Novel Protective Effects of Stem Cell Factor in a Murine Model of Acute Septic Peritonitis

Dependence on MCP-1

Cynthia L. Bone-Larson,* Cory M. Hogaboam,*
Matthew L. Steinhauser,* Sandra H. P. Oliveira,*
Nicholas W. Lukacs,* Robert M. Strieter,[†] and
Steven L. Kunkel*

From the Department of Pathology,* University of Michigan Medical School, Ann Arbor, Michigan; and the Department of Pulmonary and Critical Care Medicine,[†] University of California, Los Angeles, California

Mast cells participate in the host response during sepsis and have been shown to have a protective effect in a murine model of acute septic peritonitis and multi-organ failure initiated by cecal ligation and puncture (CLP). Stem cell factor (SCF) is a hematopoietic cytokine important in mast cell proliferation and activation. In the present study, we examined the protective effects of a single intraperitoneal injection of SCF given 2 hours before CLP surgery in mice. Four days after the CLP surgery, SCF pretreatment significantly improved mouse survival from 29 to 56% and mast cells were absolutely required for this effect. Immunoneutralization studies revealed that the SCF-stimulated release of monocyte chemoattractant protein-1 (MCP-1) into the septic peritoneal cavity contributed to the protective effect of SCF in this model. One potential cellular source of MCP-1 was the SCF-activated mast cell. In addition, SCF pretreatment significantly augmented circulating levels of SCF and the immunomodulatory cytokine interleukin-10 in septic mice, in part because the SCF pretreatment seemed to promote the release of both mediators from the liver. Additional hepatic effects of SCF treatment included an accelerated expression of hepatic levels of signal transducer and activator of transcription-3 (STAT-3) in CLP mice pretreated with SCF. Taken together, the findings from the present study demonstrate that the intraperitoneal delivery of SCF has a major protective effect in a murine model of CLP. (*Am J Pathol* 2000, 157:1177–1186)

Sepsis remains one of the leading clinical causes of mortality in the United States.¹ Approximately 40% of patients with sepsis die, but the death rate increases to

90% in patients in whom sepsis progresses to shock or multiple organ failure.² Sepsis is associated with dramatically elevated levels of inflammatory cytokines such as interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and interferon- γ . Strategies aimed at regulating these inflammatory cytokines during clinical sepsis have been fraught with limited or no success. One cellular target of recent significance is the mast cell. Previous studies have suggested that mast cells exert a beneficial role during experimental septic peritonitis partly because of the ability of this cell to quickly release preformed TNF- α .³ TNF- α is an important component of the innate host defense system that manages the lethal effects of intestinal bacteria invading the peritoneal cavity.⁴ However, mast cells are also a source of many other preformed (ie, histamine) and newly formed mediators such as arachidonic acid metabolites and chemokines that may also positively affect the outcome of sepsis.⁵

Stem cell factor (SCF) is a hematopoietic cytokine that triggers its biological effects by binding to the *c-kit* receptor.⁶ It is a primary cytokine involved in mast cell activation^{7–10} and chemotaxis.¹¹ SCF is produced by stromal cells, notably the embryonic and adult liver.^{12,13} It has multiple effects beyond mast cell activation and these include acute erythroid expansion, spermatogenesis, melanocyte development, gut motility, and response to intestinal helminth infection. More recently, the chronic subcutaneous administration of SCF to mice was shown to induce systemic mastocytosis and, more importantly, enhance innate immunity and mouse survival after cecal ligation and puncture (CLP) surgery.¹⁴ Although increased mast cell numbers and TNF- α release were critical for mouse survival after CLP surgery, this previous study alluded to other beneficial effects of SCF in this model.

Thus, the aim of the present study was to elucidate the effects of a single intraperitoneal SCF treatment in the context of experimental sepsis and CLP. To this end, we examined the effect of a single SCF treatment on the

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Address reprint requests to Steven L. Kunkel, Department of Pathology, University of Michigan Medical School, 1301 Catherine Rd., Ann Arbor, MI 48109. E-mail: slkunkel@umich.edu.

response of mice to CLP surgery. Although a single SCF pretreatment did not induce mastocytosis nor markedly increase TNF- α levels, it significantly improved mouse survival, promoted the release of the chemokine MCP-1 and the regulatory cytokine IL-10, and accelerated the hepatic nuclear expression of signal transducer and activator of transcription-3 (STAT-3) after CLP surgery.

Materials and Methods

Mice

Specific pathogen-free CD-1 mice (6- to 12-week-old females; Charles River Breeding Laboratories, Wilmington, MA) were used in the majority of the experiments. All mice were housed in specific pathogen-free conditions within the animal care facility at the University of Michigan (ULAM) until they were used in experiments. Specific pathogen-free C57BL/6J-Mgf SL-d/+ mast cell-deficient mice and appropriate controls (6- to 12-week-old females) were purchased from the Jackson Laboratories (Bar Harbor, ME) immediately before their use in separate experiments.

CLP Model

CLP has been used extensively in our laboratory.¹⁵ Briefly, all mice were anesthetized with an intraperitoneal (i.p.) injection of 3 to 3.5 mg of ketamine HCl (Ketaset; Fort Dodge Laboratories, Overland Park, KS) followed by inhaled methoxyflurane (Metaflane; Pitman-Moore Inc., Mundelein, IL) as needed. After swabbing the abdomen with 70% ethyl alcohol (EtOH), a 1- to 2-cm longitudinal incision was then applied to the lower right quadrant of the abdomen. The cecum was removed from the peritoneal cavity and the distal one-third was occluded with 3-0 silk suture, and punctured through and through with a 21-gauge needle. The cecum was then restored to the peritoneal cavity and the peritoneal incision was closed with surgical staples. All mice then were administered 1 ml of sterile saline via a subcutaneous injection to facilitate fluid resuscitation after CLP surgery. SCF, at a dose of 4 or 20 μ g/kg (Preprotech, Rocky Hill, NJ), was injected into the peritoneal cavity at 2 hours before CLP.

In additional survival experiments, 0.5 ml of anti-MCP-1 antiserum was injected i.p. into each mouse simultaneously with the SCF treatment. Control mice received 0.5 ml of rabbit serum via an i.p. injection concomitant with the SCF treatment. The biological half-life of the anti-MCP-1 antiserum used in these experiments was $\sim$ 36 hours. Polyclonal anti-murine MCP-1 antiserum was raised by multiple-site immunizations in New Zealand White rabbits with murine rMCP-1 (R&D Systems, Minneapolis, MN), and this antiserum did not cross-react with other CC and CXC chemokines in an enzyme-linked immunosorbent assay (ELISA) and demonstrated neutralizing activity against murine rMCP-1 in a chemotaxis assay.¹⁶

Murine Cytokine and Chemokine ELISAs

Murine IL-10, MCP-1, interferon- γ , IL-12, MIP-1 α , KC, MIP-2, and SCF were quantified using a modification of a double-ligand method as previously described.¹⁷ Briefly, flat-bottomed 96-well microtiter plates (Nunc Immuno-Plate I 96-F; Roskilde, Denmark) were coated with 50 μ l/well of anti-mouse cytokine antibody (1 μ g/ml in 0.6 mol/L NaCl, 0.26 mol/L H₃BO₄, and 0.08 mol/L NaOH, pH 9.6) for 16 hours at 4°C and then washed with wash buffer containing phosphate-buffered saline (PBS), pH 7.5, and 0.05% Tween-20. Nonspecific binding sites in each plate were blocked with 2% bovine serum albumin (BSA) in PBS and incubated for 90 minutes at 37°C. Plates were rinsed four times with wash buffer and diluted (neat and 1:10) peritoneal lavage fluids, serum, or cell-free supernatants (50 μ l) in duplicate were added to each plate and incubated for 1 hour at 37°C. Plates were washed four times, followed by the addition of 50 μ l/well biotinylated rabbit antibodies against the specific cytokines (3.5 μ g/ml in PBS, pH 7.5, 0.05% Tween-20, and 2% fetal calf serum), and plates incubated for 30 minutes at 37°C. After washing, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) was added and the plates were incubated for 30 minutes at 37°C. After washing again, chromagen substrate (Bio-Rad Laboratories) was added. The plates were incubated at room temperature to the desired extinction and the reaction terminated with 50 μ l/well of 3 mol/L H₂SO₄ solution. Plates were read at 490 nm in an ELISA reader. Standards were one-half log dilutions of lipopolysaccharide (LPS)-free recombinant murine cytokines (R&D Systems or Preprotech) from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected murine cytokine concentrations $>$ 25 pg/ml and ELISA specificity was confirmed for each cytokine and chemokine measured. Because many of the mice were hypotensive and dehydrated after CLP, protein concentrations were measured in serum using a Bradford assay, and cytokine levels were normalized for the amount of protein that was present in the serum.

Culture and Isolation of Mast Cells

Mast cells were derived from femur bone marrow of specific pathogen-free CBA/J mice. The mast cells obtained from cultured bone marrow cells were c-*kit*-positive but were negative for CD3, CD4, CD8, CD23, B220, and F480. Mast cells were plated in 6-well plates and exposed to 5, 10, and 50 ng/ml of SCF or medium alone 2 hours before being exposed to LPS at 500 μ g/ml. After 24 hours, supernatants were taken for ELISA analysis.

Histology and Immunohistochemistry

Livers from SCF- and saline-treated mice were removed at 4, 8, 24, and 48 hours after CLP and immediately fixed in 4% paraformaldehyde for a minimum of 12 hours. Fixed samples were subsequently embedded in paraffin, thin-sectioned, and placed on L-lysine-coated slides. Slides were deparaffinized by sequential treatment with

xylene, 100% EtOH, 90% EtOH, 70% EtOH, 50% EtOH, distilled water, and Tris-buffered saline (TBS; pH 7.68). Slides were microwaved for ~20 minutes in 10 mmol/L citric acid buffer and allowed to cool. Tissue sections were blocked in 1:50 dilution normal goat serum (blocking solution) for 2 hours. Tissue sections were treated with one of the polyclonal anti-mouse SCF antiserum, monoclonal anti-mouse IL-10 antibody (PharMingen, San Diego, CA), or the appropriate control antibody. All were diluted at 1:100 with TBS containing blocking solution (1:1) and the tissue sections were incubated overnight at 4°C in a humidified chamber. After incubation, slides were washed twice for 5 minutes in TBS. A 1:35 dilution of biotinylated goat anti-rabbit AB (BioGenex, San Ramon, CA) was placed on the slides for 2 hours at 37°C in a humidified chamber. Slides were again washed twice in TBS. Slides were incubated with a 1:35 dilution of streptavidin conjugated to horseradish peroxidase (BioGenex) for 45 minutes, followed by two washes in TBS with 50 mmol/L levamisole. Fast red chromagen (BioGenex) was placed on each slide and staining was visualized at low power until color development was complete. The staining reaction was terminated in sterile water and each slide was counterstained with Mayer's hematoxylin (0.1%; Sigma Chemical Co., St. Louis, MO).

Cytospins

Red blood cells were lysed in peritoneal washes, and red blood cell-free samples were spun for 5 minutes at 5,000 revolutions per minute in a Cytospin (Shandon, Pittsburgh, PA). Mast cells were stained with 0.5% toluidine blue O (Sigma Chemical Co.) and were counted. The mast cell number in each cytospin was expressed per 1×10^5 of the total cell number present in the peritoneum. The total cell numbers from each treatment group were not significantly different from each other.

Nuclear Extraction and Direct Lysis of Nuclei

Preparation of nuclear extracts from liver was conducted as follows. Briefly, liver samples were rapidly homogenized in PBS containing Complete™ protease inhibitor (10 mg/ml; Boehringer Mannheim, Mannheim, Germany) and washed with fresh PBS. Homogenates were then suspended in buffer A (10 mmol/L Hepes, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, 1% Nonidet P-40) for 10 minutes and centrifuged for 10 minutes at $14,000 \times g$ and the cytoplasmic supernatant was removed. The cell nuclei (found in the pellet) were suspended in buffer C (20 mmol/L Hepes, 20% glycerol, 500 mmol/L KCl, 0.2 mmol/L ethylenediaminetetraacetic acid, 0.5 mmol/L phenylmethyl sulfonyl fluoride, 0.5 mmol/L dithiothreitol, 1.5 mmol/L $MgCl_2$) for 15 minutes and centrifuged at $7,000 \times g$ for 10 minutes. The supernatant containing the nuclear proteins was removed for Western blot analysis.

Western Blot Analysis

Nuclear protein was measured using a Bradford assay (Bio-Rad) and 50 μ g of liver nuclear extracts were elec-

trophoresed on a 12% polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (Bio-Rad). Equal protein loading was confirmed by Coomassie blue staining of the gel after transfer. Membranes were blocked for 2 hours at room temperature in 5% dry milk. STAT3 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were diluted 1:1,000 and incubated overnight at 4°C. Horseradish peroxidase-linked secondary antibody (Pierce, Rockford, IL) was then added at 1:3,000 for 2 hours at room temperature and bands were detected by chemiluminescence (Bio-Rad).

Statistical Analysis

Survival curves were generated with Prism computer software (Graphpad Software, Inc., San Diego, CA), and survival was examined using the Fisher's exact test. For all other analyses, a Student's *t*-test was used to test for significance. A *P* value of ≤ 0.05 was considered statistically significant.

Results

SCF Pretreatment Increases Survival after CLP

Although it has previously been shown that multiple treatments with SCF induced mastocytosis and subsequently protected mice from CLP-induced mortality,¹⁴ we examined whether a single SCF treatment also reduced mortality in this model of sepsis. In the present study, mice were given an i.p. injection of a range of SCF doses at 2 hours before CLP surgery. In our pilot studies, we discovered that the SCF pretreatment was necessary because a SCF injection given at the time of or after CLP surgery did not improve survival (data not shown). As illustrated in Figure 1A, SCF pretreatment was associated with a dose-dependent increase in survival after CLP. A dose of 4 μ g/kg significantly increased survival above the control rate (29% versus 43%), but a dose of 20 μ g/kg of SCF produced the greatest increase in survival (29% versus 56%, $P \leq 0.05$). At a dose of 20 μ g/kg, the protective effect of SCF was observed after the first day with the survival of the SCF pretreatment group at 88% (ie, 35 of 41 animals), compared with the control group in which only 44% were alive (ie, 19 of 34 animals). By the fourth day, only 10 of the original 34 control animals had survived (29%), whereas 23 of the original 41 mice pretreated with 20 μ g/kg SCF were alive (56%). Because a dose of SCF at 20 μ g/kg produced the greatest beneficial effect on mouse survival, subsequent *in vivo* experiments used this dose of SCF.

Mast Cells and TNF- α in the Peritoneal Wash Are Not Increased by SCF Pretreatment

Mast cells are a necessary component in the host response to the bacteria introduced into the peritoneum after CLP surgery.³ Likewise, SCF is an important cyto-

Figure 1. SCF pretreatment enhanced survival after CLP surgery (**A**) dependent on mast cells (**B**). Two hours before CLP surgery, mice received an i.p. injection of 4 or 20 µg/kg of SCF suspended in saline. Controls received saline alone at the same time via the same route. The groups that received either 4 or 20 µg/kg of SCF exhibited increased survival after CLP surgery compared with the saline alone group. $P \leq 0.05$ (Fisher's exact test) for the 20 µg/kg SCF-treated group ($n = 41$) compared with the saline-treated control ($n = 34$) group. SCF pretreatment did not affect survival in *Sl^d/+* mice, which have strikingly fewer mature mast cells. Two hours before CLP surgery, 20 µg/kg of SCF or saline was administered i.p. to *Sl^d/+* mice. There were no differences in survival between the two treatment groups.

kine involved in mast cell activation,^{7–10} survival,^{18–20} and chemotaxis.¹¹ Thus, we determined whether the enhanced survival after a single SCF pretreatment was because of its effect on mast cell numbers and/or the release of preformed mediators such as TNF-α in the peritoneal wash. First, the number of granulated mast cells that stained with toluidine blue O were counted in peritoneal washes removed at 5, 10, and 15 minutes after SCF treatment or saline treatment. Other samples were removed from both treatment groups at 15 minutes, and 4, 6, and 8 hours after CLP surgery. Cytospins of peritoneal washes revealed no significant differences in the number of mast cells present between the two treatment groups at all time points examined (Table 1). Nevertheless, it is notable that stained mast cell were decreased in both treatment groups after CLP suggesting that peritoneal mast cells released their granular contents in response to the peritonitis. Furthermore, TNF-α levels were not significantly different between the two treatment groups at any time point measured before and after CLP surgery (Table 2).

Table 1. Number of Mast Cells Per 100,000 Cells in the Peritoneal Wash in Animals Pretreated with 20 µg/kg SCF or Saline, $n = 4$ to 8 in Each Group

Time	Saline treatment	SCF treatment
Before CLP		
5 minutes	40.1 ± 11.7	31.1 ± 4.8
10 minutes	37.6 ± 7.6	37.5 ± 9.2
15 minutes	76.8 ± 12.8	57.5 ± 12.8
After CLP		
15 minutes	9.6 ± 2.1	21.9 ± 14.8
4 hours	8.2 ± 3.6	1.6 ± 0.8
6 hours	1.3 ± 0.4	0.6 ± 0.2
8 hours	2.8 ± 1.2	0.3 ± 0.5

Mast Cells Are Required for the Protective Effect of SCF in the CLP Model

We next examined whether a SCF pretreatment prevented mortality in *Sl^d/+* mice. These mice are characterized by diminished levels of transmembrane SCF and markedly lower mast cell numbers, but express the *c-kit* receptor. SCF pretreatment failed to prevent mortality in these mice ($n = 5$), and by day 4 after CLP all of the *Sl^d/+* mice were dead (Figure 1B).

SCF Treatment Augments Monocyte Chemoattractant Protein-1 Levels in the Septic Peritoneum

Although peritoneal washes did not exhibit altered TNF-α levels after a single SCF injection, we nevertheless had convincing data from the survival study using *Sl^d/+* mice that mature mast cells were required for the protective effect of SCF. To further explore the relative contribution of mast cells to the protective effects of SCF in our CLP model, we next examined cytokine and chemokine levels in the peritoneal wash. The peritoneum represents the local environment in which the mast cells presumably exert part of their beneficial effect during CLP. Of the C-C and C-X-C chemokines screened in the peritoneal wash (MCP-1, MIP-2, and MIP-1α), only the pattern of expres-

Table 2. TNF-α Levels (ng/ml) in the Peritoneal Wash in Mice Pretreated with 20 µg/kg SCF or Saline, $n = 4$ to 8 in Each Group.

Time	Saline treatment	SCF treatment
Before CLP		
5 minutes	N.D.	N.D.
10 minutes	N.D.	N.D.
15 minutes	N.D.	N.D.
2 hours	N.D.	N.D.
After CLP		
5 minutes	0.31 ± 0.12	0.19 ± 0.06
10 minutes	0.31 ± 0.08	0.24 ± 0.07
15 minutes	0.07 ± 0.06	0.18 ± 0.11
4 hours	0.33 ± 0.15	0.23 ± 0.15
6 hours	0.12 ± 0.12	0.09 ± 0.05
8 hours	0.99 ± 0.82	1.01 ± 0.23
24 hours	0.13 ± 0.03	0.17 ± 0.05
48 hours	0.10 ± 0.05	0.10 ± 0.04

N.D. = not detectable

Figure 2. SCF pretreatment enhanced peritoneal levels of MCP-1 during CLP-induced sepsis (A) and promoted MCP-1 release by bone marrow-derived mast cells (B). In SCF-pretreated mice, immunoreactive levels of MCP-1 were significantly elevated before (time 0) and at 24 and 48 hours after CLP compared with saline-treated mice. However, at 4 hours after CLP, MCP-1 levels in the peritoneal wash were significantly decreased with saline-treated mice. Data shown in A are mean $\pm$ SEM of five to eight mice per group at each time point before and after CLP surgery. An asterisk indicates $P \leq 0.05$ compared with saline-treated mice. SCF dose-dependently increased MCP-1 secretion by cultured bone marrow-derived murine mast cells. Cell-free supernatants from the mast cell cultures were removed 24 hours after SCF treatment and subjected to ELISA analysis. The results shown in B are the mean $\pm$ SEM of three separate experiments.

sion for MCP-1 was markedly different between the two CLP groups. MCP-1 has been shown to be critical to the peritoneal host defense response and elicits the recruitment of mononuclear cells and neutrophils into the peritoneal cavity during CLP.¹⁶ Before CLP (time 0), MCP-1 was only present in the peritoneal wash of SCF-pretreated mice (Figure 2A). At 4 hours, both CLP groups exhibited marked levels of MCP-1 in the peritoneal wash, but the saline-treated CLP group contained twofold more MCP-1. MCP-1 levels were similar in both groups at 8 hours after CLP, but levels of this CC chemokine remained significantly elevated at 24 ($P \leq 0.0005$) and 48 ($P \leq 0.0005$) hours in the SCF-treated group compared with the saline-treated group after CLP surgery (Figure 2A). To determine whether MCP-1 was a mast cell-derived chemokine that released on SCF stimulation, cultured bone marrow-derived murine mast cells were exposed to medium alone or medium containing 5, 10, or 50 ng/ml of SCF. SCF dose-dependently increased the levels of MCP-1 in these cultures from below ELISA detec-

tion levels (untreated) to 12.8 ± 6.7 ng/ml (50 ng/ml SCF treatment) (Figure 2B). Interestingly, SCF does not promote the release of MCP-1 from untreated or LPS-stimulated peritoneal macrophages (N. W. Lukacs, personal communication). Thus, taken together these data suggested that mast cells were the primary source of MCP-1 in the peritoneal cavity.

MCP-1 Production after SCF Pretreatment Is Absolutely Essential for Increased Survival

The importance of MCP-1 in the SCF protective effect during CLP is illustrated in Figure 3. When mice received recombinant SCF and anti-MCP-1 antiserum 2 hours before CLP, the protective effect of the SCF treatment was abolished (Figure 3). At 2 days after CLP, ~81% of SCF-treated control mice were alive (13 of 16 mice), whereas the group that received SCF and anti-MCP-1 antiserum contained only 50% of the starting number of mice (eight of 16 mice). At day 7 after CLP, ~68% of the SCF-treated mice were alive, in contrast to the other group that only contained 31% of the mice subjected to CLP ($P = 0.03$). No additional protective effect is seen when SCF is given in conjunction with rabbit serum because of the beneficial effect that rabbit serum has after CLP.¹⁵ Thus, these findings demonstrated that SCF pretreatment augments MCP-1 levels within the peritoneal cavity before and after CLP surgery and these changes are absolutely required for the protective effect of SCF.

Exogenous SCF Regulates Endogenous SCF Production in the Serum and the Liver

Not only did SCF have an effect in the local peritoneal environment, there was also evidence for a beneficial systemic response to SCF treatment. Endogenous circulating levels of SCF were markedly affected by the single SCF treatment before CLP. As shown in Figure 4A, endogenous SCF was significantly increased in the serum of SCF-pretreated mice at 4 ($P = 0.044$), 8 ($P \leq 0.0005$), and 48 ($P = 0.025$) hours after CLP. Given that stromal cells in the embryonic and adult liver produce SCF,^{12,13}

Figure 3. SCF pretreatment in the absence of endogenous MCP-1 failed to enhance survival after CLP surgery. Two hours before CLP surgery, mice received an i.p. injection of 20 μ g/kg of SCF suspended in saline and normal rabbit serum (RS) or anti-MCP-1 antiserum. The group that received SCF and RS exhibited significantly increased survival after CLP surgery compared with the SCF treatment group that received anti-MCP-1 antiserum. Ten mice were examined in each treatment group. $P = 0.03$ for SCF + RS group compared with the SCF + anti-MCP-1 antiserum group (log-rank test).

Figure 4. Exogenous SCF significantly augments serum levels of endogenous SCF (**A**) and IL-10 (**B**) before and at all times examined after CLP surgery. Data shown are mean $\pm$ SEM of five to eight mice per group at each time point before and after CLP surgery. An **asterisk** indicates $P \leq 0.05$ compared with saline-treated mice.

we next ascertained whether there was also an effect in the liver. Immunohistochemical analysis of the liver is summarized in Figure 5. At 4 and 8 hours after CLP surgery in saline-treated mice, SCF was expressed in the cytoplasm and nucleus of hepatocytes localized around hepatic central veins (Figure 5, A and C). Conversely, in SCF-pretreated mice at similar times after CLP, dramatically less SCF staining was present in the cytoplasm and nucleus in hepatocytes surrounding hepatic central veins (Figure 5, B and D). Taken together, this data suggests exogenous treatment with SCF is having dramatic effects systemically on both the levels of endogenous SCF and the regulation of SCF within the liver. This may represent a beneficial positive feedback mechanism in which SCF pretreatment causes the release of endogenous SCF from the liver to maintain the levels of SCF systemically.

Systemic IL-10 Levels after CLP Were Dramatically Enhanced

The local release of cytokines is critical during the host defense response, yet when these mediators spill over into the systemic circulation, a systemic inflammatory reaction can result. IL-10 is a modulatory cytokine that enhances survival in bacterial toxin-induced shock models by inhibiting the synthesis of proinflammatory cytokines.^{21–23} IL-10 levels were measured to determine whether SCF treatment modulated levels of this cytokine.

Indeed, the dramatic changes in circulating levels of IL-10 were observed in SCF-treated mice that underwent CLP surgery. IL-10 was significantly increased at 4 ($P = 0.045$), 8 ($P = 0.041$), and 24 ($P = 0.004$) hours after CLP surgery in the SCF-pretreated group compared with the saline-treated group (Figure 4B).

We next looked to potential cellular sources of IL-10 during the CLP response. Kupffer cells are the resident macrophages of the liver that generate IL-10^{24,25} and play an important role in the release of cytokines during a septic episode. Eight hours after CLP surgery in control mice, immunoreactive IL-10 was detected in cells that morphologically resemble Kupffer cells (Figure 6A). In contrast, in mice that received a pretreatment of SCF, IL-10 staining was completely absent in these cells and hepatocytes exhibited weak IL-10 staining (Figure 6B).

Nuclear Expression of Signal Transducer and Activator of Transcription-3 (STAT-3) Is Elevated in the Liver in Response to SCF Treatment

STAT-3 is responsible for the activation of acute phase proteins²⁶ and has been positively correlated with survival from septic shock.²⁷ Therefore, we next examined whether SCF treatment had any effect on the nuclear expression of STAT-3 in the liver at 4, 8, and 24 hours after CLP using Western blot analysis. As shown in Figure 7, 4 hours after CLP, saline-treated mice exhibited very low levels of STAT-3 nuclear protein in liver nuclear extracts, whereas nuclear protein expression of STAT-3 was present in all SCF-treated mice at higher levels. Likewise, 8 hours after CLP, two of three saline-treated mice exhibited low levels of STAT-3, whereas all four of the SCF-treated mice exhibited strong STAT-3 expression. By 24 hours, all of the surviving mice in both the saline- and SCF-treated groups exhibited similar nuclear expression of STAT-3. Thus, these findings suggest that SCF can either directly through the *c-kit* receptor on hepatocytes¹³ or indirectly cause earlier nuclear translocation of STAT-3 in the liver.

Discussion

Although normally considered to be a cell primarily associated with allergy, recent studies have conclusively demonstrated that the mast cell exerts a very important role during innate immunity against pathogens. The best evidence for this novel role for mast cells comes from studies performed in a murine model of septic peritonitis because of CLP.³ These studies showed that after CLP surgery, mast cell-deficient mice showed an inability to generate sufficient levels of TNF- α to clear bacteria introduced into the peritoneum from the punctured cecum. As a result, these mice showed a significantly increased mortality rate compared to mice that have a normal complement of mast cells.³ Recently, SCF, a mast-cell growth and activating factor, has also been shown to improve the survival of mice subjected to CLP in a mast cell-dependent manner. In the present study, we elucidated the

Figure 5. Immunohistochemical localization of SCF in liver sections from saline-treated (A and C) and SCF-treated (B and D) mice with CLP-induced sepsis. Hepatocytes were strongly positive for SCF at 4 hours (A) and 8 hours (C) after CLP in saline-treated mice. In contrast, SCF staining was less intense in SCF-pretreated mice at 4 hours (B) and 8 hours (D) after CLP surgery. Original magnifications, $\times 100$ (A–D).

protective mechanism of a single SCF treatment in the absence of a mastocytosis. A single i.p. injection of SCF was not associated with increased mast cell numbers in the peritoneal wash, nor did this treatment markedly affect TNF- α levels. However, after CLP, SCF treatment was associated with markedly augmented mouse survival, enhanced MCP-1 in the peritoneal wash, an increase in systemic levels of both endogenous SCF and IL-10, and resulted in earlier expression of STAT3 in the liver. Thus, a single SCF treatment was sufficient to inhibit or abolish many of the deleterious outcomes that followed CLP surgery in mice.

Several lines of evidence in the present study suggested that SCF was protective in mice subjected to CLP through its direct effect on mast cells. First and most convincing, the protective effect of SCF was abolished in animals with decreased numbers of mature mast cells subjected to CLP, indicating that SCF did not exert protective effects independent of mast cell involvement. Next, although mast cells and TNF- α did not change in the peritoneal wash of SCF-pretreated mice before and after CLP, peritoneal levels of MCP-1 were markedly altered. Although the source of MCP-1 in SCF-treated mice was not conclusively identified, our *in vitro* studies showed that mast cells exposed to SCF for 24 hours

secreted MCP-1 whereas untreated mast cells did not. Furthermore, peritoneal macrophages did not produce MCP-1 in response to SCF and LPS (data not shown). This is in accordance with other studies that have found that depletion of mast cells reduces the production of MCP-1 in the peritoneal lavage fluids after exposure to zymosan.²⁸ The MCP-1 generation within the peritoneal cavity was absolutely essential for the beneficial effect of SCF because animals that were given SCF and antiserum to MCP-1 failed to survive. Furthermore, MCP-1 generation in the peritoneal cavity has been shown to be critical for mouse survival after infection with *Pseudomonas aeruginosa* and *Salmonella typhimurium*²⁹ and its neutralization is detrimental in the context of CLP because of its actions on peritoneal macrophages to produce LTB₄ a neutrophil chemoattractant.¹⁶ This study and others suggest that MCP-1 is the primary activator of the host response in the peritoneal cavity after infection.³⁰

The present study also suggested that there were other effects of SCF that may have contributed to its benefit after CLP. At present, it is not known whether these additional effects are downstream of the mast cell or they are independent of the mast cell. Nevertheless, SCF-pretreated mice exhibited dramatic increases in serum levels of IL-10. IL-10 is a pivotal cytokine during

Figure 6. Immunohistochemical localization of IL-10 in liver sections from saline-treated (**A**) and SCF-treated (**C**) mice with CLP-induced sepsis. In saline-treated mice, there was pronounced IL-10 staining in cells that morphologically resemble Kupffer cells at 8 hours after CLP (**inset** and **arrows**). Conversely, the Kupffer cells in SCF-treated mice were negative for IL-10 and instead hepatocytes were lightly stained for IL-10. **B** and **D** represent the negative controls for **A** and **C**, respectively. Original magnifications, $\times 200$ (**A–D**).

immune and inflammatory responses because it inhibits gene transcription thereby down-regulating a broad range of cytokines and chemokines.^{31,32} A number of studies have shown that IL-10 enhances survival during experimental toxin-induced shock models and CLP.³² Furthermore, histological findings from the present study suggested that SCF promoted the release of IL-10 from Kupffer cells. It is not likely that hepatocytes were producing IL-10 in the context of SCF treatment because isolated hepatocytes and hepatocyte cell lines fail to produce IL-10 after exposure to LPS and SCF (data not shown). Thus, these ELISA and histological findings suggest that SCF treatment before CLP promotes the rapid and prolonged release of the IL-10 from hepatic Kupffer cells, and the increase in IL-10 may also account for the protective effect of SCF in this model. These findings are consistent with previous experiments showing that IL-10 expression is autoregulated at the transcriptional level in human and murine Kupffer cells and IL-10 mRNA is dramatically increased after endotoxin exposure.²⁴

One manifestation of sepsis is liver dysfunction in which there is decreased transcription of key hepatic enzymes necessary for the survival of the animal. STAT-3

is a key transcription factor that is responsible for the transcription of many of these enzymes. Many cytokines can activate one or more STATs.³³ Specifically, MCP-1³⁴ and IL-10³⁵ can result in translocation of STAT-3 to the nucleus in both immune and nonimmune cells. The present study suggested that the SCF treatment promotes an earlier and marked nuclear translocation of STAT-3. This event has been previously associated with increased survival after CLP.²⁷ In a sublethal sepsis model, hepatic STAT-3 activation was detected at 3 hours and persisted for 3 days. In contrast, in a lethal model of sepsis, hepatic STAT-3 activation was only present for 3 to 16 hours after CLP, and at dramatically lower levels.²⁷ STAT-3 activation in SCF-treated mice could also be responsible for transcription of acute phase proteins such as C-reactive protein,²⁶ α_2 -macroglobulin,²⁷ and lipopolysaccharide-binding protein,³⁶ all of which are important during the host response to pathogens. Thus, the beneficial effect of a single SCF treatment may be related to the direct effect this factor had on the synthetic capacity of the liver during CLP.

Thus, a single SCF treatment before CLP surgery exerts a number of dramatic beneficial effects in the local

Figure 7. SCF pretreatment accelerated the nuclear translocation of STAT-3 in the liver. At 4 hours and 8 hours after CLP surgery, nuclear STAT-3 levels in liver samples were markedly greater in SCF-treated mice than in similar samples from saline-treated mice. At 24 hours after CLP, all surviving mice in both groups, had equivalent levels of STAT3 nuclear expression in the liver. Under reducing conditions, bands appeared at approximately 45 and 25 kd in the four SCF-treated mice examined at 4 hours after CLP, but these bands were very light or not present in the three saline-treated mice at this time. At 8 hours, nuclear expression of STAT3 was strongly expressed in SCF-treated mice as bands of ~45, 25, and 80 kd were visible in all four mice examined. In contrast, two of the three saline-treated mice had very low levels of nuclear STAT3 expression and only one mouse showed similar nuclear expression of STAT-3 as all of the SCF-treated mice at this time.

peritoneal environment, on the systemic levels of cytokines, and in the liver. Further studies are warranted to explore the clinical feasibility of using SCF in the treatment and prevention of bacterial peritonitis.

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