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The Effects of Sorafenib on Liver Regeneration in a Model of Partial Hepatectomy

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Abstract

Background—Sorafenib is currently approved for advanced hepatocellular carcinoma (HCC) and is presently being studied as an adjuvant treatment for HCC following resection. The effects of sorafenib on liver regeneration have not been clearly defined. Our objective was to identify the effects of sorafenib on liver regeneration in a murine partial hepatectomy (PH) model.

Materials and Methods—We performed PH in C57Bl/6 mice treated with a range of sorafenib doses with assessments at several time points. Liver sinusoidal endothelial cells (LSEC) and hepatocyte DNA synthesis and proliferation were assessed with 5-bromo-2-deoxyuridine (BrDU) and Ki67 by flow cytometry and immunohistochemistry (IHC).

Results—Treatment with sorafenib did not result in any deaths following PH. When we measured BrDU uptake to assess DNA synthesis, there was a statistically significant increase at 48 HR post-PH for non-fibrotic LSEC following treatment with 60 mg/kg of sorafenib. However, BrDU and Ki67 staining among LSEC and hepatocytes was not significantly affected by sorafenib at any of the other doses or time points. BrDU and Ki67 flow cytometry data correlated with IHC findings and post-operative liver weights.

Conclusion—In a murine PH model, sorafenib did not alter the repair response of normal or fibrotic livers following PH as measured by changes in liver weight, DNA synthesis, and cellular proliferation. These findings suggest sorafenib administered following hepatic resection does not impair liver regeneration.

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Keywords

Sorafenib; liver regeneration; partial hepatectomy; BrdU; Ki67

INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) has increased within the United States and most patients with HCC will succumb to their disease. Currently, HCC is the fifth most common cancer worldwide with more than 626,000 new cases and 600,000 deaths annually^{1,2}. The only potentially curative option for HCC is surgical resection but only 20–30% of patients diagnosed with the disease in western countries are suitable surgical candidates^{3,4}. Even following potentially curative resection, recurrence rates approach 80%, highlighting the need for effective systemic agents^{5,6}. For those who undergo resection of HCC, successful postoperative outcomes are dependent on the ability of the liver to regenerate and function. Therefore, adjuvant therapy for HCC should not interfere with the ability of the liver to restore its functional volume following resection.

Sorafenib, a multi-kinase inhibitor, targets serine/threonine kinases as well as the receptor tyrosine kinases vascular endothelial growth factor receptor 2 (VEGFR2), VEGFR3, platelet derived growth factor receptor (PDGFR), FLT3, Ret, and c-KIT^{7–11}. In a phase 3 trial, sorafenib prolonged median overall and progression-free survival in patients with advanced HCC¹². Sorafenib is currently approved for advanced HCC treatment, and is now being studied as an adjuvant agent in phase III trials (NCT00692770). The molecular pathways targeted by sorafenib mediate angiogenesis and cell division, both of which are integral components of liver repair and regeneration^{8,13–15}. The effects of sorafenib on liver regeneration remain to be clearly defined. As sorafenib may soon be used in the adjuvant setting following resection of HCC, we must develop an understanding of the effects on liver regeneration. We have investigated the effects of sorafenib on liver regeneration in a murine partial hepatectomy (PH) model in the presence or absence of liver fibrosis. Our study provides insight into the potential implications of sorafenib therapy on recovery of liver mass following hepatic resection.

MATERIALS AND METHODS

Mice

Adult 6–10 week old male C57Bl/6 were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were maintained in the animal care facility at the Roger Williams Medical Center. Procedures were approved by the Institutional Animal Care and Use Committee (IACUC). For serum chemistry analysis, blood was harvested from the heart prior to sacrifice. Histologic sections with routine staining or immunohistochemistry (IHC) were performed by the University of Massachusetts Department of Pathology.

Sorafenib

Sorafenib was generously provided by Bayer HealthCare Pharmaceuticals (Morristown, NJ)/Onyx Pharmaceutical (San Francisco, CA). Sorafenib was diluted in Cremophore (Sigma-Aldrich, St. Louis, MO), ethanol (Pharmco Products Inc, Brookfield, CT), and distilled water per manufacturer recommendations and administered via orogastric tube (Harvard Apparatus, Holliston, MA) one day prior to liver resection and then once daily. Animals were randomly assigned to one of six groups: sham operation, PH + placebo (cremophore + ethanol + water), PH + 15 mg/kg sorafenib, PH + 30 mg/kg sorafenib, PH + 60 mg/kg sorafenib, and PH + 90 mg/kg sorafenib, with 4 mice per dose group for each experiment.

Surgical Procedures

Mice were anesthetized with inhaled anesthetic. Abdomens were shaved and prepared in sterile fashion. An upper-midline laparotomy incision was made and the liver exposed. The left and mid upper lobes were resected to remove approximately 70% of the liver, with the cut liver edges secured with 6-0 silk ties (Ethicon, Somerville, NJ). The peritoneum and fascia were closed with absorbable suture, followed by skin clips. All steps excluding liver resection were performed for sham operations, which were used for the purpose of establishing baseline Ki67 and BrdU staining for IHC and flow cytometry.

Cell Preparation

Liver nonparenchymal cells (NPC) and hepatocytes were isolated as previously described, with modifications¹⁶. Briefly, animals were euthanized and the portal vein injected with 3 ml of 1% (wt/vol) collagenase IV (Sigma, St. Louis, MO) in HBSS. The liver was mechanically disrupted prior to incubation in 10 ml of 1% collagenase at 37°C for twenty minutes. The resulting cell suspension was passed through sterile 100 µm nylon mesh filters (Falcon, BD Biosciences) and centrifuged three times at 50g for 5 minutes and the hepatocytes were collected. The NPC were then isolated from the supernatant and washed in complete media (RPMI 1640, 10% FBS, 2mM L-glutamine, 0.1% 2-mercaptoethanol, 100 u/ml penicillin, 100 ug/ml streptomycin). The pellet containing NPC was resuspended in 40% (wt/vol) Optiprep (Sigma) to enrich the cells. The suspension was layered under 4 ml of GBSS and spun at 500g for 15 minutes. The NPC at the interface were then harvested. Liver NPC were incubated with 1ug/1e6 cells FcR block (BUF041B, AbD Serotec, Raleigh, NC), and CD45- liver sinusoidal endothelial cells (LSEC) were purified using immunomagnetic beads and separation columns (Miltenyi Biotech, Auburn, CA)¹⁶.

Flow cytometry

Flow cytometry was performed on an LSR-II flow cytometer (BD Biosciences, San Jose, CA). Voltages were set based on unstained cells and compensation was adjusted using single-stained controls. For assessment of DNA synthesis, animals were injected with intra-peritoneal (i.p.) 5-bromo-2'-deoxyuridine (BrdU) two hours prior to sacrifice. We measured BrdU incorporation by hepatocytes and LSEC as division of these cell types accounts for the majority of new liver tissue following regeneration^{14,15,17-19}. Samples were incubated with Fc block prior to staining with antibodies against BrdU (51-235AK, BD Biosciences), Ki67 (556027, BD Biosciences) and CD31 (MEC 13.3, BD Biosciences) which were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), and allophycocyanin (APC), respectively.

Statistics

Liver weight was normalized as follows: $[(\text{liver mass}^{\text{EXPERIMENTAL}})/(\text{body mass}^{\text{EXPERIMENTAL}})] / [(\text{liver mass}^{\text{CONTROL}})/(\text{body mass}^{\text{CONTROL}})]$. Liver mass is expressed relative to body mass to control for differences in animal size. The corrected liver mass from experimental animals was normalized to that control animals to demonstrate the extent of liver weight recovery following PH. Body mass values were adjusted by subtracting the weight of skin clips placed during the surgical procedure (0.06 g per clip) from the body weight. Statistical analyses were performed using two-tailed t-tests (Prism 5.00 for Windows, GraphPad Software, San Diego, CA). A p-value < 0.05 was deemed statistically significant.

RESULTS

Effect of Sorafenib on the Hepatocyte Response to Liver Resection

We chose to analyze hepatocytes and LSEC as proliferation of these two cell types accounts for the majority of new liver parenchymal mass following PH^{15,19}. To determine the effects of sorafenib on hepatocyte proliferation, we isolated hepatocytes at 48 HR, 96 HR, and 360 HR (15D) following PH for assessment of Ki67 and BrdU staining. When we measured BrdU uptake to assess DNA synthesis, we found that at 48 HR, 96 HR, and 15 days, hepatocyte proliferation was preserved following treatment with 15 mg/kg, 30 mg/kg, 60 mg/kg, or 90 mg/kg of sorafenib ($p=0.06-0.9$). Likewise, Ki67 staining to quantify cell proliferation revealed that there were no significant differences among the four sorafenib dose groups compared to control animals at all time points ($p=0.08-0.8$). Despite non-significant increases in DNA synthesis and cell proliferation at the higher sorafenib doses at 48 and 96 HR, the values equilibrated at the 15D time point (Figure 1).

Effect of Sorafenib on the LSEC Response to Liver Resection

BrdU uptake by LSEC (CD45- NPC) was similar among controls, 15 mg/kg, 30 mg/kg, 60 mg/kg, or 90 mg/kg doses of the drug. Of note, the 60 mg/kg dose led to a statistically significant increase in LSEC BrdU uptake at 48 hours ($p=0.009$). LSEC Ki67 expression in the 48 HR, 96 HR, and 15 D groups, was not significantly altered by the 15 mg/kg, 30 mg/kg, 60 mg/kg, or 90 mg/kg doses ($p=0.2-0.9$). The non-significant variations in BrdU and Ki67 uptake at 48 HR and 96 HR equilibrated at longer time point of 15 days (Figure 2). Finally, we performed IHC to confirm our flow cytometry data and found that Ki67 staining was similar among dose groups (Figure 3).

Recovery of liver mass following resection and perioperative treatment with sorafenib

To confirm that sorafenib did not negatively impact liver regeneration following PH, we measured liver weight following resection with or without treatment with sorafenib. At 48 HR, 96 HR, and 15D following PH, liver weight recovery was not significantly limited by the 15 mg/kg, 30 mg/kg, 60 mg/kg, or 90 mg/kg of sorafenib when compared to the control group ($p=0.09-0.9$). A trend toward decreased recovery of liver mass in the groups treated with sorafenib for 15 D was not statistically significant (Figure 4). Serum albumin and total protein levels were not diminished in mice treated with sorafenib (not shown).

Perioperative treatment with sorafenib following induction of fibrosis

We expanded the clinical relevance of our data by studying liver regeneration in the setting of liver fibrosis, as many patients with HCC are cirrhotic. To induce liver fibrosis, we injected CCl₄ twice weekly for 8 weeks prior to PH. We chose the 30mg/kg sorafenib dose at assess the liver regeneration at 48 HR, 96 HR, and 15D post-operatively. We found that hepatocyte DNA synthesis as demonstrated by BrdU uptake and cell proliferation as reflected by Ki67 uptake was similar to control at the 48 HR, 96 HR, and 15 D time points ($p=0.09-0.9$). Analysis of LSEC in fibrotic livers revealed no significant effect of sorafenib treatment with respect to Ki67 or BrdU staining ($p=0.3-0.9$). Liver weight recovery was also similar between the control and treated groups at 48 HR, 96 HR, and 15D ($p=0.4-0.5$) (Figure 5).

DISCUSSION

Patients can tolerate resection of up to 70% of liver volume, assuming normal hepatic parenchyma. The ability of the liver to restore its preoperative volume and physiologic function within a short period of time is an important consideration for major hepatic resections¹³⁻¹⁵. This study was designed to define the effects of sorafenib on liver

regeneration in a murine model. As sorafenib may soon be used in the adjuvant setting following resection of HCC, understanding the effects on liver regeneration will be important to determine if perioperative outcomes may be affected.

In our study, multiple doses of sorafenib were tested over a range of time intervals to assess the effects of the drug on the post-resection liver regenerative response. Sorafenib did not impair hepatocyte or LSEC proliferation as measured by Ki67 and BrDU staining with flow cytometry or IHC. Consistent with our cellular proliferation and DNA synthesis data, liver weight was not adversely affected by treatment with sorafenib following PH. Although a trend toward lower liver weight was noted following 15 days of treatment, these results were not significantly different from animals treated with placebo. We cannot exclude the possibility that the results would have reached statistical significance in a study with a larger number of animals. Moreover, although we did find that albumin levels were not affected by sorafenib, measurement of proteins with shorter half-lives may yield more information²⁰.

A previous study by Hora indicated that sorafenib administration impaired liver regeneration when given postoperatively but not when given preoperatively²¹. Several methodological differences between their study and the present one may account for the discrepant findings. We studied normal and fibrotic livers, employed a wider range dose, tested numerous time points, and use additional markers to assess cell proliferation. Importantly, we normalized our liver weight measurements to animals receiving placebo for each time point following partial hepatectomy rather than unmanipulated mice at one time point.

The model used in the present study does not address how sorafenib may affect liver regeneration in animals with intrahepatic tumor. The interactions between tumor cells and native liver cell populations may alter the liver regeneration pathway with possible changes at the transcriptional level^{22,23}. Furthermore, the effects of sorafenib on hepatic immune and inflammatory cell biology remain to be defined. Alterations in the phenotype and function of liver nonparenchymal cells may not only impact the repair and regenerative responses to PH, but may have immunologic implications as well.

As we incorporate adjuvant targeted molecular agents into multi-disciplinary cancer care paradigms, the anti-tumor effects must be considered in the context of potential adverse effects on normal physiology and the response to surgical trauma. Our pre-clinical study suggests that sorafenib does not significantly impair liver regeneration following partial hepatectomy. Correlation with data from ongoing clinical trials will be useful to confirm our findings.

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Figure 1. Hepatocyte BrdU and Ki67 staining following partial hepatectomy

Animals were treated with sorafenib at varying doses or placebo and euthanized at several time points. Liver cells were isolated and stained with anti-BrDU or anti-Ki67 to measure the effects of sorafenib on DNA synthesis and cell proliferation, respectively using flow cytometry. Data represent mean values from 4 mice per dose level. The experiments shown are representative of 4 repetitions.

Figure 2. LSEC BrdU and Ki67 staining following partial hepatectomy

Animals were treated with sorafenib at varying doses or placebo and euthanized at several time points. Liver cells were isolated and stained with anti-BrDU or anti-Ki67 to measure the effects of sorafenib on DNA synthesis and cell proliferation, respectively using flow cytometry. Data are expressed as percentages of cells positive for each marker relative to animals treated with control. Data represent mean values from 4 mice per dose level. The experiments shown are representative of 4 repetitions.

Figure 3. Immunohistochemistry analysis of Ki67 uptake in normal liver (100× and 400×)
A. Sham operation + placebo B. PH + Placebo C. PH + Sorafenib 90mg/kg D. PH + 60mg/kg E. PH + 30mg/kg F. PH + 15mg/kg. Data shown represent 48 hours time point.

Figure 4. Normalized liver weight following partial hepatectomy

Post-operative liver weights were normalized to body weight and expressed relative to control animals. Animals were treated with sorafenib at varying doses or placebo and euthanized at several time points. Data represent mean values from 4 mice per dose level. The experiments shown are representative of 4 repetitions.

Figure 5. Hepatocyte and LSEC Ki67 staining and liver weight following 96 hours post partial hepatectomy in CCl_4 induced liver fibrosis

Animals with CCl_4 induced liver fibrosis were treated with sorafenib or placebo and euthanized at several time points. Liver cells were isolated and stained with antibodies specific for BrDU or Ki67 to measure the effects of sorafenib on DNA synthesis and cell proliferation, respectively using flow cytometry. Data are expressed as percentages of cells positive for each marker relative to animals treated with control. Normalized liver weight data is shown as well. Data represent mean values from 4 mice per dose level. The experiments shown are representative of 3 repetitions.