Published in final edited form as:

Surgery. 2008 June ; 143(6): 790–802.

Stem cell factor and its receptor, c-kit, are important for hepatocyte proliferation in wild type and TNF receptor-1 knock out mice after 70% hepatectomy

Xiaodan Ren, Bin Hu, and Lisa Colletti

Department of Surgery, University of Michigan Medical School, Ann Arbor, MI 48109

Abstract

Background—Stem cell factor (SCF) has well-known proliferative effects on hematopoietic cells. SCF also has effects on differentiation and proliferation in other cell types. Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF) have proliferative effects in the liver. Recent studies in our laboratory have linked SCF's hepatoproliferative actions to those of IL-6, demonstrating that IL-6-induced hepatocyte proliferation is dependent, at least in part, on SCF. We now hypothesize that TNF's hepatoproliferative effects are also dependent on SCF.

Methods and Results—In vitro studies using primary mouse hepatocytes show that SCF is induced by TNF; anti-SCF antibody treatment in this system inhibits TNF-induced hepatocyte proliferation, suggesting that TNF-induced hepatocyte proliferation is also SCF-dependent. Additional in vivo experiments were performed in which wild type and/or TNF receptor-1 knock out mice (TNFR1 $-/-$) were subjected to 70% hepatectomy or sham laparotomy. TNFR1 $-/-$ mice are known to have delayed hepatic regeneration after partial hepatectomy. Initial experiments demonstrated that the SCF receptor, c-kit, is up-regulated after partial hepatectomy in wild type mice, further emphasizing the importance of this system in the restoration of hepatic mass. SCF administration to TNFR1 $-/-$ mice in the context of partial hepatectomy restores hepatocyte proliferation to normal. Further, SCF administration to TNFR1 $-/-$ mice prior to hepatectomy increases p-stat-3 levels, suggesting that SCF-induced increases in hepatocyte proliferation may also be stat-3-mediated.

Conclusions—These data suggest that TNF-induced hepatocyte proliferation is dependent, at least in part, on SCF and that SCF and its receptor, c-kit, are important for the liver's regenerative processes.

Introduction

The regenerative response of the liver can be triggered by surgical resection or toxic, ischemic, inflammatory, or traumatic hepatic injury and is characterized by the rapid onset of hepatocyte proliferation, resulting in recovery of a functional liver mass within a period of weeks to

Address all correspondence to: Lisa M. Colletti, M.D., 2922G Taubman, Box 0331, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0331, Phone: 734-936-7944, Fax: 734-936-5830, Email: colletti@umich.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

This PDF receipt will only be used as the basis for generating PubMed Central (PMC) documents. PMC documents will be made available for review after conversion (approx. 2-3 weeks time). Any corrections that need to be made will be done at that time. No materials will be released to PMC without the approval of an author. Only the PMC documents will appear on PubMed Central -- this PDF Receipt will not appear on PubMed Central.

months. Cell proliferation ceases once the liver reaches a species- and age-specific percentage of the total body mass.

Stem cell factor (SCF) is a hematopoietic factor, inducing leukocyte maturation and differentiation¹. Additional studies have suggested that this molecule is not only important for hematopoiesis, but also for gametogenesis and melanogenesis². Recent studies in our laboratory have suggested that SCF has mitogenic properties in the liver after partial hepatectomy³. Other investigators have also shown that SCF plays a role in the liver's recovery from a toxic injury, specifically an acetaminophen-induced hepatic injury⁴. SCF is initially found as a transmembrane protein that is enzymatically cleaved from the cell surface during injury and inflammation¹. Thus, it appears that SCF can be expressed by several different cells populations and be quickly released after cellular injury; since it is stored in a transmembrane form, some tissues, such as the liver, may have large reservoirs of available SCF^{1,4}.

SCF exerts its biological effect by binding to its receptor, c-kit. Significant reservoirs of both SCF and c-kit have been documented in the liver³⁻⁸. SCF and c-kit also have documented actions in neoplastic processes, further suggesting a regulatory function during cellular proliferation⁹⁻¹¹. Since the liver is a unique organ in that it is the only organ in the human body that repairs itself with functional hepatic tissue as opposed to scar, it is also not unexpected that it has significant reservoirs of both c-kit and SCF, consistent with the observations that these factors play a role in hepatic regeneration following partial hepatectomy³⁻⁸.

Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF) have proliferative effects in the liver¹²⁻²³. Recent studies in our laboratory have linked SCF's hepatoproliferative effects to those of IL-6³. In these studies, SCF was shown to have proliferative effects on hepatocytes, both in vitro and in vivo after partial hepatectomy³. Further, IL-6 was shown to induce hepatocyte production of SCF and treatment of IL-6 knock out mice with SCF after partial hepatectomy restored liver regeneration to normal³. Other investigators have also documented this relationship between IL-6 and SCF in other cellular systems²⁴.

Investigations have shown a link between the actions of IL-6 and TNF during liver regeneration. Mice lacking TNF receptor-1 (TNFR1 -/-) have decreased IL-6 levels, decreased stat-3 binding, and the defects in hepatic regeneration that are seen in these animals can be reversed by IL-6 administration^{12-19, 26}. In the current study, we hypothesize that there is a link between TNF and SCF in the setting of hepatic regeneration following partial hepatectomy in the mouse, possibly mediated via IL-6.

Materials and Methods

Seventy percent hepatectomy model

All experiments were performed in compliance with the standards for animal use and care set by the University of Michigan's Committee for the Use and Care of Animals. Experiments were conducted using six to eight week old male CBA/J mice, SCF-deficient mice (Sl/Sld), TNF receptor-1 knock out mice (TNFR1 -/-), and their appropriate wild type controls (Jackson Breeding Laboratories, Bar Harbor, ME). Since complete SCF knock out mice are very fragile animals which do not tolerate general anesthesia or laparotomy, our experiments utilized Sl/Sld mice; these mice are "partial" SCF knock outs, ie are heterozygous for the gene deletion, express low levels of SCF, and are much more tolerant to general anesthesia and surgery. Partial (70%) hepatectomy was performed as previously described^{27,28}. Anatomically, this consists of resection of the median and left lateral hepatic lobes. Anesthesia was induced with subcutaneous ketamine hydrochloride (100 mg/kg) and maintained with isoflurane inhalation. Unless otherwise noted, a minimum of five animals were used per treatment group per time point for all in vivo studies.

Administration of exogenous SCF or TNF

In vivo studies were conducted to measure the effects of exogenous SCF or TNF on liver regeneration after partial hepatectomy. Exogenous murine recombinant TNF (Peprotech, Rocky Hill, NJ), exogenous murine recombinant SCF (Peprotech, Rocky Hill, NJ), or vehicle (sterile phosphate buffered saline (PBS)) administration was accomplished by bolus tail vein injection. SI/Sld and TNFR1 $-/-$ mice received a dose of 0.4 μ g SCF/mouse (each mouse weighed approximately 20 grams) two hours prior to operation. For the experiments involving administration of exogenous TNF, recombinant murine TNF (Peprotech, Rocky Hill, NJ) was injected in a dose of 5 ng/gram of body weight two hours prior to operation and 24 hours after operation; this dosing regime has been used in previously described experiments in the literature related to the hepatoproliferative effects of TNF^{14,16-18}. In both cases, control animals received the same volume of vehicle³.

Production of anti-SCF, anti-TNF and control antibodies²⁷

Rabbit anti-murine SCF or anti-TNF antibodies were elicited from New Zealand White Rabbits as previously described using murine recombinant SCF or recombinant TNF (Peprotech, Rocky Hill, NJ) in complete Freund's adjuvant²⁷. Direct ELISA was used to titer the resultant polyclonal antibodies and the IgG portion of the serum was purified over a protein A column. Serum from unimmunized rabbits was also purified over protein A column and this purified IgG portion of serum was utilized as control antibody. As previously described, the quality of the antibodies was investigated using an in vitro assay in which the ability of the antibody to block SCF-induced mast cell activation and migration is measured²⁹.

Liver weight/total body weight ratios

For the liver weight/body weight ratios, liver weights are expressed as a percentage of total body weight. Since SI/Sld and TNFR1 $-/-$ mice are generally smaller than their wild type controls and also regain body weight after laparotomy more slowly than wild type animals, liver weight/body weight ratios are utilized instead of liver weights alone.

Primary hepatocyte isolation and culture

Primary mouse hepatocyte isolation was accomplished by collagenase perfusion as previously described²⁷. After isolation, the cells are plated in Media 199 with 10% fetal calf serum, 10% horse serum, 10 mM Hepes, 10⁵ U/L penicillin/streptomycin, 1.6 U/L insulin, and 4 $\times$ 10⁻⁷ M dexamethasone and incubated at 37°C under 5% CO₂. Trypan blue exclusion is used to determine hepatocyte viability, which was generally 85-95%. Hepatocyte purity is typically 90-95%, as determined by LDL staining.

IL-6 and HGF ELISA

The serum and hepatic levels of IL-6 and HGF were measured as per the manufacture's instructions using ELISA kits purchased commercially (Quantikine mouse IL-6 and mouse HGF DuoSet ELISA Development kit, R&D Systems, Minneapolis, MN). Hepatic IL-6 and HGF levels were measured after homogenization of 0.03g of frozen liver tissue in 300 μ l of PBS buffer (pH7.4) containing a cocktail of protease inhibitors (Complete™, Boehringer Mannheim). IL-6 and HGF levels were normalized to total protein levels in the liver tissue, as measured by BCA protein assay kit (Pierce, Rockford, IL). Serum levels of IL-6 were normalized per ml of serum.

SCF ELISA

A modification of a double ligand ELISA technique was used to quantify SCF as previously described^{27,29}. Initial in vitro studies were performed to measure cellular production of SCF.

For these studies, cell-free supernatants were collected to quantitate soluble SCF. Because SCF is found in both soluble and transmembrane forms, additional in vitro experiments were conducted in which both soluble and transmembrane (bound) SCF were measured. In these experiments, cells and supernatants were collected and sonicated, in order to quantitate both forms of SCF. All in vitro studies were performed in triplicate and repeated a minimum of three times. ELISA assays were also performed in triplicate. Standards were created with 1/2 from 10 pg/ml to 100 ng/ml log dilutions of recombinant mSCF; SCF levels were expressed as ng/ml.

In vitro measurement of hepatocyte DNA synthesis by ^3H -thymidine incorporation²⁷

In vitro primary hepatocyte proliferation was measured by incorporation of ^3H -thymidine as previously described²⁷. Cells were incubated with 0.5 uCi of ^3H -thymidine for 18 hours, were then harvested onto glass wool filters, and ^3H -thymidine incorporation measured by liquid scintillation counting on a Beckman counter (Fullerton, CA). Each experiment repeated was repeated a minimum of three times and each ^3H assay was performed in triplicate.

Flow cytometric determination of c-kit expression after 70% hepatectomy

Animals underwent hepatectomy or sham laparotomy and were sacrificed 24, 48, or 72 hours post-operatively; hepatocytes were immediately harvested and studied with flow cytometry for c-kit expression. Once harvested, cells were washed twice in staining buffer (DIFCO, Detroit, MI), resuspended in 100 μl staining buffer, and incubated for 30 minutes at 4°C in the dark with PE labeled anti-CD117 or PE labeled rat IgG2b κ (BD PharMingen, San Diego, CA) diluted in 100 μl of staining buffer. Final antibody concentrations were 1-2 $\mu\text{g}/100$ cells. After incubation, cells were washed twice in staining buffer, and analyzed immediately. Flow cytometry was performed using a FACScan cytometer (Becton Dickinson, Mountain View, CA). Data were collected and analyzed using CellQuest software. A minimum of 10,000 viable cells were analyzed to determine cell-surface c-kit receptor expression.

Hepatic immunohistochemical staining for SCF

Liver tissues were fixed in 10% buffered formalin over night and embedded in paraffin. Five- μm sections from tissue blocks were cut and mounted on Superfrost Plus slides (Fisher Scientific, Itasca, IL), deparaffinized in xylene, and then rehydrated into distilled H_2O through graded alcohols. Antigen retrieval was enhanced by microwaving slides in citrate buffer (pH 6.0, Biogenex, San Ramon, CA) for 10 minutes at 50% power. Endogenous peroxidase activity was quenched by incubation with 6% hydrogen peroxide in methanol. Slides were then incubated with primary rabbit polyclonal SCF antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:20 overnight at 4°C. Slides were washed three times in PBS and then incubated with goat biotinylated anti-rabbit secondary antibody for 30 minutes at room temperature. Antigen-antibody complexes were detected with the avidin-biotin-peroxidase method using diaminobenzidine as a chromogenic substrate (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) per the manufacturer's protocol. Tissue sections were lightly counter-stained with hematoxylin and then examined by light microscopy.

In vivo measurement of hepatocyte DNA synthesis by bromodeoxyuridine (BrdU) incorporation

For these experiments, animals underwent partial hepatectomy or sham laparotomy; two hours before sacrifice, animals were injected intraperitoneally with 30 μg BrdU per gram of body weight. Animals were sacrificed, liver specimens were obtained and fixed in 4% paraformaldehyde for 24 hours, processed for histological analysis, and stained using the Amersham cell proliferation kit, as per the manufacture's instructions (Amersham Pharmacia Biotech Limited, United Kingdom). For the BrdU experiments, there were three mice per

treatment group per time point and five separate low power fields were analyzed for each animal.

Western blot analysis

Whole cell lysates were prepared from frozen liver samples as previously described³. BCA protein assay Kit (Pierce, Rockford, IL) was used to quantitate total protein levels. Western blot analysis was also performed as previously described³. Briefly, 60 µg of total cell lysate were electrophoresed on a 12% polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories Inc., Hercules, California). Membranes were blocked in 5% dry milk and then incubated with primary antibody for phosphotyrosine signal transducer and activator of transcription at 1:100 (p-stat-3; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or Pro-TGF- α at 1:1000 (Cell Signaling Technology, Beverly, MA). Antibodies were diluted in 5% dry milk in Tris-buffered saline with 0.1% Tween 20. Membranes were washed and the appropriate secondary antibodies in a dilution of 1:5000 were then added. The enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Piscataway, NJ) was used to detect antigen-antibody complexes. In order to have an internal protein loading control, these blots were then stripped and reanalyzed using anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) monoclonal antibodies (Chemicon International, Inc., Temecula, CA). The maximum OD for each well was normalized to the maximum OD for the same well for GAPDH to correct for slightly unequal protein loading. Each experiment was repeated a minimum of five times. Densitometry was utilized to further assess each gel. Representative gels are illustrated and the accompanying densitometry represents the mean values from five gels from five separate animal groups.

Statistical analysis³⁰

Data is expressed as the mean $\pm$ standard error of the mean (SEM). Groups of data were analyzed by analysis of variance by the methods of Student-Newman-Keuls to indicate groups with significant differences³⁰. P values of less than 0.05 were considered to indicate a statistically significant difference. A PowerPC 7100 computer using the Statview II statistical software package (Abacus Concepts, Inc.) was used for data analysis.

Results

Hepatocyte SCF production in response to TNF

Primary mouse hepatocytes were isolated and allowed to adhere overnight. They were then stimulated with 20ng/ml TNF and harvested at 1, 2, 4, 8, 12, and 24 hours of incubation. Soluble and soluble plus bound SCF were then measured by ELISA. As illustrated in Figure 1, primary mouse hepatocytes produce significant amounts of both soluble and bound SCF in response to stimulation with TNF. Levels of soluble and soluble plus bound SCF are significantly increased as compared to media alone. TNF stimulation also results in a significant increase in the production of bound SCF, as estimated by the differences in SCF levels between the soluble as compared to the soluble plus bound groups.

Hepatocyte proliferation in response to TNF and SCF

It is well-documented that TNF induces hepatocyte proliferation^{16-18,20,26}. Previous investigations in our laboratory have shown that SCF also induces hepatocyte proliferation³. Because TNF induces hepatocyte SCF production and because both TNF and SCF are capable of inducing hepatocyte proliferation, we were interested in investigating whether the proliferative actions of these molecules were linked. Previous studies in our laboratory have shown that in vitro doses of SCF in the range of 10-25 ng/ml result in significant hepatocyte proliferation³. Similar dose response experiments have demonstrated that antibody doses of

10 micrograms/liter results in adequate blockade of the molecules in question³. Therefore, hepatocyte proliferation was measured following stimulation with media alone, TNF (20 ng/ml), SCF (20 ng/ml), TNF (20 ng/ml) plus anti-SCF antibody (10 micrograms/liter), or SCF (20 ng/ml) plus anti-TNF antibody (10 micrograms/liter). As illustrated in Figure 2, both TNF alone and SCF alone induce significant hepatocyte proliferation, as compared to media alone. When hepatocytes were incubated with TNF and anti-SCF antibody the previously seen increase in hepatocyte proliferation was obliterated; a parallel decrease in proliferation was not seen when cells were incubated with SCF and anti-TNF antibody. These data, coupled with the data in Figure 1, suggests that TNF up regulates SCF and that some of the proliferative effects of TNF in the liver are likely related to SCF.

Administration of SCF to wild type and TNFR1 $-/-$ mice prior to partial hepatectomy increases both hepatic and serum levels of IL-6

Wild type and TNFR1 $-/-$ mice were treated with SCF or vehicle control as previously described. Animals were sacrificed kinetically and serum and liver specimens were obtained for IL-6 and HGF measurement by ELISA. These investigations demonstrated that SCF increases both serum and hepatic levels of IL-6; these effects were statistically significant 1 hour following hepatectomy; while levels were increased in SCF-treated mice as compared to vehicle-treated mice out to 24 hours, statistical significance was only seen 1 hour post-hepatectomy (Figure 3A and 3B).

Administration of SCF to wild type and TNFR1 $-/-$ mice prior to partial hepatectomy does not alter hepatic or serum HGF levels, but does increase hepatic TGF- α levels

SCF administration to wild type or TNFR1 $-/-$ mice prior to hepatectomy did not alter hepatic or serum HGF levels, as measured by ELISA (data not shown). In contrast, SCF administration prior to partial hepatectomy did increase hepatic TGF- α levels in both wild type and TNFR1 $-/-$ mice. TGF- α levels were measured by Western blot analysis. In the TNFR1 $-/-$ mice, this effect is most marked at 1 hour post-hepatectomy, but is still present 4 hours post-resection and diminishes at 8 and 24 hours (Figure 4). In the wild type mice, the increase in TGF- α is also noted at 1 hour post-resection, but persists out to 8 hours post-resection (Figure 4). Interestingly, minimal to no TGF- α is detected in the wild type mice at 8 and 24 hours post-operatively. In contrast, significant levels of TGF- α are noted out to 24 hours post-operatively in the TNFR1 $-/-$ mice, with and without treatment with SCF (Figure 4).

Expression of c-kit on hepatocytes post-hepatectomy in wild type and TNFR1 $-/-$ mice

Hepatocyte c-kit expression was measured by flow cytometry. Primary murine hepatocytes were isolated from both wild type mice and TNFR1 $-/-$ mice at various time points after 70% hepatectomy. As shown in Figure 5, a gradual rise in c-kit expression was seen in both wild type and TNFR1 $-/-$ mice undergoing partial hepatectomy, with significant increases in c-kit expression occurring 24, 48, and 72 hours post-hepatectomy as compared to sham operated control animals. In general, c-kit expression was higher in wild type mice undergoing partial hepatectomy as compared to TNFR1 $-/-$ mice undergoing partial hepatectomy, with this difference reaching statistical significance at 16 hours post-operatively (Figure 5). The increase in c-kit expression parallels our previous findings related to hepatic SCF expression post-hepatectomy. In these experiments, hepatic SCF levels initially decrease after partial hepatectomy, with a concurrent increase in serum SCF levels, followed at 24-48 hours by a decrease in serum SCF levels and an increase in hepatic SCF levels to supranormal levels³. The currently observed increase in hepatic c-kit expression directly parallels the previously documented increases in hepatic SCF production.

Hepatic immunohistochemical staining for SCF after partial hepatectomy in wild type and TNFR1 $-/-$ mice

Wild type and knock out mice underwent 70% hepatectomy; immunohistochemical staining for SCF was then performed on liver tissue obtained at 24, 36, 48 and 60 hours after resection. In wild type mice, SCF expression was increased within 24 hours of partial hepatectomy and peaked at 48 hours post-operatively. Although a similar pattern of SCF expression occurred in livers of TNFR1 $-/-$ mice, overall SCF expression was obviously lower than that seen in the wild type mice (Figure 6). Staining for SCF protein was clearly demonstrated in the hepatocyte (Figure 6: insets for Panels E and F), which is in contrast to what has been published by other investigators⁸.

In vivo hepatocyte proliferation after partial hepatectomy in TNFR1 $-/-$ mice with and without SCF treatment

Prior investigations in our laboratory have documented that SCF is important for hepatocyte proliferation after partial hepatectomy in mice³. In addition, these studies also suggested that IL-6 upregulates SCF, and that SCF is responsible for at least some of the proliferative effects of IL-6 in this model³. Based on additional data in the literature that suggests that TNF and IL-6 have overlapping functions and because they appear to be linked^{16-18,26}, we were interested in investigating whether there are additional inter-relationships between TNF and SCF.

Therefore, our next experiments investigated whether SCF administration to TNFR1 $-/-$ mice could augment hepatic regeneration following partial hepatectomy. Initial experiments evaluated liver weight as a percentage of total body weight after 70% hepatectomy (Figure 7). There were no significant differences at any time point in wild type mice treated with vehicle or wild type mice treated with SCF. As would be expected based on prior published data, liver regeneration is delayed in TNFR1 $-/-$ mice after partial hepatectomy; liver weight as a percentage of total body weight is significantly decreased at 7 days post-hepatectomy as compared to wild type mice treated with vehicle or wild type mice treated with SCF. However, TNFR1 $-/-$ mice treated with SCF after partial hepatectomy had an increased rate of hepatic weight gain; liver weight as a percentage of total body weight in these animals was not significantly different from liver weight as a percentage of total body weight in wild type animals treated with vehicle or wild type animals treated with SCF after partial hepatectomy (Figure 7). Following partial hepatectomy, TNFR1 $-/-$ mice treated with SCF had significantly increased liver weight as a percentage of total body weight as compared to TNFR1 $-/-$ mice treated with vehicle after partial hepatectomy (Figure 7).

Based on the results of these initial experiments, the effects of SCF on hepatic proliferation in TNFR1 $-/-$ mice was further investigated by BrdU staining as an estimate of in vivo hepatocyte proliferation. These results are illustrated in Figure 8A, B, and C. As shown in Figure 8A, and as has been previously published, SI/SId mice have significantly delayed hepatocyte proliferation after partial hepatectomy and this effect is reversed by SCF administration³. SCF has no obvious effects in wild type animals³. Figure 8B illustrates that TNFR1 $-/-$ mice also have delayed hepatocyte proliferation after partial hepatectomy, with significantly decreased BrdU staining 48 and 60 hours post-hepatectomy, as compared to wild type animals treated with either vehicle or SCF; this has also been previously published¹⁶⁻¹⁸. TNFR1 $-/-$ mice treated with SCF in the context of partial hepatectomy demonstrate a significant increase in hepatocyte proliferation. TNFR1 $-/-$ mice treated with SCF after partial hepatectomy not only have normalization of hepatocyte proliferation, but these rates were also significantly increased as compared to wild type animals treated with vehicle (Figure 8B). Of significance, treatment of SI/SId mice with TNF had no effect on hepatocyte proliferation after partial hepatectomy (Figure 8C).

Enhance hepatocyte proliferation in response to SCF in TNFR1 $-/-$ mice is stat-3-mediated

Prior investigations have suggested that TNF-induced hepatocyte proliferation following hepatectomy may occur via a signal transducer and activator of transcription-3 (stat-3)-mediated pathway¹⁶⁻¹⁸. Since prior investigations in our laboratory suggest that SCF-induced hepatocyte proliferation may also occur via a stat-3-mediated pathway, we next investigated whether stat-3 may also be involved in the SCF-induced increases in hepatocyte proliferation observed in TNFR1 $-/-$ mice. Figure 9 illustrates a representative Western blot for cytosolic p-stat-3. The densitometric analysis of 5 separate Western blots from 5 different animals is also shown in this figure. These experiments demonstrate that by 3 hours post-hepatectomy, there is a significant decrease in p-stat-3 levels in the TNFR1 $-/-$ mice as compared to wild type control. Further, treatment of both wild type and TNFR1 $-/-$ mice with SCF prior to hepatectomy significantly increases cytosolic p-stat-3, as compared to both untreated groups at one hour post-hepatectomy; this effect disappeared by 3 hours post-hepatectomy in the wild type animals, but persists in the TNFR1 $-/-$ mice and restores p-stat-3 levels to those of wild type controls at this time point (Figure 9). SCF's effects on p-stat-3 occurred at early time points following partial hepatectomy; no effects on p-stat-3 levels were seen at later time points (after 6 hours; data not shown). Despite the increases p-stat-3 that are observed in wild type animals treated with SCF, no increases were observed in proliferation levels.

Discussion

Previous investigations have shown that TNF and IL-6 are important for liver regeneration^{12,13,15,17-23,26}. Kupffer cells appear to be the most important source of TNF in this setting, while IL-6 can be produced by many cells within the liver¹⁵. Some of the proliferative effects of TNF may be related to upregulation of transforming growth factor- α (TGF- α)³¹. Experiments in primary mouse hepatocytes have suggested that at least some of TNF's proliferative effects are related to upregulation of TGF- α ³¹; these results emphasize the complex and overlapping systems that are involved in hepatic repair and regeneration. Our results also suggest that TGF- α may be involved in TNF-mediated hepatocyte proliferation post-hepatectomy; SCF treatment of both wild type and TNFR1 $-/-$ mice after partial hepatectomy resulted in increases in hepatic TGF- α levels. One of the earliest TNF-dependent effects to occur during liver regeneration is the activation of NF- κ B via the TNFR1 receptor^{14,17-19}. Prior investigations have documented that mice deficient in TNF-receptor 1 regenerate their livers more slowly than normal animals after a variety of injuries and that stat-3 binding was also decreased in these animals¹⁶⁻¹⁸. More importantly, following hepatic injury, these animals also showed decreased levels of IL-6 protein and mRNA; IL-6 injection into these animals restored hepatocyte proliferation to normal¹⁶⁻¹⁸. Other investigations have also documented that TNF-induced hepatocyte proliferation is related to IL-6 upregulation^{17,18,26}. In these studies, treatment with anti-TNF antibodies in the setting of partial hepatectomy decreased hepatocyte proliferation and also decreased hepatic IL-6 levels²⁶. The experiments outlined in this study further support the inter-relationships between TNF, IL-6, and SCF. Our prior investigations demonstrated that SCF restored hepatocyte proliferation in IL-6 knock out mice after partial hepatectomy³; prior investigations by Fausto and colleagues showed that IL-6 administration to TNFR1 $-/-$ mice also restored hepatocyte proliferation to normal in the context of carbon tetrachloride-induced liver injury¹⁶. Since our current study documents that SCF also restores hepatocyte proliferation to normal in TNFR1 $-/-$ mice, this suggests that SCF's proliferative effects are inter-related with those of both TNF and IL-6; a possible cascade is TNF upregulates IL-6, which in turn upregulates SCF, which may function, at least in part, via a stat-3-dependent mechanism. Bone-Larson and colleagues have also shown that SCF treatment in the context of acute septic peritonitis results in accelerated expression of hepatic stat-3 levels⁵. While activation of NF- κ B via the TNFR1 receptor has been documented to be an important pathway in hepatic

regeneration, the stat-3 pathway is also important. Prior investigations have suggested that both SCF and IL-6 function via a stat-3-dependent pathway; this is also corroborated by this study, particularly by the differences in p-stat-3 expression in wild type versus TNFR1 $-/-$ mice after partial hepatectomy and the restoration of p-stat-3 levels in the TNFR1 $-/-$ mice by treatment with SCF prior to hepatectomy. These data suggest that stat-3 may be involved in the hepatoproliferative effects of SCF and TNF in this model, although this does not definitively illustrate that stat-3 is critical to the end effects of these mediators.

Recent studies have shown that TNF may initiate liver regeneration, with most of these studies based on investigations using antibody neutralization of TNFF or TNFR1 $-/-$ mice^{17,18,26}. Studies using TNFR1 knock out mice have shown dramatic decreases in the NF-KB and stat-3 binding that usually occur within the first few hours after partial hepatectomy; concurrent with this, liver regeneration is significantly impaired^{17,18}. Similar decreases in stat-3 binding after partial hepatectomy are also observed in IL-6 knock out mice¹⁹. In both TNFR1 $-/-$ mice and IL-6 knock out mice, a single injection of IL-6 at the time of partial hepatectomy causes normalization of stat-3 binding and restores liver regeneration to normal¹⁷⁻¹⁹. Further, the impaired hepatic regeneration that is seen following partial hepatectomy in TNFR1 $-/-$ mice correlates with persistently low IL-6 levels in both the plasma and the liver. TNFR1 activation is a potent inducer of hepatic NF-KB, whereas stat-3 is directly activated by IL-6^{18,19}. The link between these two events is that NF-KB is a transactivator of IL-6³². This suggests that signaling via the TNFR1 receptor initiates hepatic regeneration via an IL-6-dependent pathway that is regulated via a stat-3 signal transduction mechanism. Our studies also suggest that SCF is part of this pathway and also may function via a stat-3-mediated pathway. In addition, other studies have also shown that SCF is up regulated by TNF, particularly in the context of hepatic injury³³. The current study suggests that SCF may upregulate IL-6 and TGF-alpha. When SCF is given to TNFR-1 $-/-$ mice, both IL-6 and TGF-alpha are increased relative to levels seen in vehicle-treated control animals. This suggests that SCF-induced normalization of hepatocyte proliferation in the TNFR-1 $-/-$ mice may also be at least partially related to upregulation of both IL-6 and TGF-alpha.

Stem cell factor has been described to signal via the jak-stat-3 pathway, which is also a critical pathway in hepatic regeneration³⁴. More recently, SCF and c-kit have also been shown to be involved in the proliferation and differentiation of potential hepatocyte progenitor cells within the liver, substantiating the data presented here^{35,36}. Recent studies in our laboratory have documented that SCF is produced and released in response to hepatic resection and that SCF induces hepatocyte proliferation and is critically important for liver regeneration following 70% hepatectomy³. In these studies, SCF production was induced by IL-6 and IL-6-induced hepatocyte proliferation was blocked by the administration of anti-SCF antibodies. This is somewhat in contrast to the current study, which suggests that SCF upregulates IL-6 in the TNFR-1 $-/-$ mice. Further, SCF administration to IL-6 knock out mice after partial hepatectomy restored liver regeneration to normal, and was mediated via a stat-3 signal transduction mechanism³. The current study supports these data in that we have also documented parallel increases in the SCF receptor c-kit. These previous data suggest that IL-6-induced hepatocyte proliferation in the context of 70% hepatectomy is SCF-dependent and mediated via stat-3. In addition, the current study also suggests that there is a large reservoir of hepatic SCF that is mobilized at the time of hepatic resection. Our in vitro studies document increases in both soluble and bound SCF in response to stimulation with TNF. In addition, in vivo flow cytometry also documents a significant increase in c-kit receptor expression after partial hepatectomy.

Our prior investigations support the data that is presented in the current study. The current data suggest that TNF also functions via an IL-6- and SCF-dependent mechanism. In addition, our current data also suggests that TGF-alpha may also be involved in this pathway. Prior studies have shown that defective liver regeneration in TNFR1 $-/-$ mice is reversed by IL-6

administration^{17,18}. Prior studies in our laboratory demonstrate that impaired liver regeneration in IL-6 knock out mice is reversed by SCF³. The current study suggests that there may be a pathway which involves TNF, IL-6 and SCF in the context of hepatic regeneration and that this may at least partially involves stat-3.

Acknowledgements

This work was supported by National Institutes of Health grant number 1R01 DK58106

References

1. Galli SJ, Zsebo KM, Geissler KM. The kit ligand, stem cell factor. *Adv Immunol* 1994;55:1–96. [PubMed: 7508174]
2. Reith, AD.; Bernstein, A. Molecular biology of the W and Steel loci. In: Davis, KE.; Tilghman, S., editors. *Genes and phenotypes*. 3. New York: Cold Spring Harbor Laboratory; 1991. p. 105-133.
3. Ren X, Hogaboam C, Carpenter A, Colletti LM. Stem cell factor restores hepatocyte proliferation in IL-6 knock out mice following 70% hepatectomy. *J Clin Invest* 2003;112:1407–1418. [PubMed: 14597766]
4. Simpson K, Hogaboam CM, Kunkel SL, Harrison DJ, Bone-Larson C, Lukas NW. Stem cell factor attenuates liver damage in a murine model of acetaminophen-induced hepatic injury. *Lab Invest* 2003;83:199–206. [PubMed: 12594235]
5. Bone-Larson CL, Hogaboam CM, Steinhauser ML, Oliveira SHP, Lukacs NW, Strieter RM, et al. Novel protective effects of stem cell factor in a murine model of acute septic peritonitis: Dependence on MCP-1. *Am J Pathol* 2000;157:1177–1186. [PubMed: 11021822]
6. Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin FH, Atkins HL, et al. Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell* 1990;63:213–224. [PubMed: 1698556]
7. Fujio K, Evarts RP, Hu A, Marsen ER. Expression of stem cell factor and its receptor, c-kit, during liver regeneration from putative stem cells in adult rat. *Lab Invest* 1994;70:511–516. [PubMed: 7513770]
8. Fujio K, Hu Z, Evarts RP, Marsden ER, Nui CH, Thorgeirsson SS. Co-expression of stem cell factor and c-kit in embryonic and adult liver. *Exp Cell Res* 1996;224:243–250. [PubMed: 8612701]
9. Ricotti E, Fagioli F, Garelli E, Linari C, Crescenzo N, Horenstein AI, et al. C-kit is expressed in soft tissue sarcoma of neuroectodermic origin and its ligand prevents apoptosis of neoplastic cells. *Blood* 1998;91:2397–2405. [PubMed: 9516139]
10. Papadimitriou CA, Topp MS, Serve H, Oelmann E, Koenigsmann M, Maurer J, et al. Recombinant human stem cell factor does exert minor stimulation of growth in small cell lung cancer and melanoma cell lines. *Eur J Cancer* 1995;31:2371–2378.
11. Turner AM, Zsebo KM, Martin F, Jacobsen FW, Bennett LG, Broudy VC. Nonhematopoietic tumor cell lines express stem cell factor and display c-kit receptors. *Blood* 1992;80:374–381. [PubMed: 1378316]
12. Selzner N, Selzner M, Odermatt B, Tian Y, Van Rooijen N, Clavien PA. ICAM-1 triggers liver regeneration through leukocyte recruitment and Kupffer cell-dependent release of TNF-alpha/IL-6 in mice. *Gastroenterol* 2003;124:692–700.
13. Selzner N, Selzner M, Tian Y, Kadry Z, Clavien PA. Cold ischemia decreases liver regeneration after partial liver transplantation in the rat: A TNF-alpha/IL-6-dependent mechanism. *Hepatology* 2002;36:812–818. [PubMed: 12297828]
14. Chaisson ML, Brooling JT, Ladiges W, Tsai S, Fausto N. Hepatocyte-specific inhibition of NF-kappaB leads to apoptosis after TNF treatment, but not after partial hepatectomy. *J Clin Invest* 2002;110:193–202. [PubMed: 12122111]
15. Iwai M, Cui TX, Kitamura H, Saito M, Shimazu T. Increased secretion of tumor necrosis factor and interleukin-6 from isolated, perfused liver of rats after partial hepatectomy. *Cytokine* 2001;13:60–64. [PubMed: 11145844]

16. Yamada Y, Fausto N. Deficient liver regeneration after carbon tetrachloride injury in mice lacking type 1, but not type 2 tumor necrosis factor receptor. *Am J Pathol* 1998;152:1577–1589. [PubMed: 9626061]
17. Yamada Y, Webber EM, Kirillova I, Peschon JJ, Fausto N. Analysis of liver regeneration in mice lacking type 1 or type 2 tumor necrosis factor receptor: requirements for type 1 but not type 2 receptor. *Hepatology* 1998;28:959–970. [PubMed: 9755232]
18. Yamada Y, Kirillova I, Peschon JJ, Fausto N. Initiation of liver growth by tumor necrosis factor: Deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. *Proc Natl Acad Sci USA* 1997;94:1441–1446. [PubMed: 9037072]
19. Cressman DE, Greenbaum LE, DeAngelis RQA, Ciliberto G, Furth EE, Poli V, et al. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. *Science* 1996;274:1379–1383. [PubMed: 8910279]
20. Iocca HA, Ison HC. Tumor necrosis factor-alpha acts as a complete mitogen for primary rat hepatocytes. *Am J Pathol* 2003;163:465–476. [PubMed: 12875968]
21. Peters M, Blinn G, Jostock T, Schirmacher P, Buschenfelde KHMZ, Galle PR, et al. Combined interleukin-6 and soluble interleukin-6 receptor accelerates murine liver regeneration. *Gastroenterology* 2000;119:1663–1671. [PubMed: 11113088]
22. Zimmers TA, McKillop IH, Pierce RH, Yoo JY, Koniaris LG. Massive liver growth in mice induced by systemic interleukin-6 administration. *Hepatology* 2003;38:326–334. [PubMed: 12883476]
23. Blindenbacher A, Wang X, Langer I, Savino R, Terracciano L, Heim MH. Interleukin-6 is important for survival after partial hepatectomy in mice. *Hepatology* 2003;38:674–682. [PubMed: 12939594]
24. Peters MS, Goldschmidt F, Schirmacher J, Rose-John S. Interleukin-6 and the soluble interleukin-6 receptor induced stem cell factor and Flt-3L expression in vivo and in vitro. *Exper Hematol* 2001;29:146–155. [PubMed: 11166453]
25. Fong Y, Tracey KJ, Moldawer LL, Hesse DG, Manogue KB, Kenney JS, et al. Antibodies to cachectin/tumor necrosis factor reduce interleukin 1 beta and interleukin-6 appearance during lethal bacteremia. *J Ex Med* 1989;170:1627–1633.
26. Akerman P, Cote P, Yang SQ, McClain C, Nelson S, Bagby GJ, et al. Antibodies to tumor necrosis factor-alpha inhibit liver regeneration after partial hepatectomy. *Am J Physiol* 1992;263:G579–G585. [PubMed: 1415718]
27. Colletti LM, Green M, Burdick M, Kunkel SL, Strieter RM. Proliferative Effects of CXC Chemokines in Rat Hepatocytes In Vitro and In Vivo. *Shock* 1998;10:248–257. [PubMed: 9788656]
28. Higgins GM, Anderson RM. Restoration of the liver of the white rat following partial surgical removal. *Arch Path* 1931:186–202.
29. Lukacs NW, Strieter RM, Lincoln PM, Brownell E, Pullen DM, Schock HJ, et al. Stem cell factor (c-kit ligand) influences neutrophil recruitment and histamine levels in allergic airway inflammation. *J Immunol* 1996;156:3945–3951. [PubMed: 8621935]
30. Glantz, SA. Alternatives to analysis of variance and the t test based on ranks. In: Glantz, SA., editor. *Primer of Biostatistics*. New York: McGraw-Hill; 1987. p. 287-331.
31. Gallucci RM, Simeonova PP, Toriumi W, Luster MI. TNF-alpha regulates transforming growth factor-alpha expression in regenerating murine liver and isolated hepatocytes. *J Immunol* 2000;164:872–878. [PubMed: 10623834]
32. Libermann TA, Baltimore D. Activation of interleukin-6 gene expression through the NF-KB transcription factor. *Mol Cell Biol* 1990;10:2327–2334. [PubMed: 2183031]
33. Brito JM, Borojevic R. Liver granulomas in schistosomiasis: mast cell-dependent induction of SCF expression in hepatic stellate cells is mediated by TNF-alpha. *J Leuk Biol* 1997;62:389–396.
34. Jacobs-Helber SM, Penta K, Sun Z, Lawson A, Sawyer ST. Distinct signaling from stem cell factor and erythropoietin in HCD57 cells. *J Biol Chem* 1997;272:6850–6853. [PubMed: 9054369]
35. Baumann U, Crosby HA, Ramani P, Kelly DA, Strain AJ. Expression of the stem cell factor receptor c-kit in normal and diseased pediatric liver: identification of a human hepatic progenitor cell? *Hepatology* 1999;30:112–117. [PubMed: 10385646]
36. Matsuoka S, Tsujimura T, Toyosaka A, Nakaso K, Sugihara A, Okamoto E, et al. Role of c-kit receptor tyrosine kinase in development of oval cells in the rat 2-acetylaminofluorene/partial hepatectomy model. *Hepatology* 1999;29:670–676. [PubMed: 10051467]

Figure 1. Primary mouse hepatocyte SCF production in response to TNF in vitro

Primary mouse hepatocytes were stimulated with TNF or media alone and ELISA was used to determine soluble or soluble+bound levels of SCF over time. There were no significant differences noted in levels of soluble as compared to soluble+bound SCF in cells incubated in media alone. In contrast, primary mouse hepatocytes produce significant amounts of both soluble and bound SCF in response to stimulation with TNF. Levels of soluble and soluble plus bound SCF are significantly increased as compared to media alone. (+p<0.05 and *p<0.01 versus both media alone groups). TNF stimulation also results in a significant increase in the production of bound SCF, as estimated by the differences in SCF levels between the soluble as compared to the soluble plus bound groups (*p<0.05 versus soluble levels).

Figure 2. In vitro primary mouse hepatocyte proliferation in response to SCF or TNF with and without treatment with anti-TNF or anti-SCF antibody

Treatment of primary mouse hepatocytes with TNF alone or SCF alone results in a significant increase in hepatocyte proliferation at 24 hours of incubation (+ $p < 0.05$ media alone versus all other treatment groups). Next, cells were treated with SCF and anti-TNF antibody; this treatment did not result in a significant change in hepatocyte proliferation. In contrast, when cells were treated with TNF and anti-SCF antibody, a significant decrease in hepatocyte proliferation was observed (* $p < 0.05$ versus all other groups except media alone).

Figure 3. Administration of SCF to wild type and TNFR1 $-/-$ mice prior to partial hepatectomy increases both hepatic and serum levels of IL-6

Administration of SCF prior to partially hepatectomy increases both serum and hepatic levels of IL-6 as compared to animals treated with vehicle; these effects were statistically significant at 1 hour following hepatectomy (* $p < 0.05$ and ** $p < 0.01$); while levels were increased in SCF-treated mice as compared to vehicle-treated mice out to 24 hours, statistical significance was only seen at 1 hour post-hepatectomy. Figure 3A: Serum IL-6 levels, Figure 3B: Hepatic IL-6 levels.

Figure 4. Administration of SCF to wild type and TNFR1 $-/-$ mice prior to partial hepatectomy increase hepatic TGF- α levels as measured by Western blot analysis
 Representative Western blots are illustrated; GAPDH levels are also shown to demonstrate equal loading of the gels. SCF administration prior to partial hepatectomy increases hepatic TGF- α levels in both wild type and TNFR1 $-/-$ mice, particularly at early time points.

c-kit expression after partial hepatectomy

Figure 5. Hepatocyte c-kit expression following partial hepatectomy in wild type and TNFR1 $-/-$ mice

Primary hepatocytes were isolated after 70% hepatectomy and c-kit expression was measured by flow cytometry. Following partial hepatectomy, a gradual rise in c-kit expression was seen in both wild type and TNFR1 $-/-$ mice, with significant increases in c-kit expression occurring in both groups at 24, 48, and 72 hours post-hepatectomy, as compared to sham operated control animals (* $p < 0.05$ for wild type sham laparotomy versus wild type partial hepatectomy, ** $p < 0.05$ for TNFR1 $-/-$ sham laparotomy versus TNFR1 $-/-$ partial hepatectomy). While in general, c-kit expression was higher in wild type mice undergoing partial hepatectomy as compared to TNFR1 $-/-$ mice undergoing hepatectomy, this difference reached statistical significance at the 16 hour time point (***) $p < 0.05$).

Figure 6. Hepatic immunohistochemical staining for SCF after partial hepatectomy in wild type and TNFR1 $-/-$ mice

Panels A, C, E, and G are from wild type mice. Panels B, D, F, H are from TNFR1 $-/-$ mice. Time points are listed on the left side of each set of representative panels. All photomicrographs were taken at 200X; inserts in Panels E and F are at 400X. In general, SCF expression begins to increase at 24 hours post-hepatectomy, with maximal increases seen at 48 hours post-hepatectomy. Wild type mice show an obvious increase in hepatic SCF expression as compared to TNFR1 $-/-$ mice.

Figure 7. Liver weight as a percentage of total body weight in wild type and TNFR1 $-/-$ mice after 70% hepatectomy with and without treatment with SCF

There were no significant differences in liver weight/body weight ratios for wild type animals +vehicle as compared to wild type animals+SCF at any time point. At 7 days post-hepatectomy, TNFR1 $-/-$ mice treated with vehicle have a significantly decreased liver weight/body weight ratio as compared to all other groups (* $p < 0.05$ versus wild type+vehicle, wild type+SCF, and TNFR1 $-/-$ + SCF). For TNFR1 $-/-$ undergoing hepatectomy and treatment with SCF, at 7 days post-hepatectomy, liver weight/body weight is restored to that of wild type controls; there are no significant differences between this group and either of the wild type groups.

Figure 8. BrdU staining following partial hepatectomy in: 8A. SI/Sld or wild type mice treated with vehicle or SCF, 8B. TNFR1 $-/-$ or wild type mice treated with vehicle or SCF, and 8C. SI/Sld or wild type mice treated with TNF or vehicle

Figure 8A compares the effects of SCF or vehicle on hepatocyte proliferation following 70% hepatectomy in SI/Sld mice or wild type mice and shows that BrdU staining is significantly decreased in SI/Sld mice treated with vehicle ($*p < 0.05$ as compared to wild type+vehicle, wild type+SCF, and SI/Sld+SCF). Hepatocyte proliferation is restored to normal levels in SI/Sld mice treated with SCF prior to hepatectomy. Figure 8B illustrates the effects of SCF treatment on TNFR1 $-/-$ mice undergoing hepatectomy. TNFR1 $-/-$ mice undergoing hepatectomy and treatment with vehicle had significantly decreased BrdU staining as compared to all other groups ($*p < 0.05$ versus wild type+vehicle, wild type+SCF, and TNFR1 $-/-$ + SCF). TNFR1 $-/-$ mice treated with SCF had significantly increased BrdU staining as compared to wild type controls and TNFR1 $-/-$ mice treated with vehicle ($+p < 0.05$ versus wild type+vehicle and TNFR1 $-/-$ + vehicle). In contrast, TNF administration to SI/Sld mice after partial hepatectomy did not increase hepatocyte proliferation (Figure 8C). SI/Sld mice treated with vehicle or TNF had significantly decreased BrdU staining as compared to both wild type groups ($*p < 0.05$ versus wild type+vehicle and wild type+TNF).

Figure 9. Representative Western blot and associated densitometric analysis for cytosolic p-stat-3 levels in wild type and TNFR1 $-/-$ mice undergoing hepatectomy with and without SCF treatment These experiments demonstrated that there is a significant decrease in cytosolic p-stat-3 levels in TNFR1 $-/-$ mice treated with vehicle at 3 hours post-hepatectomy as compared to wild type animals undergoing hepatectomy and treatment with vehicle, as well as both TNFR1 $-/-$ mice and wild type mice treated with SCF (* $p < 0.05$ vs all other treatment groups at 3 hours); this is notable in that SCF restored p-stat-3 levels to that of wild type controls at 3 hours post-hepatectomy. Further at 1 hour post-hepatectomy, treatment of TNFR1 $-/-$ mice and wild type mice with SCF significantly increased cytosolic p-stat-3 levels after hepatectomy (+ $p < 0.05$).

versus untreated wild types and untreated TNFR1 ^{-/-} mice at 1 hour post-hepatectomy); this effect disappeared by 3 hours post-hepatectomy.