Published in final edited form as:

J Surg Res. 2008 April ; 145(2): 192–198. doi:10.1016/j.jss.2007.04.002.

A Critical Role for Matrix Metalloproteinases in Liver Regeneration

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Abstract

BACKGROUND—Matrix metalloproteinases (MMPs), TNF- α and IL-6 are mediators of liver regeneration. To determine whether MMPs are required for normal hepatic regeneration, we performed 67% hepatectomies on mice, treated with a broad-spectrum MMP-inhibitor, and assessed the effect on liver regeneration and urinary MMP activity.

METHODS—Mice were subjected to sham operations, 67% hepatectomy, or 67% hepatectomy plus treatment with the broad-spectrum MMP inhibitor MarimastatTM. Urine collected preoperatively and for 8 days postoperatively was tested for MMP-2 and MMP-9 activity using zymography. Serum AST, ALT, alkaline phosphatase, bilirubin, TNF- α , IL-6, and hepatocyte growth factor (HGF) levels were measured. Liver sections were analyzed by CD31 immunohistochemistry and microvessel density. Mitotic index (MI) and proliferating cell nuclear antigen (PCNA) labeling index were determined.

RESULTS—The mean regenerating liver weight on post-operative day 8 was 0.72 $\pm$ 0.01 grams for the hepatectomy MarimastatTM group, and 0.83 $\pm$ 0.02 grams for the hepatectomy control group ($p < 0.001$). Urinary MMP-9 activity was elevated during hepatic regeneration, and decreased on postoperative day 8 when the liver returned to its preoperative mass. In contrast, urine from hepatectomy MarimastatTM mice, in which liver regeneration was successfully inhibited, showed consistently low levels of MMP-2 and MMP-9 activity. The hepatectomy MarimastatTM group also exhibited elevated serum IL-6 levels on post-operative day 8, while serum TNF- α soluble receptor II levels were unchanged. HGF levels were not significantly different between the control hepatectomy and hepatectomy MarimastatTM groups at days 2, 4, and 8. Liver microvessel density

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was reduced in the hepatectomy MarimastatTM group at day 4. MI and PCNA index were significantly decreased in the MarimastatTM hepatectomy group at post-operative day 2.

CONCLUSIONS—The broad-spectrum MMP-inhibitor MarimastatTM inhibits liver regeneration. Microvessel density is reduced at day 4. Furthermore, urinary MMP-9 is elevated during liver regeneration, and this effect is not observed when regeneration is inhibited by the broad-spectrum MMP-inhibitor MarimastatTM.

Keywords

matrix metalloproteinase; MMP; liver regeneration; IL-6; TNF-alpha; microvessel density; metalloproteinase inhibitor; MarimastatTM

INTRODUCTION

Liver regeneration is an essential physiologic response after hepatic injury, resection, or transplantation. The liver is a unique organ in that regeneration occurs by DNA replication and mitosis, whereas most other organs simply hypertrophy (1). Stimulators of hepatic regeneration include hepatocyte (HGF) and epidermal growth factor (EGF), transforming growth factor- α (TGF- α), and the potent stimulators of angiogenesis, fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) (2). In addition, interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) are associated with liver regeneration (3–5).

IL-6 and TNF- α are both considered to be priming factors for regeneration because they prepare hepatocytes for replication (6). IL-6 initiates the acute phase response in the liver, and activates STAT3, a transcription factor that initiates hepatocyte replication (7). Therefore, it appears that IL-6 plays both a priming and mitogenic role in hepatic regeneration. Further demonstrating the importance of this cytokine in the regenerative process, IL-6 deficient mice demonstrate abnormal regeneration after partial hepatectomy (8). Although it has been difficult to test the result of giving mice large doses of IL-6 due to systemic toxicity, lower doses administered over a long period of time have resulted in livers that regenerate beyond their original mass (6). While IL-6 activates STAT3, TNF- α activates NF κ B, a similar transcription factor (9, 10). The main action of TNF- α is thought to be the release of IL-6, resulting in the cascade described above. While TNF- α deficient mice do not regenerate their livers successfully, preoperative administration of IL-6 produces normal regeneration, suggesting that TNF- α affects hepatic regeneration principally through its effects on IL-6 (11). Lastly, the metalloproteinase, ADAM 17, actively releases TNF- α from the cell surface.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that work in concert with their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) to control the rate and degree of extracellular matrix degradation (12, 13). The effects of this activity are widespread and include tissue regeneration, embryologic development, ovulation, tumor growth and metastasis (14–16). Extracellular matrix (ECM) remodeling by MMPs is critical for the growth of new capillaries from pre-existing vessels (angiogenesis) (15, 17, 18).

In a recent study, we investigated whether urinary MMPs or TIMPs could predict the status of liver regeneration after partial hepatectomy. Rodent livers are known to regenerate to their original mass 8 days after a 67% hepatectomy (19, 20). We showed that during hepatic regeneration in mice, there is an elevation in MMP-9 activity in the urine. At post-operative day 8, loss of MMP-9 activity was consistent with the cessation of hepatic regeneration (14).

Because MMPs appear to be a key mediator of hepatic regeneration, we more recently hypothesized that a broad-spectrum MMP inhibitor would reduce the rate of organ regeneration. Successful inhibition of hepatic regeneration through this route would support the concept that liver regeneration is dependent on MMP activity and would provide additional insight into the complex mechanisms of hepatic regeneration. In order to test our hypothesis, mice underwent a partial hepatectomy and were subsequently treated with Marimastat™, a broad-spectrum MMP inhibitor.

MATERIALS AND METHODS

Partial Hepatectomy

Experiments were performed on seven to eight week old male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). The mice were housed five animals to a cage in a barrier room. Mice were acclimated to their environment for at least 72 hours prior to the initiation of each experiment and allowed food and water *ad libitum*. Animal protocols complied with the NIH Animal Research Advisory Committee guidelines and were approved by the Children's Hospital Institutional Animal Care and Use Committee. Mice underwent the surgical removal of 67% of the liver under isoflurane anesthesia. The left upper, right upper, and left lower lobes of the liver were ligated with 5-0 silk ties and then excised, as previously described (2, 21).

Groups of mice (n=5) were placed into sham control, sham treatment, hepatectomy control, and hepatectomy treatment groups, and were sacrificed on postoperative days 4 and 8. Animals in the sham group underwent a midline laparotomy in which their liver was manipulated with a cotton swab and blunt forceps. The sham control group was given methylcellulose via orogastric gavage every 12 hours. The sham treatment group received Marimastat™ (Vernalis, BB-2516) at a dose of 200 mg/kg/day in methylcellulose vehicle every 12 hours by orogastric gavage (22, 23). The hepatectomy control group underwent a 67% hepatectomy and was administered methylcellulose via orogastric gavage. The hepatectomy treatment group also underwent a 67% hepatectomy and received Marimastat™. Mice were weighed and urine was collected pre-operatively and on postoperative days 2, 4, 6, and 8. Urine was immediately placed on ice, aliquoted, and frozen at -80 °C, as previously described (14, 23). Serum samples were obtained preoperatively and on post-operative days 4 and 8 for liver function tests (LFTs), and on post-operative days 2, 4, and 8 for HGF levels. At sacrifice, the livers were weighed and prepared for histological analysis.

Sample Preparation and Substrate Gel Electrophoresis

Gelatin zymography was performed on urine samples as previously described (24, 25). Samples were thawed on ice and 30 microliters of each urine sample was mixed with buffer consisting of 4% SDS, 0.15 M Tris (pH 6.8), 20% (v/v) glycerol, and 0.5% (w/v) bromophenol blue. Samples were added to wells of a 4% acrylamide Laemmli stacking gel/10% and SDS-acrylamide separating gel containing 0.1% (w/v) gelatin (Bio-rad laboratories, Inc., Hercules, CA) on a mini-gel apparatus. Gels were run at 15 mA/cm of gel during stacking and at 20 mA/cm of gel during the resolving phase at room temperature. After electrophoresis, gels were soaked in 2.5% Triton X-100 with gentle shaking for 30 minutes at ambient temperature with one change of detergent solution. The gels were then rinsed and incubated overnight at 37 °C in substrate buffer [50 mM Tris-HCL buffer (pH 8), 5 mM CaCl₂, and 0.02% NaN₃]. After incubation, gels were stained for 15–30 minutes in 0.5% Coomassie Blue R-250 in acetic acid, isopropyl alcohol, and water (1:3:6), destained in acetic acid, ethanol, and water (1:3:6), photographed, and dried for permanent record.

Both proenzyme and activated proteinases appeared as zones of substrate clearing. All experiments were repeated twice.

Interleukin-6, TNF- α Soluble Receptor II, Hepatocyte Growth factor Levels

At the time of euthanasia, mice were anesthetized with 300 μ l of 2.5% Avertin (Tribromoethanol, Sigma-Aldrich Corporation, St. Louis, MO) by intraperitoneal injection. Approximately 400 μ l of blood was collected from each mouse via retro-orbital puncture. The specimens were then placed into serum separator tubes (Becton Dickinson, Franklin Lakes, New Jersey), and centrifuged and 4°C at 8000 rpm for 10 minutes to separate serum. Serum was frozen at -80°C and delivered to the Clinical Laboratory at Children's Hospital, Boston for measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, and total and direct bilirubin levels.

Circulating IL-6, TNF- α soluble receptor II levels (Quantikine®, R&D systems, Inc., Minneapolis, MN), and hepatocyte growth factor (HGF) levels (B-Bridge International, Inc., Mountain View, CA) were determined in serum using commercial ELISA kits. Optical density was read at 450 nm and analyzed with Softmax® PRO Software (Molecular Devices Corporation, Sunnyvale, CA).

Immunohistochemistry

Livers of hepatectomy control and hepatectomy Marimastat™ mice on postoperative days 4 and 8 were fixed at 4°C in 10% formalin overnight and washed with phosphate buffered saline (PBS). The specimens then were embedded in paraffin and cut into 5 μ m sections. Endothelial cells were identified by antibody staining against the endothelial surface antigen CD31 (PECAM-1). After deparaffinization and rehydration, sections underwent antigen retrieval with AR10 solution (Biogenex, San Ramon, CA) at pH 10 in a pressure cooker for 1.5 minutes. Endogenous peroxidase activity was blocked with H₂O₂, and non-specific binding sites were blocked for 30 minutes at room temperature (Power Block, Biogenex, San Ramon, CA). Sections were then incubated with primary CD31 antiserum (Biogenex, San Ramon, CA) for 1 hour at room temperature. Slides were washed twice with PBS, and secondary antibody (Super Sensitive Detection System, Biogenex, San Ramon, CA) was then applied. Slides were again washed with PBS and developed for 15 minutes with diaminobenzidine (DAB) chromagen (Biogenex, San Ramon, CA). Slides were washed with water, counterstained with Gill's hematoxylin and placed in 0.5% ammonium hydroxide.

Microvessel Density Analysis

To quantify vessel density, CD31 immunostained sections underwent digital image analysis using a Nikon Eclipse TE300 inverting photomicroscope with an attached Spot RT (Diagnostic Instruments Inc., Sterling Heights, MI) video camera linked to a personal computer. Images were imported directly to the image analysis program IP Laboratory (Scanalytics Inc., Fairfax, VA). Sequential images were captured at 200 X for the CD31 sections without overlapping. To calculate the volume fraction of stained regions, a color threshold was chosen for each image that distinguished between the stained areas and background. The proportion of the selected color was calculated as a percentage of total area and used as a measure of vessel density. Vascularization was scanned and quantified in five representative fields per section. The scores of the five fields were then averaged to give a final score for each section.

Regeneration Indices

Five-micron thick sections were cut from the paraffin blocks and stained with hematoxylin and eosin (H&E). The mitotic index was calculated by the number of positive cells per high

power field (400x), expressed as per 100 cells. Ten sections per mouse were counted, and 3 mice per timepoint were used.

For PCNA staining, liver sections were incubated with Citra buffer (Dako, Denmark) for 10 minutes at 90°. Non-specific binding sites were then blocked for 30 minutes at room temperature with TNB blocking buffer (NEN Life Sciences, Boston, MA). Primary PCNA anti-serum 1:150 (Signet Laboratories, Dedham, MA) was added to the sections overnight at 4°C. Secondary anti-mouse IgG 1:400 antibody was then added for 30 minutes at room temperature. Next, the slides were incubated with streptavidin alkaline phosphatase (AP) solution (1:100), followed by biotinyl tyramide amplification diluent (1:50). After repeat incubation with AP solution (1:100) for 30 minutes at room temperature, the chromagen New Fuchsin (Biogenex, San Ramon, CA) was added. The slides were counterstained with Gill's hematoxylin. The proliferation index of PCNA stained tissue was quantified as the mean of positive staining cells per high powered field (400x).

Statistical Analysis

Data are presented as mean $\pm$ standard error of the mean. Statistical analysis of all results was performed using the Student's two-tailed, unpaired *t* test or the Mann-Whitney Rank Sum Test for comparisons between groups (SigmaStat, SPSS, Chicago, IL). Differences were considered significant when $p < 0.05$.

RESULTS

Mouse and Liver Weights

There was no statistical difference in mouse weights between any of the groups when comparing preoperative weights and post-hepatectomy day 8 weights (data not shown). There was no statistical difference in mean liver weights in the sham control and sham MarimastatTM mice at post operative day 4 and day 8 (data not shown). Mean post-hepatectomy liver weights after sacrifice at day 4 were 0.84 ± 0.07 grams for the hepatectomy control group and 0.74 ± 0.03 grams for the hepatectomy MarimastatTM animals ($p = 0.004$) (Figure 1A). Post-hepatectomy liver weights after sacrifice at day 8 were 0.83 ± 0.02 grams for the hepatectomy control animals and 0.72 ± 0.01 grams for the hepatectomy MarimastatTM animals ($p < 0.001$) (Figure 1B).

MMP Activity

To determine the presence of MMP activity during regeneration, urine was analyzed for MMPs using substrate gel electrophoresis (zymography). In the hepatectomy control mice, MMP-9 activity was elevated in the urine starting at postoperative day 2, increased through postoperative day 6, and decreased significantly on postoperative day 8 (Figure 2A). In hepatectomy MarimastatTM mice, MMP-9 activity, while present, was at a consistent, low level over the 8-day experiment (Figure 2B). There was no evidence of increased MMP-2 activity during the regenerative period in either of these groups.

Liver Function Tests

On postoperative day 4, LFTs were slightly elevated in the sham control, hepatectomy control, hepatectomy MarimastatTM groups. There was no difference in elevation in aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, or direct bilirubin between the hepatectomy control and hepatectomy MarimastatTM animals (data not shown). In the hepatectomy control group, alkaline phosphatase level was 81.3 ± 2.3 U/L versus 103.3 ± 7.8 U/L in the hepatectomy MarimastatTM-treated group ($p = 0.009$). At postoperative day 8, the AST and ALT levels in both groups were elevated further, without a significant difference between the hepatectomy control and the hepatectomy MarimastatTM-treated

groups. Alkaline phosphatase levels for the hepatectomy Marimastat™ group fell to 69 U/L compared to 163 U/L in the hepatectomy control group ($p=0.002$) and 113 U/L in the sham control group ($p=0.003$) (Figure 3A). Mean total bilirubin was 0.10 mg/dl for the hepatectomy Marimastat™ group, 0.28 mg/dl for the hepatectomy control group ($p=0.008$), and 0.120 for the sham control group ($p=0.639$) (Figure 3B).

Cytokines

On postoperative day 4, IL-6 levels for the hepatectomy control and hepatectomy Marimastat™ groups were comparable, with means of 37.5 pg/mL and 28.8 pg/mL, respectively. However, at postoperative day 8, mean IL-6 levels were 21.3 pg/mL for the sham control group, 29.1 pg/mL for hepatectomy control group, and 56.8 pg/mL for the hepatectomy Marimastat™-treated group. A statistically significant difference was found when comparing IL-6 levels of the hepatectomy control group versus the hepatectomy Marimastat™ group ($p=0.022$) (Figure 4). TNF- α levels were not statistically significant between groups at day 4 or day 8 (data not shown).

Hepatocyte Growth Factor

HGF serum levels among the hepatectomy control group at day 2, 4, and 8 were not statistically different. HGF serum levels increased over time in the Marimastat™ hepatectomy group ($d2=0.572$, $d4=0.946$, $d8=0.990$) and this became statistically significant between day 2 and day 4 ($p=0.039$). Differences in HGF serum levels between hepatectomy control and hepatectomy Marimastat™ groups were not statistically significant at day 2, 4, or 8 ($p=0.474$, $p=0.331$, $p=0.539$, respectively).

Microvessel Density

On postoperative day 4, hepatic microvessel density in the hepatectomy Marimastat™-treated group (7.45%) was significantly lower than that of the hepatectomy control group (8.86%) ($p=0.022$) (Figure 5A). On postoperative day 8, no significant difference in hepatic microvessel density was seen between the hepatectomy Marimastat™ (16.4%) and hepatectomy control groups (17.1%) ($p=0.485$) (Figure 5B).

Regeneration Indices

The mitotic index of the hepatectomy control mice was 2.2, 0.15, 0.19 on post-operative days 2, 4, and 8, respectively. The mitotic index of the hepatectomy Marimastat™ group was 1, 0.20, and 0 on days 2, 4, and 8, respectively. The statistically significant difference was seen only on post-operative day 2 ($p=0.002$, SD 0.0135). The median PCNA index in the hepatectomy control mice and the hepatectomy Marimastat™ group at day 2 was 37.5 (IQR 33.0–42.0) and 18.5 (IQR 15.0–24.0), respectively ($p<0.001$). At day 4 the mean PCNA index for the hepatectomy control mice and the hepatectomy Marimastat™ mice was 3.0 (IQR 2.0–5.0) and 2.0 (IQR 1.0–3.0), respectively ($p=0.019$). At day 8 the median PCNA index for the hepatectomy control and the hepatectomy Marimastat™ mice was 1.0 (IQR 1.0–3.0) and 1.0 (IQR 0.0–1.0), respectively ($p=0.026$).

Discussion

It has become increasingly clear that organ regeneration, like tumor growth, is dependent upon angiogenesis. The endothelial cell proliferation and apoptosis that accompany hepatic regeneration, for example, can be modulated through the administration of angiogenic agents (2). In this report we show that matrix metalloproteinases are also a critical requirement in the normal hepatic regenerative process. This hypothesis was founded on the fact that MMPs are active in both extracellular matrix remodeling and angiogenesis. We

have previously shown that MMPs are expressed in the urine after partial hepatectomy and that their profile correlates with the stage of the regenerative process (12). In addition, urinary MMP expression changes when liver regeneration is modified with angiogenesis agents (12). Thus, in order to determine whether hepatic regeneration requires MMP activity, in addition to being endothelial dependent, we treated mice undergoing liver regeneration with Marimastat™, a broad-spectrum MMP inhibitor. Because hepatic regeneration was inhibited by the administration of Marimastat™, it appears that MMPs are critically important for organ regeneration in this partial hepatectomy model.

MMPs may facilitate hepatic regeneration due to their direct effect on extracellular matrix remodeling, a process that is integral to tissue regeneration (26–28). Alternatively, MMPs may influence the regenerative process indirectly, through their effects on angiogenesis. MMPs promote angiogenesis by facilitating endothelial migration through the degraded extracellular matrix. In addition, MMPs release pro-endothelial growth factors from the extracellular matrix (29). Thus, Marimastat™ may inhibit hepatic regeneration by preventing extracellular matrix remodeling or limiting capillary formation.

The endothelial-mediated action of MMPs is supported by the finding that microvascular density was reduced in the livers of animals treated with the MMP inhibitor. Peak endothelial proliferation occurs four days after partial hepatectomy in the regenerating liver (2). When hepatic regeneration is inhibited by a specific antiangiogenic agent, the peak in endothelial proliferation is blunted (2). Similarly, while inhibiting liver regeneration, Marimastat™ also reduced endothelial activity in the liver at postoperative day four, suggesting that MMPs influence hepatic regeneration through an endothelial pathway.

Not only may their matrix degradation and endothelial properties be important regulators of hepatic regeneration, but MMP-mediated alterations in IL-6 may contribute to the control of hepatic regeneration as well. The cytokine profile of the animals was significant for an elevation in IL-6 levels in the Marimastat™-treated group. Since IL-6 has been shown to be necessary for liver regeneration, one might first expect decreased liver regeneration to be accompanied by low levels of IL-6. While the reason for higher levels of IL-6 in Marimastat™-treated mice in these experiments is not known, we hypothesize that IL-6 levels are increased in an effort to “turn on” regeneration. IL-6 activates STAT3, a transcription factor that causes hepatocytes to enter the cell growth cycle. Since regeneration is slowed, there may not be a negative feedback on IL-6 production. This would imply that liver regeneration modulated by MMPs is not IL-6 dependent.

Our laboratory has previously demonstrated that urinary MMP activity is changed by the administration of an angiogenesis inhibitor during hepatic regeneration (14). These earlier studies showed that MMP-2 and MMP-9 were up-regulated in the normal regenerating liver for the first 4 postoperative days, and remained high through day 8. After day 8, when hepatic regeneration was complete, MMP levels returned to normal. In the current experiment, MMP activity, specifically that of MMP-9, was elevated in the control hepatectomy animals and was reduced at day 8 when the liver had completely regenerated. In animals treated with Marimastat™, there was inhibited regeneration and no measurable rise in MMP-9 activity throughout the 8-day experiment.

Mouse weights of the sham control, sham treatment, hepatectomy control, and hepatectomy Marimastat™ groups did not differ over the course of the study. This indicates that, while there may be a decrease in liver regeneration, the mice remained healthy. There was no evidence of a toxic effect of Marimastat™ on the liver, as the liver weights and histology in the sham control and sham treatment group were not significantly different at day 2, 4, or day 8.

There was a statistical difference between the weights of the livers of the hepatectomy control and hepatectomy Marimastat™ groups at both the day 4 and day 8 sacrifice time points. Under normal conditions, mitotic cells and expression of proliferating nuclear antigen are elevated on days 1 and 2 after hepatectomy (30, 31). At day 2 the hepatectomy Marimastat™ mice demonstrated a statistically significant lower MI and PCNA index, indicating inhibition of regeneration. The PCNA index continued to be lower on days 4 and 8. Although the median PCNA index was the same in both groups, there was a statistically significant difference in the range of numbers. However, HGF levels between these 2 groups at each time point was not different, indicating that the mechanism of regeneration inhibition by Marimastat™ is not through inhibition of hepatocyte growth factor.

This study demonstrates that liver regeneration is dependent on the proper functioning of MMPs, and can be impaired when an MMP inhibitor is administered. MMPs may direct hepatic growth through their degradation of the extracellular matrix, influence on angiogenesis, or effects on IL-6. The vast number of known physiologic and pathologic processes that are MMP dependent is growing rapidly. Urinary MMP levels have been shown to correlate with the presence of cancer metastases, and likewise, may be used in the future as a marker for normal hepatic regeneration (14).

Acknowledgments

JEV is supported by the American Society of Transplant Surgeons-Roche Laboratories Scientist Research Award and the Robert E. Wise Research Foundation. MP is supported by the CHMC Surgical Foundation and by NIH grant DK069621-01. IPJA is supported by the Dutch Cancer Society. MAM is supported by P01 CA45548 and 1 P50 DK065298.

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Figure 1.

Figure 2.

Figure 3.

Day Eight Post Hepatectomy Serum IL-6 Levels **Day Four Post Hepatectomy CD31 Microvessel Density** **Day Eight Post Hepatectomy CD31 Microvessel Density** **Figure 4.**

Figure 5.