine its direct and indirect effects on colorectal carcinoma metastasis. Our data provide preclinical evidence that MMP inhibitors may be effective in reducing metastatic tumor burden when administered following hepatic resection.

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References

- Anasagasti MJ, Alvarez A, Martin JJ, Mendoza L, Vidal-Vanaclocha F. Sinusoidal endothelium release of hydrogen peroxide enhances very late antigen-4-mediated melanoma cell adherence and tumor cytotoxicity during interleukin-1 promotion of hepatic melanoma metastasis in mice. *Hepatology* 1997;25:840–6.
- Chari R, Callery M. Colorectal cancer metastatic to the liver: resection and intraarterial chemotherapy. 7th ed. In: Gery L, editor. *Current surgical therapy*. St. Louis: Mosby; 2001. p. 375–80.
- Higashiyama A, Watanabe H, Okumura K, Yagita H. Involvement of tumor necrosis factor α and very late activation antigen 4/vascular cell adhesion molecule 1 interaction in surgical-stress-enhanced experimental metastasis. *Cancer Immunol Immunother* 1996;42:231–6.
- Doi K, Horiuchi T, Uchinami M, et al. Hepatic ischemia-reperfusion promotes liver metastasis of colon cancer. *J Surg Res* 2002;105:243–7.
- Ku Y, Kusunoki N, Shiotani M, et al. Stimulation of haematogenous liver metastases by ischaemia-reperfusion in rats. *Eur J Surg* 1999;165:801–7.
- Guller U, Zajac P, Schneider A, et al. Disseminated single tumor cells as detected by real-time quantitative polymerase chain reaction represent a prognostic factor in patients undergoing surgery for colorectal cancer. *Ann Surg* 2002;236:768–75.
- Koch M, Kienle P, Sauer P, et al. Hematogenous tumor cell dissemination during colonoscopy for colorectal cancer. *Surg Endosc* 2004;18:587–91.
- Yamaguchi K, Takagi Y, Aoki S, Futamura M, Saji S. Significant detection of circulating cancer cells in the blood by reverse transcriptase-polymerase chain reaction during colorectal cancer resection. *Ann Surg* 2000;232:58–65.
- Vidal-Vanaclocha F, Alvarez A, Asumendi A, Urcelay B, Tonino P, Dinarello CA. Interleukin 1 (IL-1)-dependent melanoma hepatic metastasis *in vivo*; increased endothelial adherence by IL-1-induced mannose receptors and growth factor production *in vitro*. *J Natl Cancer Inst* 1996;88:198–205.
- Hanazaki K, Kajikawa S, Shimoza N, et al. Survival and recurrence after hepatic resection of 386 consecutive patients with hepatocellular carcinoma. *J Am Coll Surg* 2000;191:381–8.
- Ohlsson B, Stenram U, Tranberg KG. Resection of

- colorectal liver metastases: 25-year experience. *World J Surg* 1998;22:268–76.
12. Pringle JH. Notes on the arrest of hepatic hemorrhage due to trauma. *Ann Surg* 1908;48:541.
 13. Horiuchi T, Muraoka R, Tabo T, Uchinami M, Kimura N, Tanigawa N. Optimal cycles of hepatic ischemia and reperfusion for intermittent pedicle clamping during liver surgery. *Arch Surg* 1995;130:754–8.
 14. Kimura N, Muraoka R, Horiuchi T, et al. Intermittent hepatic pedicle clamping reduces liver and lung injury. *J Surg Res* 1998;78:11–7.
 15. Uchinami M, Muraoka R, Horiuchi T, et al. Effect of intermittent hepatic pedicle clamping on free radical generation in the rat liver. *Surgery* 1998;124:49–56.
 16. Anderson CD, Belous A, Pierce J, et al. Mitochondrial calcium uptake regulates cold preservation-induced Bax translocation and early reperfusion apoptosis. *Am J Transplant* 2004;4:352–62.
 17. Belous A, Knox C, Nicoud IB, et al. Altered ATP-dependent mitochondrial Ca^{2+} uptake in cold ischemia is attenuated by ruthenium red. *J Surg Res* 2003;111:284–9.
 18. Belous A, Knox C, Nicoud IB, et al. Reversed activity of mitochondrial adenine nucleotide translocator in ischemia-reperfusion. *Transplantation* 2003;75:1717–23.
 19. Chen CF, Leu FJ, Chen HI, Wang D. Oxygen radicals and matrix metalloproteinases mediate reperfusion liver injury. *Transplant Proc* 2005;37:4547–9.
 20. Cursio R, Mari B, Louis K, et al. Rat liver injury after normothermic ischemia is prevented by a phosphinic matrix metalloproteinase inhibitor. *FASEB J* 2002;16:93–5.
 21. Khandoga A, Kessler JS, Hanschen M, et al. Matrix metalloproteinase-9 promotes neutrophil and T cell recruitment and migration in the postischemic liver. *J Leukoc Biol* 2006;79:1295–305.
 22. Yoshida M, Horiuchi T, Uchinami M, et al. Intermittent hepatic ischemia-reperfusion minimizes liver metastasis in rats. *J Surg Res* 2003;111:255–60.
 23. Corbett TH, Griswold DP, Jr., Roberts BJ, Peckham JC, Schabel FM, Jr. Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. *Cancer Res* 1975;35:2434–9.
 24. Vu TH, Shipley JM, Bergers G, et al. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 1998;93:411–22.
 25. Acuff HB, Carter KJ, Fingleton B, Gorden DL, Matrisian LM. Matrix metalloproteinase-9 from bone marrow-derived cells contributes to survival but not growth of tumor cells in the lung microenvironment. *Cancer Res* 2006;66:259–66.
 26. Anderson CD, Pierce J, Nicoud IB, Belous A, Knox C, Chari RS. Modulation of mitochondrial calcium management attenuates hepatic warm ischemia-reperfusion injury. *Liver Transpl* 2005;11:663–8.
 27. Gujral JS, Bucci TJ, Farhood A, Jaeschke H. Mechanism of cell death during warm hepatic ischemia-reperfusion in rats: apoptosis or necrosis? *Hepatology* 2001;33:397–405.
 28. Hirsch J, Hansen KC, Choi S, et al. Warm ischemia induced alterations in oxidative and inflammatory proteins in hepatic Kupffer cells in rats. *Mol Cell Proteomics* 2006;5:979–86.
 29. Noh J, Behrends M, Choi S, et al. Glutamine does not protect against hepatic warm ischemia/reperfusion injury in rats. *J Gastrointest Surg* 2006;10:234–9.
 30. Pozzi A, LeVine WF, Gardner HA. Low plasma levels of matrix metalloproteinase 9 permit increased tumor angiogenesis. *Oncogene* 2002;21:272–81.
 31. Chen X, Su Y, Fingleton B, et al. Increased plasma MMP9 in integrin $\alpha 1$ -null mice enhances lung metastasis of colon carcinoma cells. *Int J Cancer* 2005;116:52–61.
 32. Kim TH, Mars WM, Stolz DB, Michalopoulos GK. Expression and activation of pro-MMP-2 and pro-MMP-9 during rat liver regeneration. *Hepatology* 2000;31:75–82.
 33. Pozzi A, Moberg PE, Miles LA, Wagner S, Soloway P, Gardner HA. Elevated matrix metalloprotease and angiostatin levels in integrin $\alpha 1$ knockout mice cause reduced tumor vascularization. *Proc Natl Acad Sci U S A* 2000;97:2202–7.
 34. O'Donnell SM, Hansberger MW, Connolly JL, et al. Organ-specific roles for transcription factor NF- κ B in reovirus-induced apoptosis and disease. *J Clin Invest* 2005;115:2341–50.
 35. Hardingham JE, Hewett PJ, Sage RE, et al. Molecular detection of blood-borne epithelial cells in colorectal cancer patients and in patients with benign bowel disease. *Int J Cancer* 2000;89:8–13.
 36. Uotani H, Yamashita I, Nagata T, Kishimoto H, Kashii Y, Tsukada K. Induction of E-selectin after partial hepatectomy promotes metastases to liver in mice. *J Surg Res* 2001;96:197–203.
 37. van der Bilt JD, Kranenburg O, Nijkamp MW, et al. Ischemia/reperfusion accelerates the outgrowth of hepatic micrometastases in a highly standardized murine model. *Hepatology* 2005;42:165–75.
 38. Sarraf-Yazdi S, Mi J, Dewhirst MW, Clary BM. Use of *in vivo* bioluminescence imaging to predict hepatic tumor burden in mice. *J Surg Res* 2004;120:249–55.
 39. Smakman N, Martens A, Kranenburg O, Borel Rinkes IH. Validation of bioluminescence imaging of colorectal liver metastases in the mouse. *J Surg Res* 2004;122:225–30.
 40. McCawley LJ, Matrisian LM. Matrix metalloproteinases: multifunctional contributors to tumor progression. *Mol Med Today* 2000;6:149–56.
 41. de Hingh IH, Waas ET, Lomme RM, Wobbes T, Hendriks T. Circulating matrix metalloproteinase-9 is transiently elevated after colorectal surgery. *Int J Colorectal Dis* 2004;19:446–50.
 42. Ishida H, Murata N, Tada M, et al. Determining the levels of matrix metalloproteinase-9 in portal and peripheral blood is useful for predicting liver metastasis of colorectal cancer. *Jpn J Clin Oncol* 2003;33:186–91.
 43. Murray D, Morrin M, McDonnell S. Increased invasion and expression of MMP-9 in human colorectal cell lines by a CD44-dependent mechanism. *Anticancer Res* 2004;24:489–94.
 44. Tutton MG, George ML, Eccles SA, Burton S, Swift RI, Abulafi AM. Use of plasma MMP-2 and MMP-9 levels as a surrogate for tumour expression in colorectal cancer patients. *Int J Cancer* 2003;107:541–50.
 45. Waas ET, Lomme RM, DeGroot J, Wobbes T, Hendriks T. Tissue levels of active matrix metalloproteinase-2 and -9 in colorectal cancer. *Br J Cancer* 2002;86:1876–83.
 46. Zeng ZS, Guillem JG. Unique activation of matrix metalloproteinase-9 within human liver metastasis from colorectal cancer. *Br J Cancer* 1998;78:349–53.
 47. Lubbe WJ, Zhou ZY, Fu W, et al. Tumor epithelial cell matrix metalloproteinase 9 is a target for antimetastatic therapy in colorectal cancer. *Clin Cancer Res* 2006;12:1876–82.
 48. Mook OR, Frederiks WM, Van Noorden CJ. The role of gelatinases in colorectal cancer progression and metastasis. *Biochim Biophys Acta* 2004;1705:69–89.
 49. Waas ET, Wobbes T, Lomme RM, Hendriks T. Plasma gelatinase activity does not reflect disease activity after operation for colorectal cancer. *Oncology* 2005;68:256–62.
 50. Zucker S, Cao J, Chen WT. Critical appraisal of the use of matrix metalloproteinase inhibitors in cancer treatment. *Oncogene* 2000;19:6642–50.