

Hepatic Resection but Not Radiofrequency Ablation Results in Tumor Growth and Increased Growth Factor Expression

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Objective: The purpose of this study was to examine the effects of radiofrequency ablation (RFA) on tumor growth and growth factor expression in a murine model.

Background: Surgical excision remains the only potentially curative therapy for hepatic malignancies. Tumor growth in the remaining liver may be accelerated after resection. The mechanism of this enhanced tumor growth remains unexplained, although growth factors that are released after hepatic resection (which facilitate liver regeneration) may play a role in residual tumor growth. RFA has become a viable alternative for patients who are not candidates for a curative resection. The effect of RFA on tumor growth and growth factor expression has not been studied.

Methods: Hepatic tumors were established by direct injection with CT-26, a murine adenocarcinoma. Tumors were treated by either partial hepatic resection (PH) or RFA. Hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF) expression was measured at selected time intervals post-treatment. Tumor growth was measured by reinjection of CT-26 into the residual liver after treatment. Nine days after reinjection, tumor volume was calculated and compared with nontreated controls.

Results: HGF and bFGF expression was significantly higher at baseline in the CT-26 tumor-bearing mice when compared with non-tumor-bearing controls ($P = 0.00001$ and $P = 9 \times 10^{-7}$, respectively). There was an increase in HGF and bFGF expression at 24 hours ($P = 0.005$, and $P = 0.001$) in the PH group. In the RFA group, there was a decrease in HGF and bFGF expression at 24 and 72 hours ($P = 0.001$ and $P = 0.002$). Tumor growth comparisons revealed an increase in tumor growth in the hepatectomy group ($P = 0.006$) but not the RFA group ($P = 0.2$).

Conclusions: Baseline growth factor expression in tumor-bearing mice is exponentially higher when compared with non-tumor-bearing controls. HGF and bFGF expression are increased posthepatectomy, and decreased post-RFA. Partial hepatectomy results in an increase in tumor growth in the residual liver. RFA did not increase tumor growth after treatment. While hepatectomy is the only curative option for patients with hepatic malignancies, it may accelerate growth of microscopic residual disease.

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Primary and metastatic hepatic tumors are a common cause of death worldwide. Chemotherapy and radiation therapy are relatively ineffective against these tumors.¹ Resection has traditionally been the primary curative option available for patients presenting with hepatic malignancies.² After tumor resection, the liver is the most frequent site of recurrence. This implies that microscopic residual disease is a common occurrence. A number of authors have suggested that residual tumor growth is accelerated after resection^{3–5}; however, the exact mechanism of this enhanced tumor growth remains unexplained.

The liver exhibits a remarkable potential to regenerate after partial hepatectomy (PH). Advances in molecular and cellular biology have provided clues to the mechanism by which the liver regenerates. Growth factors are released in a timely and controlled fashion immediately after resection. TGF- α , EGF, IL-6, FGF, TNF- α , and insulin like growth factor expression are all increased after resection; however, inhibiting these growth factors does not inhibit liver regeneration.⁶

Hepatocyte growth factor (HGF), also known as scatter factor, was first described in 1984.⁷ HGF is produced by the fat storing Ito cells of the liver within 1 hour after PH,⁸ peaks by 24 hours, and remains elevated for 7 days before returning to baseline.⁵ The inhibition of HGF results in failure of the remaining liver to regenerate after resection.⁹ Hepatocyte growth factor is a potent stimulator of growth in normal hepatocytes and some neoplasms. It is also a stimulus for increased motility of malignant cells by both a paracrine and autocrine mechanisms.¹⁰ The treatment of tumor cells in vivo with HGF results in an increase in the metastatic potential of the treated cells.^{11–13} C-met, the 190-kDa transmembrane receptor for HGF, is present in virtually all human colorectal cell lines.¹¹

Basic fibroblast growth factor (bFGF) also plays an important role in liver regeneration following resection. It rises exponentially and its peak coincides with the peak of hepatocyte DNA synthesis at 24 hours following PH. This elevation lasts for several days before returning to baseline at 7 days. bFGF promotes angiogenesis and vascularization in the regenerating liver,¹⁴ and is also responsible for promoting tumor growth by similar mechanisms.⁵ The gene for bFGF is a potential oncogene and influences tumor growth by promoting vascularization.¹⁵ Administration of bFGF to nude mice bearing human colon carcinoma xenografts resulted in increased tumor growth and decreased hypoxic fractions of tumors.⁵ The elevated expression these growth factors fol-

lowing resection may explain the accelerated growth of residual tumors after PH.

While resection remains the gold standard for treatment of hepatic carcinomas, only 5% to 20% of such tumors have clinical and pathologic features that are favorable for resection at the time of presentation.^{16,17} Factors limiting surgical resection include size, site, number of tumors, vascular and extrahepatic involvement, poor general condition, and poor liver function.¹⁸ The use of local ablative techniques (radio-frequency ablation [RFA], cryoablation, and microwave ablation), has expanded the population of patients with treatable tumor.

RFA is the most popular minimally invasive thermal ablation technique.^{1,18–29} To perform RFA an electrode is introduced into the tumor. This can be done via an open approach, laparoscopy, or percutaneously. Tissue surrounding the electrode is heated by ionic friction from RF current, which induces coagulation necrosis once tissue temperature exceeds 50°C.³⁰ However, it is unknown what effect RFA has on residual tumor growth or growth factor response after treatment. The purpose of this study was 2-fold: 1) a comparison of residual tumor growth in mice treated with either RFA or partial hepatic resection, and 2) a measurement of the growth factor response in each of these groups to their respective treatment modality.

MATERIALS AND METHODS

Animal Tumor Model

CT-26, an undifferentiated murine adenocarcinoma that was induced by rectal injection of N-nitroso-N-methylurethane in BALB/c mice, was provided by Dr. Nicholas Restifo (Surgery Branch, National Cancer Institute). The cell line was maintained in RPMI 1640 (Biowhittaker, Walkersville, MD) cell culture media supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1% minimal Eagle medium nonessential amino acids (Biowhittaker), and 1× Antibiotic-Antimycotic (Gibco BRL, Grand Island, NY) in a humidified atmosphere of 5% CO₂ at 37°C. For these experiments, all tumors were created by direct hepatic injection of 1 × 10⁵ of the CT-26 tumor cells in 50 μL of PBS.

BALB/c female mice, 6 to 8 weeks of age, were obtained from the Harlan-Sprague-Dawley animal facility (Indianapolis, IN) and Taconic Farms (Germantown, NY). Animals were housed and fed standard mouse chow and water ad libitum. All animal experiments were conducted in accordance with principles stated in *Guide for the Care and Use of Laboratory Animals* (NIH publication 86-23, National Institutes of Health, Bethesda, MD, 1985). These experiments were approved by the University of Wisconsin Animal Care Committee, Madison, WI. For induction of tumor, 80 BALB/c mice were anesthetized by an intraperitoneal injection of ketamine 100 μg/xylozine 10 μg/g of the mouse. After sedation, a small right subcostal incision was made the liver was exposed and then injected with the tumor solution.

CT-26 tumor cells were injected in all mice for 9 days before treatment. This time interval was assigned based upon previous in vivo studies (data not shown). Nine days postin-

jection consistently created reproducible and measurable tumor volumes. Fewer than 9 days produced smaller tumors; and when tumor was left for more than 9 days, mice were more likely to have extrahepatic metastasis and less likely to tolerate treatment.

Growth Factor Expression

Growth factor expression was evaluated in normal liver before and after hepatectomy or RFA. Liver samples were taken from 10 mice to measure baseline HGF and bFGF expression in the non-tumor-bearing population. Seventy mice underwent injection of CT-26 tumor cells into the right or left lobe of the liver. Nine days after tumor implantation, the animals underwent repeat laparotomy. Samples were taken from 10 mice to measure baseline HGF and bFGF expression in the tumor-bearing population. RFA (n = 30) or PH (n = 30) was then performed. Mice were then divided into groups of 10 for both RFA and PH. Samples were taken at designated time intervals: 2, 4, 6, 12, 24, 48, 72, 120, and 168 hours post-treatment. This allowed for a plot of exponential increase. Graphically, only time-points taken at 24 (n = 10), 72 (n = 10), and 168 (n = 10) hours for each RFA and PH group are shown. All liver samples were limited to 0.5 mg of tissue to avoid excess manipulation of the liver and to minimize unintentional growth factor release.

RFA and PH

A 22 needle was used as an RF electrode and was inserted into the tumor under direct visualization. An RF generator (Radionics, Burlington, MA) was connected to the electrode and provided power of 3 W for 3 minutes under temperature control. Grounding pads were placed under the shaved back of the animals.

Partial hepatic resection was performed via subcostal incision. The tumor-bearing hepatic lobe was resected distal to a 5-0 silk suture. All tumors in the PH group were completely excised, and all tumors in the RFA group were completely ablated. This was confirmed pathologically after sectioning.

Real-Time Quantitative PCR

The total RNA of the liver tissues were purified by Qiagen RNA purification kit and 2 μg of total RNA were reverse transcribed by using a Moloney murine leukemia virus reverse transcription (Promega). Transcribed cDNA was purified by QIAquick nucleotide removal kit. (Qiagen). The concentration of cDNA was obtained using spectrophotometry; 10 ng of cDNA was amplified by real-time PCR under the following conditions: (94°C, 30 seconds; 65°C, 30 seconds; and 72°C, 30 seconds) for 50 cycles (iCycler, iQmix cyber green Bio-Rad). The following sequences were used as primers: HGF (sense) 5' ATGTCCTCCTGCACCTCCTCCT 3' and (antisense) 5' GTAAGAGTAGTTTTTGCTGACT 3'. bFGF (sense) 5' CACCTGCACGGCATCCTG 3' and (antisense) 5' GCTGTGATCCTGCCGGGT 3' (National Center for Biotechnology Information).

Normal mouse liver cDNA was used for real-time quantitative PCR standard. House keeping gene primers were supplied by commercial source of mouse GAPDH, which is

ubiquitous to all tissues (Catalog No. GMO 0104. Biosource International, Inc., Camarillo, CA). A 10-fold serial dilution of the cDNA from 1 μg to 1×10^{-7} μg was used for generating a standard curve. Cycle threshold was plotted against starting concentration in micrograms. The log of the resultant values yielded a linear correlation, which allowed for the comparison of unknown samples to known concentrations of housekeeping genes.

A standard real-time quantitative PCR block is capable of processing 63 samples at a given session. Each sample was repeated multiple times, and at each new PCR block a new serial dilution of house keeping genes was also created in triplicate. Data presented for growth factor expression represent an average of the means and resultant standard error of hundreds of samples per given specimen.

Tumor Growth

The effect of hepatectomy or RFA on tumor growth was assessed after treatment. Hepatectomy or RFA was performed on both animals with tumor and on non-tumor-bearing animals. All animals then had implantation of tumor into the residual liver.

Control

The animals underwent anesthesia ($n = 10$) and laparotomy in the same fashion as previously described. After sedation, CT-26 tumor cells were injected into the right lobe of the liver. Nine days after tumor implantation, the animals were killed, tumor volume was measured, and normal liver was sampled for growth factor measurement.

Group 2

Nine days after tumor implantation, 11 mice underwent repeat laparotomy. Tumors were treated by RFA ($n = 5$) or PH ($n = 6$) and mice were then reinjected with tumor cells into the right/left hepatic lobe. Nine days after the second tumor injection, all mice were killed, tumor volume was measured, and normal liver was sampled for growth factor measurement.

Group 3

RFA ($n = 5$) or PH ($n = 5$) was performed on non-tumor-bearing mice. Immediately after treatment, the nontreated liver was injected with tumor cells. Nine days after tumor implantation, the animals were killed, tumor volume was measured, and normal liver was sampled for growth factor measurement.

Tumor Volume Measurement

After death, the livers were removed and sectioned at 2-mm intervals. Tumor maximum and minimum diameters were measured at each interval and used to calculate areas for each section. The total volume per tumor was calculated by multiplying the area for each lesion by the slice thickness, and summing each individual volume: $V_{\text{total}} = (A_1 \times T) + (A_2 \times T) \dots (A_n \times T)$ where A = the area of each individual slice, T = slice thickness, and n = total number of slices.

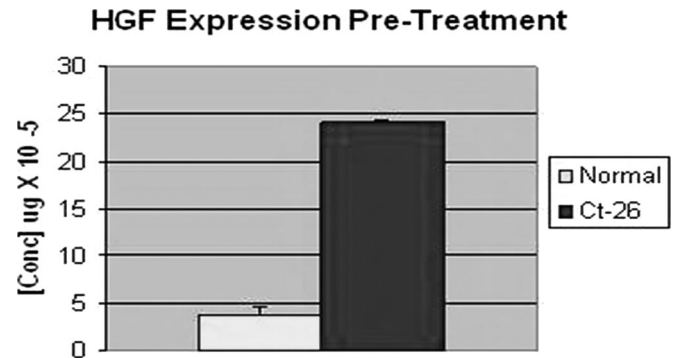


FIGURE 1. Comparison of baseline HGF expression between tumor-bearing mice and non-tumor-bearing controls. Time reflected in hours.

Statistical Analysis

Data were analyzed using Student t test assuming unequal variance. Data were considered significant if $P < 0.05$.

RESULTS

Growth Factor Expression

HGF and bFGF expression in normal liver was evaluated in both tumor and non-tumor-bearing mice without treatment (Figs. 1 and 2). There was a 5-fold increase in HGF expression at baseline in the CT-26 tumor-bearing mice when compared with non-tumor-bearing controls ($P = 0.00001$). Likewise, there was 7-fold increase in bFGF expression in the CT-26 tumor-bearing mice ($P = 9 \times 10^{-7}$).

Growth factor expression was measured at 24, 72, and 168 hours post-treatment (Figs. 3–6). After PH, HGF expression was increased at 24 and 72 hours ($P = 0.005$, and $P = 0.01$, respectively) with a peak at 24 hours before returning to baseline at 168 hours ($P = 0.41$) in the PH group. In the RFA group, there was a decrease in HGF expression at 24 hours ($P = 0.001$). The HGF levels at 72 hours and 168 hours were lower than baseline pretreatment levels, but the difference was not statistically significant ($P = 0.07$).

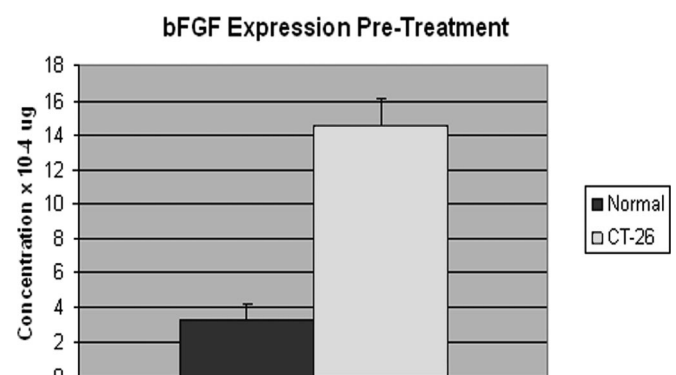


FIGURE 2. Comparison of baseline bFGF expression between tumor-bearing mice and non-tumor-bearing controls. Time reflected in hours.

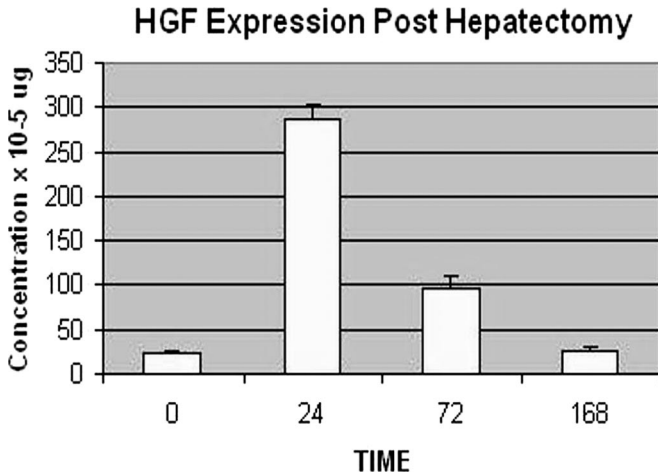


FIGURE 3. HGF expression posthepatic resection demonstrates significant increase at 24 hours.

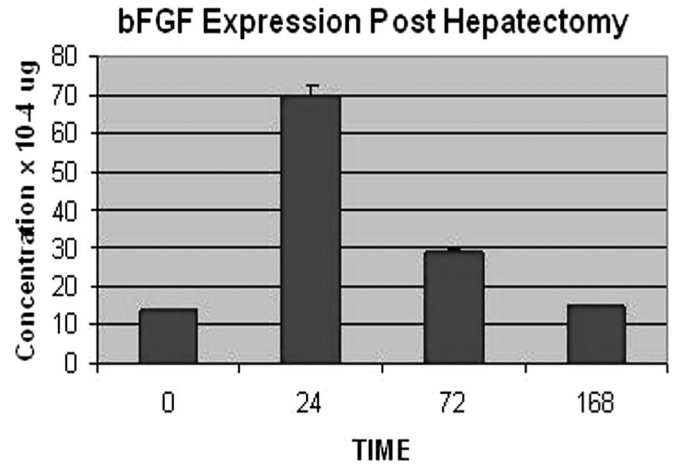


FIGURE 5. Hepatic resection results in an increase in bFGF expression at 24 hours.

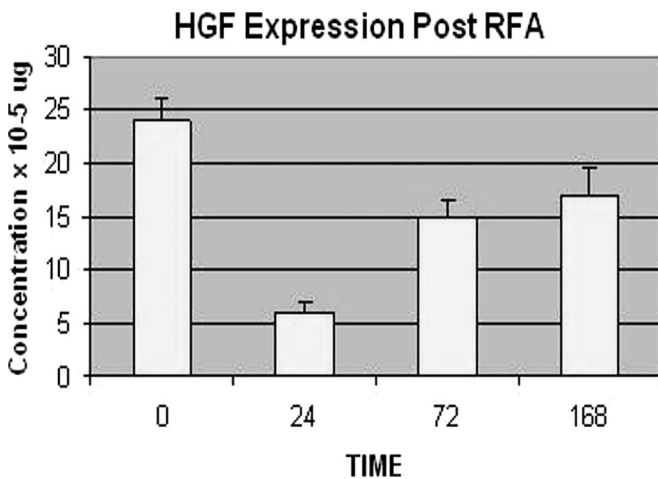


FIGURE 4. HGF expression post-RFA with a significant decrease seen at 24 hours post-treatment.

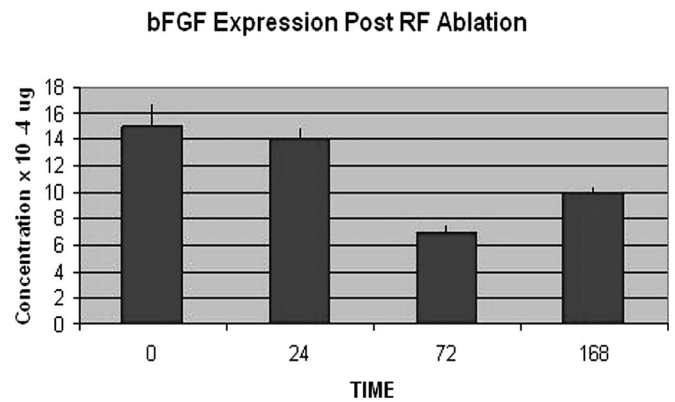


FIGURE 6. bFGF expression post-RFA demonstrates a decrease in expression at 72 hours.

After PH bFGF expression was increased at 24 and 72 hours ($P = 0.001$ and $P = 0.0003$, respectively) with a peak at 24 hours before returning to baseline at 168 hours ($P = 0.12$). In the RFA group, bFGF expression was unchanged at 24 hours ($P = 0.32$) but decreased at 72 hours ($P = 0.002$). There was a trend to return to baseline (CT-26 positive) levels at 168 hours ($P = 0.34$). However, similar to HGF expression, bFGF expression did not fully return to pretreatment levels of expression.

Tumor Growth

The average tumor volume for the control group was $2.84 \pm 0.63 \text{ cm}^3$. Group 2 (injection + treatment + reinjection) had an average tumor volume of $2.71 \pm 0.83 \text{ cm}^3$ for the RFA group and $11.96 \pm 2.12 \text{ cm}^3$ for the PH group. Group 3 (treatment + injection) had an average tumor volume of $8.24 \pm 1.62 \text{ cm}^3$ in the PH group and $1.2 \pm 0.65 \text{ cm}^3$ in the RFA group (Fig. 7).

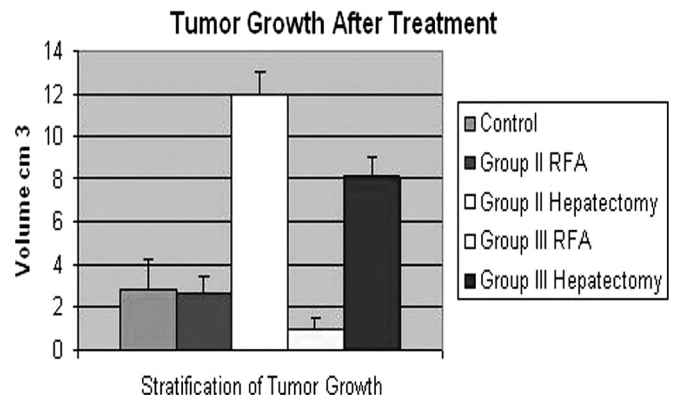


FIGURE 7. Comparison of tumor growth between mice undergoing treatment with radiofrequency ablation or partial hepatic resection.

In group 2, there was a statistically significant increase of residual tumor growth in mice after PH ($P = 0.006$), but not after RFA ($P = 0.2$). Also in group 3, there was a significant increase in tumor growth for mice who underwent PH ($P = 0.03$) but not after RFA ($P = 0.4$).

All mice who underwent PH (groups 2 and 3 combined) had a statistically significant increase in tumor growth ($P = 0.006$). This effect was not seen in any mice who underwent RFA ($P = 0.2$).

DISCUSSION

The most common site of recurrence after hepatic resection for metastatic colorectal cancer is the liver, implying that microscopic residual disease is a common occurrence.^{3,31} Several studies have suggested that surgical resection may increase residual tumor growth.^{2-5,31,32}

The liver is unique when compared with other solid organs due to its remarkable ability to regenerate. Specific growth factors are produced in a predictable and timely fashion following resection. However, it is not well understood what effect these cytokines have on residual tumor growth. HGF and bFGF are cytokines that play a key role, not only in regeneration of the resected liver, but also have been linked to the growth of neoplastic tissues.⁵ Inhibition of HGF by NK4, an HGF-antagonist/angiogenesis inhibitor, suppresses liver metastasis and invasive growth of colon cancer in mice.³³

We found that baseline CT-26 tumor-bearing mice had a significant increase in both HGF and bFGF expression when compared with non-tumor-bearing controls. We can only hypothesize that the tumor is stimulating the residual liver to regenerate despite the lack of hepatic parenchymal loss. However, we have no kinetics postinjection to monitor the rise in this growth factor expression. HGF and bFGF expression increased exponentially (above the baseline elevation) after hepatic resection and peaked at 24 hours before returning to baseline at 7 days. This increase after hepatectomy is similar to previous reports.^{5,6,9} RFA, unlike hepatectomy, resulted in a decrease in HGF expression at 24 hours and bFGF expression at 72 hours. This seems counterintuitive given that RFA also results in trauma to the liver and should result in an increase in growth factor release. A possible explanation for the decrease in HGF and bFGF is that eradication of the tumor removed the tumor stimulus for growth factor expression and that ablation unlike resection was not a stimulant of HGF and bFGF expression. Partial hepatectomy resulted in a relative increase in tumor growth in all experiments. The mice in group 2 (CT-26 injection, followed by PH and reinjection) had a 6-fold increase in tumor volume ($11.96 \pm 2.12 \text{ cm}^3$) compared with RFA-treated animals ($2.71 \pm 0.83 \text{ cm}^3$) or controls ($2.84 \pm 0.63 \text{ cm}^3$). Tumor growth in RFA-treated animals and controls was not different. The CT-26 tumor-bearing mice already had a higher baseline level of growth factor expression, but this was the same in both resection and RFA-treated animals. Adding an additional stimulator of growth factor release (resection) allowed for an increase in HGF and bFGF available not only to the regenerating liver, but also to the newly implanted tumor cells. We postulate that this increase in growth factor expression resulted in enhanced tumor growth in the resection animals.

We were concerned that the elevated baseline levels of HGF and bFGF (although perhaps in the most clinically

relevant model) may contribute to tumor growth. A third group of animals (group 3) with no prior tumor and normal baseline HGF and bFGF levels was thus included. The mice in group 3 (treated with hepatectomy or RFA and subsequent CT-26 injection) had significantly larger tumor volumes ($8.24 \pm 1.62 \text{ cm}^3$) when compared with RFA-treated animals ($1.2 \pm 0.65 \text{ cm}^3$) or controls ($2.84 \pm 0.63 \text{ cm}^3$) at the same time point. No RFA-treated animals in group 3 had increased tumor growth compared with controls. Tumor volumes were larger in group 2 compared with their counterparts in group 3, implying that the higher baseline levels of growth factors resulted in enhanced tumor growth.

The impact of RFA on tumor growth in the residual liver seems initially to be at odds with clinical data indicating that RFA has a higher intrahepatic recurrence rate compared with PH. In this report, the initial tumor burden and the completeness of tumor treatment were the same in all cohorts. In contrast to this murine study, patients treated clinically with RFA are different from patients treated with PH. RFA patients are typically not surgical candidates because of either underlying liver dysfunction or technical inability to completely remove all tumor volume surgically. Many other factors also have an impact on intrahepatic recurrence, including adequacy of complete tumor treatment, tumor size, experience of the treating clinician, the heat sink effect of vascular flow, and underlying tumor biology. These factors have translated into higher local recurrence rates in RFA patients in the area of the ablation, perhaps not because of an increase in HGF and bFGF but despite an increase in HGF and bFGF in hepatectomy patients.

There are limitations to this study. We did not standardize the volume of liver ablated to the volume of liver resected. Our goal was a comparison of both growth factor release and tumor growth between the 2 treatment modalities, which mimicked as closely as possible clinical scenarios. Hepatic resection often involves removal of a substantial segment of normal hepatic parenchyma anatomically to achieve margins. The goal of RFA is to completely kill tumor and minimize the damage to surrounding tissues. Therefore, the volume of resection and volumes of ablations are rarely equal. The RFA volume was approximately 75% of the resection volume. We did not perform experiments in which ablated volume equaled resection volume as we thought this would weaken the clinical relevance. The striking differences we demonstrate seem unlikely to be a result of the size of the resection or ablation. Also, we did not perform survival analysis between mice undergoing RFA and partial hepatic resection. However, the design of this study lends to easily perform survival studies in the future. Given the paucity of data comparing survival between the 2 treatment modalities, these experiments may help identify differences that may be applicable to human outcomes.

CONCLUSION

Mice bearing CT-26 tumors have exponentially increased baseline HGF (5-fold) and bFGF (7-fold) expression when compared with non-tumor-bearing mice. HGF and

bFGF expression increased exponentially after PH, whereas this was not seen in the mice treated with RFA. Rather, there was a decrease in both HGF and bFGF expression at 24 and 72 hours, respectively, following treatment with RFA. Hepatectomy resulted in an increase in tumor growth compared with RFA-treated animals and controls. There was no difference in tumor volumes in any of the mice treated with RFA compared with controls. Growth factors active in normal liver regeneration may play a role in tumor growth. Hepatic tumors may place the liver in a state of chronic regeneration, allowing its use of intrinsic growth factors to potentiate tumor progression. While hepatectomy is the only curative option for patients with hepatic malignancies, it could potentially accelerate the growth of unrecognized residual disease. RFA does not appear to induce accelerated growth of residual tumor.

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