의 학 박 사 학 위 논 문

새로운 절제 방법으로 시행한 90% 부분 흰쥐 간절제 모델에서 간재생에 영향을 주는 stem cell factor 와 granulocyte macrophage colony-stimulating factor 동시 투여의 효과

2016 년 2 월

서울대학교 대학원

의학과 외과학 전공

이 승 덕

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2016년

이승덕

Abstract

The combined effect of stem cell factor and granulocyte macrophage colony-stimulating factor administration after 90% partial hepatectomy in rats using a modified ligation technique

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Background: After major hepatectomy or liver transplantation, the ability of the liver to regenerate regeneration has been used to prevent postoperative hepatic failure. Stem cell factor (SCF) and granulocyte macrophage colony-stimulating factor (GM-CSF) are known to play important roles in liver regeneration with

synergistic effects in a previous *in vitro* study. The purpose of this study was to identify the impact of exogenous SCF and GM-CSF administration in combination after 90% major hepatectomy in rats.

Methods: Sprague-Dawley rats underwent 90% major hepatectomy using a newly modified ligation technique of bile duct-sparing portal pedicle ligation under microscopy. The rats were divided into two groups: group 1 (phosphate-buffered saline treatment) and group 2 (SCF+GM-CSF treatment, each 25 mcg/kg). Treatment was administrated immediately after operation through the inferior vena cava. Liver regeneration capacity and molecules related to hematopoietic stem cell migration were evaluated on postoperative days 1, 2, 4, and 7. We sacrificed five rats at each time point for obtaining statistical power.

Results: The survival rate after 90% hepatectomy using this technique was 95% compared with 55% for the conventional ligation technique (p = 0.004). Group 2 exhibited a significantly increased liver regeneration index on postoperative days 2 and 4 compared to group 1 (day 2: 287.5 ± 19.6 vs. 513.9 ± 67.1, p = 0.025 and day 4: 647.6 ± 108.8 vs. 941.7 ± 53.9, p =

0.046). Serum liver enzyme levels, including total bilirubin, aspartate aminotransferase, and alanine aminotransferase, were significantly lower in group 2 than in group 1 on postoperative day 1. The expression of Ki-67 on immunohistochemistry was significantly higher in group 2 than in group 1 on postoperative day 4 (343.3 ± 35.4 vs. 535.7 ± 56.4, p = 0.045). Group 2 displayed significant increases of interleukin (IL)-6 and Transforming growth factor (TGF)-β expression within 24 h after hepatectomy. Furthermore, C-X-C motif chemokine 12 (CXCL12)/C-X-C chemokine receptor type 4 (CXCR4) and matrix metalloproteinases 2 and 9 levels in the liver tissue of group 2 animals were also significantly upregulated according to quantitative polymerase chain reaction on postoperative days.

Conclusions: Our data suggest that the administration of SCF+GM-CSF after major hepatectomy can enhance liver regeneration by modulating IL-6/TGF-β, CXCL12/CXCR4 pathway as well as by matrix remodeling. These findings suggest the possibility of therapeutic treatment using a combination of SCF and GM-CSF in clinical setting to promote liver regeneration.

Key words: liver regeneration, SCF, GM-CSF, partial hepatectomy, rat

Student Number : 2010-30536

Contents

LIST OF FIGURES

Figure 5. The change of liver size according to postoperative days

The liver volume after 90% PH was increased rapidly, and then original liver volume was restored until postoperative day 7.

Figure 6. The change of liver regeneration index between two groups

The liver regeneration index was statistically significantly increased in SCF+GM-CSF group at postoperative day 2 and 4 (287.5 ± 19.6 vs. 513.9 ± 67.1, $p = 0.025$ at day 2; 647.6 ± 108.8 vs. 941.7 ± 53.9, $p = 0.046$ at day 4)

Figure 7. The results of liver function tests between two groups

The liver function test including total bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) at preoperative status, postoperative day 1, 2, 4, and 7 between control and SCF+GM-CSF group (*: $p < 0.05$).

Figure 8. The results of immunohistochemistry using Ki-67 between two groups

SCF+GM-CSF group showed significant increased Ki-67 expression at postoperative day 4 compared with control group (*: $p < 0.05$).

Figure 9. Western blotting using cyclin D1 antibody

SCF+GM-CSF group showed a higher cyclin D1 expression than the control group on postoperative days 1 and 2.

Figure 10. The results of ELISA assays using IL-6 and TGF- β between two groups

The trend of TGF- β and IL-6 after 90% PH in control and SCF+GM-CSF group (*: $p < 0.05$).

Figure 11. The relative expression of CXCL12 and CXCR4 between two groups

The relative expression of CXCL12 and CXCR4 after 90% PH in control and SCF+GM-CSF group analyzed by quantitative PCR (*: $p < 0.05$).

Figure 12. The relative expression of MMP-2, MMP-9, and TIMP-3 between two groups

The relative expression of MMP-2, MMP-9, and TIMP-3 after 90% PH in control and SCF+GM-CSF group analyzed by quantitative PCR (*: $p < 0.05$).

LIST OF ABBREVIATIONS

ALT: alanin aminotrasferase

AST: aspartate aminotransferase

CXCL12: C-X-C motif chemokine 12

CXCR4: C-X-C chemokine receptor type 4

EPO: erythropoietin

EGF: epidermal growth factor

ELISA: enzyme-linked immunosorbent assay

G-CSF: granulocyte colony-stimulating factor

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GM-CSF: granulocyte macrophage colony-stimulating factor

HGF: hepatocyte growth factor

HSC: hematopoietic stem cells

IL-6: interleukin-6

MMP: matrix metalloproteinase

MSC: mesenchymal stem cells

PBS: phosphate-buffered saline

PH: partial hepatectomy

SCF: stem cell factor

SE: Standard error

TGF-β: transforming growth factor-β

TNF-α: tumor necrosis factor-α

TIMP-3: metalloproteinases inhibitor 3

Introduction

Liver regeneration is an essential component of the reparative process following liver injury, resection, and transplantation.(1) The capacity of hepatic regeneration after surgical resection is an important issue for surgeons to determine the extent of resection for safe recovery. Post-hepatectomy liver failure and small-for-size graft after liver transplantation are among the most serious complications associated with liver surgery.(2, 3) If an adequate healthy remnant liver (approximately 30%-35% of the initial hepatic mass) remains after the surgical resection, the patient usually recovers without liver failure.(4) In particular, in patients with cirrhotic, cholestatic, or severe fatty livers, the remnant hepatic volume should be greater than that required for patients with normal livers. However, in clinical situations, surgeons often decide the small remnant volume for curative resection and encounter small grafts during liver transplantation according to donor selection. At that time, drugs promoting liver regeneration are needed to prevent postoperative liver failure.

Liver regeneration is a complex phenomenon and the mechanisms of this process are not fully clarified. It has been studied by performing surgical resection in rodents (rats and mice), a conventional technique known as 2/3 partial hepatectomy (PH).(5, 6) The 70% PH model involves the resection of the two anterior lobes (median and left lateral lobes). Although ligature en-bloc at the base of the lobe is the most commonly used technique, it carries the highest risk for injuries because the mass ligature may compromise elements of other particles. In particular, the risk of vena cava stenosis and liver congestion is high when only one ligation is performed for both the median and left lateral lobes. Therefore, in cases of extreme resection models such as 90 or 95% PH, the survival rate is decreased to approximately 50%. Kubota et al. reported the new technique of pedicle ligation before lobe resection under microscopic view.(7, 8) They demonstrated an 100% survival rate after 90% PH in rats. The advantages of this technique are reduced risks of bleeding from the stump and vena cava constriction. However, this portal pedicle ligation technique including the bile duct has the possibility of biliary complications including extrahepatic bile duct stenosis, biloma, and biliary leakage.

With the advance of surgical techniques for rodent liver resection, many hemodynamic and molecular-based studies for liver regeneration have been reported.(9) The liver has been confirmed as the target organ for many cytokines and some cytokines including hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), insulin and noradrenaline appear to play important roles in liver regenerative capacity in response to the loss of hepatic parenchyma.(10-13) Although many cytokines have proliferative effects on hepatocytes both *in vitro* and *in vivo*, no single molecule has been reported to be the only factor responsible for promoting proliferation *in vivo*. Moreover, when liver damage occurs or hepatocyte proliferation is inhibited, a facultative cellular compartment of hepatic oval cells, located within the smallest branches of the intrahepatic biliary tree, is activated which leads to liver repair.(14, 15) Several groups have demonstrated that bone marrow-derived hematopoietic stem cells (HSCs) may contribute to liver repair.(16-18) Therefore, after liver injury or resection, three levels of proliferating cells were included: 1) hepatocyte, 2) endogenous ductular progenitor cells or hepatic oval cells, and 3) pluripotent

stem cells derived from circulating bone marrow cells.(19)

Focused on stem cells that induce liver regeneration, several cytokines have been identified as candidates in the liver remodeling processes, including stem cell factor (SCF), erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF). These factors regulate the production of circulating red cells, white cells, and platelets by bone marrow and act on stem cells, leading to lineage-specific differentiation.(20) SCF regulates the differentiation of $CD34^+$ stem cells and has a more generalized role in inducing cellular maturation and proliferation in a variety of cell types.(21, 22) Investigations have documented significant hepatic SCF expression possibly associated with hepatocyte proliferation.(21-23) Furthermore, other factors, such as EPO, G-CSF, and GM-CSF, modulate the synthesis of more specific cell types.(24) CSFs are associated with hepatic inflammation via direct effects on the vascular endothelium and they participate in neutrophil recruitment, activation, hepatic repair, and regeneration.(25) These factors can stimulate HSCs located within the canals of Hering and biliary epithelia. Recently, Meng

et al. reported significant increases in the expression of key remodeling molecules, such as S100 calcium-binding protein A4 and miR-181b, after SCF plus GM-CSF administration in *in vitro* cholangiocyte cultures.(26) In addition, they reported that SCF promotes synergistic cellular proliferation in combination with GM-CSF, but not EPO or G-CSF, in human hepatocytes and small murine cells with biliary markers.

The current study elucidated the possible role of the combined *in vivo* administration of SCF and GM-CSF in a 90% PH rat model using a new bile duct-sparing portal pedicle ligation technique.

Materials and Methods

1) Animal protocols and 90% PH model

Male Sprague-Dawley rats, aged 6—8 weeks (200—250 g), were purchased from Orientbio Company (South Korea) after approval from the Institutional Animal Care and Use Committee of the National Cancer Center Research Institute, South Korea. The rats were fasted for 12 h before the procedure and routine food and water care was provided in the postoperative period. The 90% PH technique was performed according to the following procedures. Under general anesthesia using isoflurane, we made a bilateral subcostal incision and pushed out the liver after detaching it from the surrounding ligaments. Afterwards, we dissected the hilum under microscopy (10× magnification) and divided the bile duct and portal vein/hepatic artery branches entering the median/left lateral and right lateral lobes, saving the caudate lobe. We checked the ischemic area of liver lobes. The pedicles of the lobes were divided into two or three parts and ligated via piercing before the lobes were resected (Fig. 1). In the control group, we directly administrated 0.2 ml phosphate-

buffered saline (PBS) into the inferior vena cava immediately after 90% PH (Fig. 2). In the experimental group, we administrated the same volume of PBS containing both rat SCF and GM-CSF (each 25 mcg/kg, Prospec-Tany, Rehovot, Israel). At the same time (postoperative days 0, 1, 2, 4, and 7), we sacrificed five rats of each group and obtained a liver specimen and blood samples.

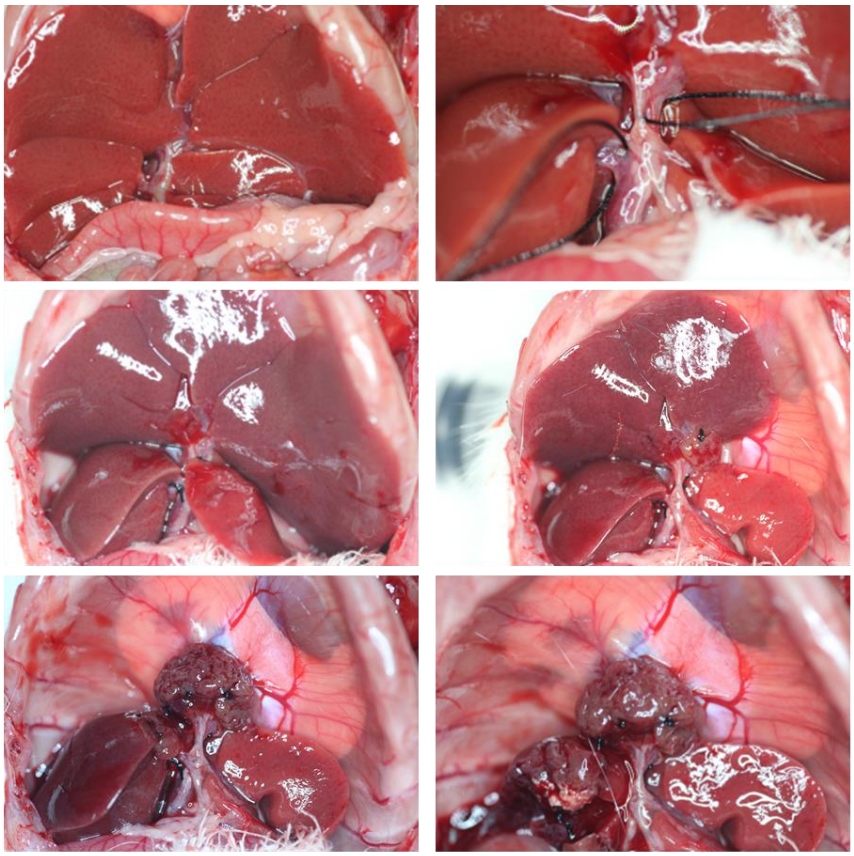

Fig. 1. Surgical technique of 90% partial hepatectomy (PH)
The 90% PH was performed to dissect hilum under microscopy and divide bile duct and portal vein/hepatic artery pedicle entering median/left lateral lobe and right lateral lobe. The pedicles of the lobes were divided into two to three parts and ligated by piercing before the lobes were resected.

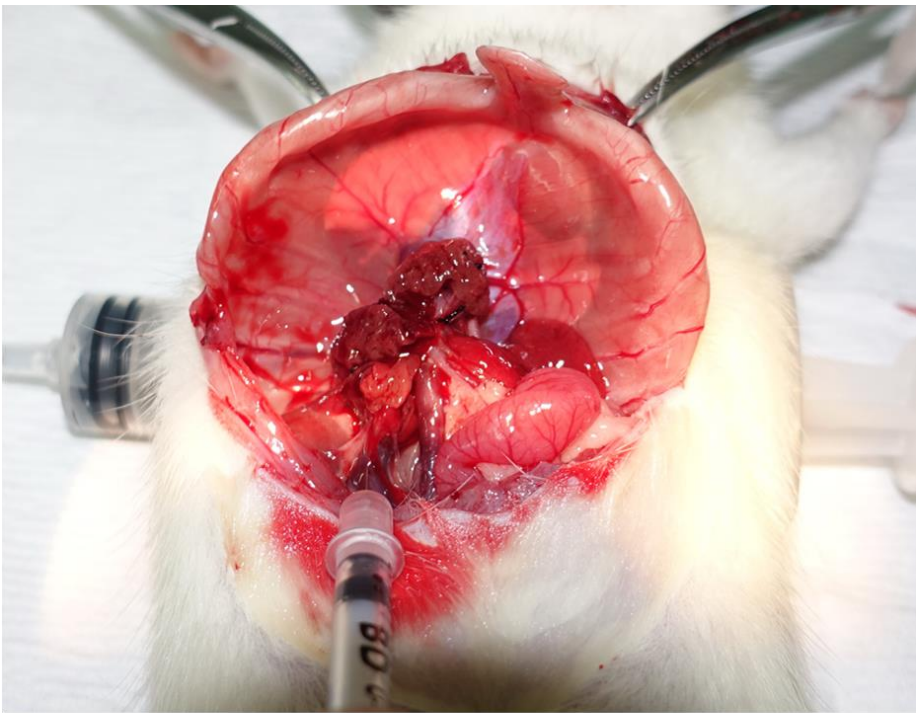

Fig. 2. Injection route of drugs
PBS (0.2 ml) or PBS with SCF+GM-CSF (25mcg/kg, each) was administrated directly into inferior vena cava immediately after 90% PH.

2) *In vitro* cell proliferation and migration assay using Hep3B and SNU449 cells

Commercially available kits (Promega Cell Titer 96® Aqueous One Solution) were used for the proliferation and migration assays in Hep3B and SNU449 cells.

3) Liver regeneration index

The liver regeneration index was calculated using the following formula:

$R\ (\%) = 100 \times (RLs - RLo)/RLo,$

where RLs is the liver weight of the rat at sacrifice and RLo is the remnant liver weight of the rat at surgery, which was calculated as the resected liver weight divided by 9 in the 90% PH model. This provides a percentage value of regeneration based on liver weight of rats at the times of hepatectomy and death.

4) Liver function test

Plasma biochemical analysis was performed within 24 h of blood collection. Blood was collected from the rats, which were sacrificed under general anesthesia. Afterwards, the blood samples were centrifuged after allowing clotting (1—3 h) at 2000 revolutions per min for 15 min. The sera were assayed for serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin levels using commercial kits (Fuji Photo Film Co, Tokyo, Japan).

5) Immunohistochemistry of Ki-67

Immunostaining of liver specimens was performed using a mouse anti-rat Ki-67 antibody (Abcam, USA). Tissue sections were inspected at high power (400× magnification) and positive nuclei were counted in 5-10 randomly chosen fields that approximate 1000 hepatocytes per section. The intensity of the staining was evaluated as negative, medium and high, the latter two being accepted as positive.

6) Western Blotting of Cyclin D1

For western blotting, the 16mcg protein samples of the rat liver were loaded. The antibody (beta-actin diluted 1:500, Santacruz, USA/ cyclin D1 diluted 1:500, CellSingaling, USA) was used to check protein expression.

7) Enzyme-linked immunosorbent assay (ELISA) of IL-6 and TGF-β

Rat blood samples were collected under general anesthesia using a vacuum tube without anticoagulants. Samples were centrifuged

for 5 min at 2000× g and the serum was stored in 0.5 ml aliquots at −80° C until assayed. The levels of rat IL−6 and TGF−β were assessed using sandwich ELISA (R&D system, Oxford, UK). ELISAs were performed according to the manufacturer s protocol.

8) Quantitative real−time polymerase chain reaction (PCR) of CXCL12, CXCR4, MMP−2, MMP−9, and TIMP−3

The mRNA level of C−X−C motif chemokine 12 (CXCL 12, stromal cell−derived factor 1α), C−X−C chemokine receptor type 4 (CXCR 4), matrix metalloproteinases (MMPs) 2 and 9, and tissue inhibitor of metalloproteinases−3 (TIMP−3) were analyzed using quantitative real−time PCR. One microgram of total RNA was extracted from cultured cells using RNeasy Mini kit (Qiagen, German), and cDNA was synthesized using the SuperScript™ First−Strand Synthesis System for use with the RT−PCR kit (Invitrogen, USA), according to the manufacturer' s protocol. Quantitative real−time PCR was performed using the First Essential DNA Probes Master on a LightCycler® 480 Real−

Time PCR System (Roche, Swiss). Experiments were performed in triplicate, and the mean value of the three experiments was used as a relative quantitation value. The glyceraldehyde-3-phosphate dehydrogenase gene was used for control and normalization.

9) Statistical analysis

We sacrificed five rats at each time point for obtaining statistical power. Survival rates were estimated using the Kaplan—Meier method and survival curves were compared using the log-rank test. Continuous variables were expressed as the mean ± standard error (SE). The differences between groups were analyzed using the Student s t-test. $P < 0.05$ was used to indicate statistically significant differences.

Results

1) Survival rates using the modified vessel-oriented surgical method

Before the start of this study, we practiced the 90% PH technique in 20 consecutive rats using a conventional mass ligation technique. We obtained 7-day survival rates of 55% and identified complications including bleeding, abscess formation, congestion of the caudate lobe, and biloma. With this experience, we changed the technique for 90% PH to bile duct-sparing portal pedicle ligation under microscopy before lobe ligation. Using this new procedure, 7-day survival rates were significantly increased to 95% in the subsequent 20 rats (Fig. 3). This technique can minimize blood loss and constriction of the inferior vena cava. Furthermore, it can reduce biliary complications, such as extrahepatic bile duct compromise and disturbance of bile flow by the remnant liver.

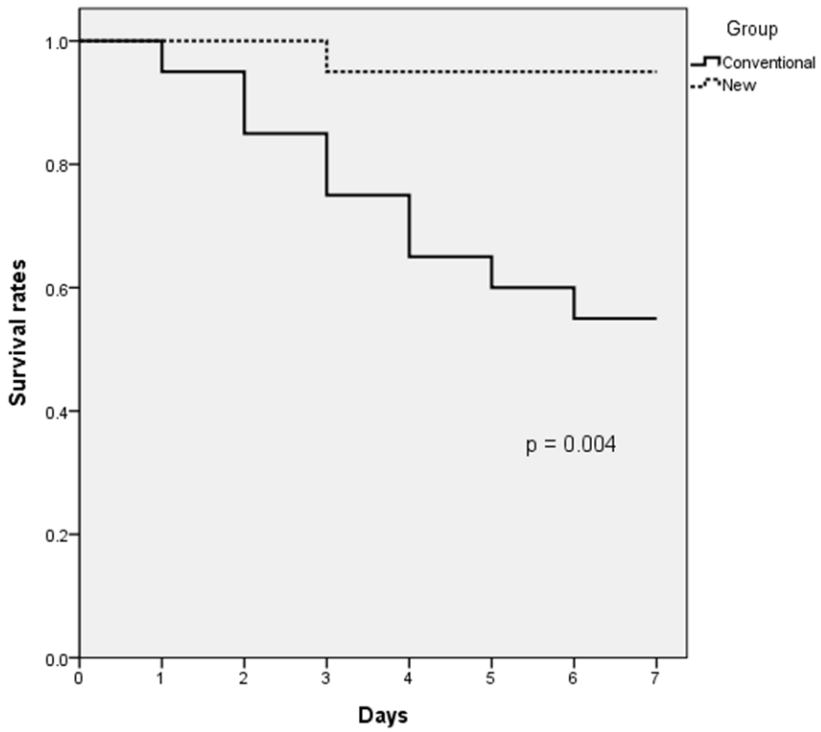

Fig. 3. Comparison of 7 days survivals between two techniques
Survival rates between conventional ligation technique and bile duct sparing portal pedicle ligation technique under microscopy were compared. (7-day survival rates: 95% *vs.* 55%, $p = 0.004$)

2) Synergistic role of SCF and GM-CSF in the *in vitro* proliferation of Hep3B and SNU 449 cells

We evaluated the *in vitro* effects of SCF and GM-CSF on the proliferation of human hepatoma cell lines such as Hep3B and SNU 449 cells. Cells were exposed to medium alone or medium containing SCF and GM-CSF (both at 1, 10, or 100 ng/ml). Proliferation was measured at 72 h by the MTS assay (Fig. 4). Exposure to both 10 ng/ml SCF and 10 ng/ml GM-CSF resulted in a significant increase in the proliferation of cells of the

hepatocytic lineage compared to that of cells exposed to SCF or GM-CSF alone. Based on this result of the synergistic effects of SCF in combination with GM-CSF, we administered the combination drugs to 90% PH rats in *in vivo* setting.

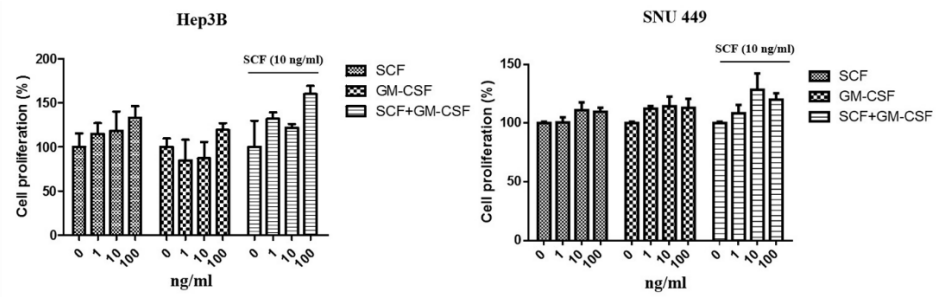

Fig. 4. MTS assays using Hep3B and SNU 449 cell lines
The synergistic effects using SCF and GM-CSF were showed in MTS assay in *in vitro* cell line of Hep3B and SNU 449.

3) Increased liver regeneration according to liver volume and liver function test data

Rats that underwent 90% PH were sacrificed to collect blood and check liver weight on postoperative days 1, 2, 4, and 7. The liver volume was increased rapidly after the surgical procedure and the original volume was restored until postoperative day 7 (Fig. 5). The liver regeneration index was statistically significantly increased in the SCF+GM-CSF group on postoperative days 2 and 4 (day 2: 287.5 ± 19.6 vs. 513.9 ± 67.1, p = 0.025; day 4: 647.6 ± 108.8 vs. 941.7 ± 53.9, p = 0.046; Fig. 6).

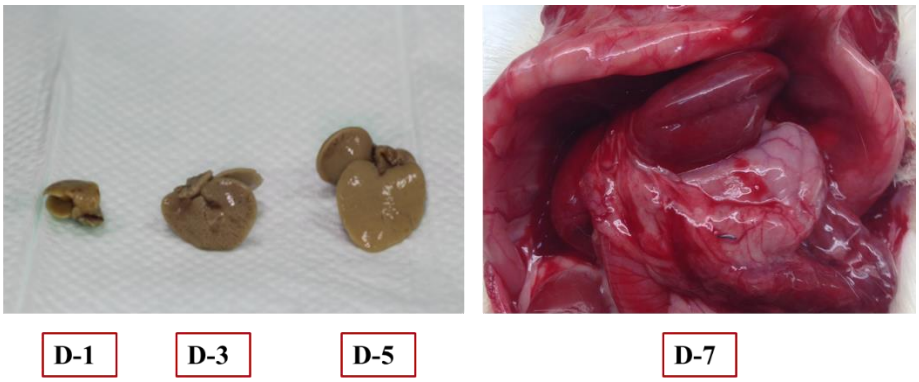

Fig. 5. The change of liver size according to postoperative days
The liver volume after 90% PH was increased rapidly, and then original liver volume was restored until postoperative day 7.

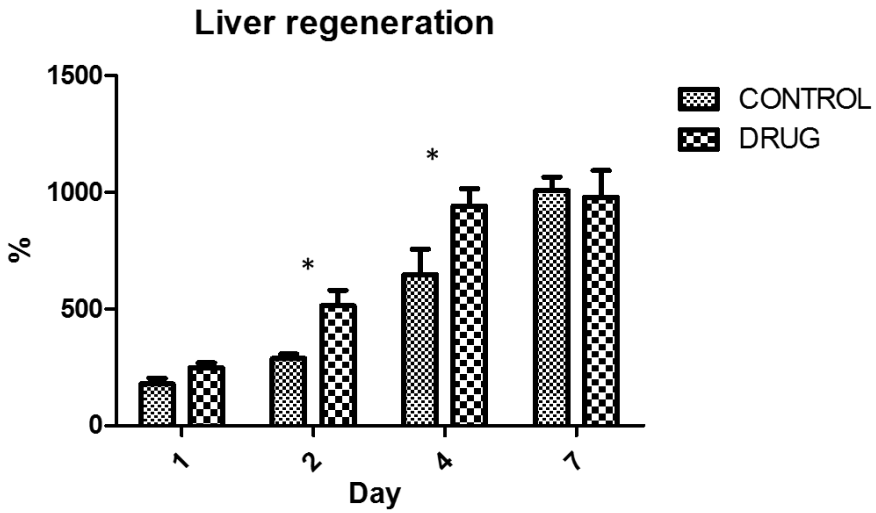

Fig. 6. The change of liver regeneration index between two groups
The liver regeneration index was statistically significantly increased in SCF+GM-CSF group at postoperative day 2 and 4 (287.5 ± 19.6 vs. 513.9 ± 67.1, $p = 0.025$ at day 2; 647.6 ± 108.8 vs. 941.7 ± 53.9, $p = 0.046$ at day 4)

Liver enzyme levels including total bilirubin, AST, and ALT were compared between the control and SCF+GM-CSF group at baseline and on postoperative days 1, 2, 4, and 7 (Fig. 7). For total bilirubin, the level on postoperative day 1 was significantly different between the two groups (2.96 ± 0.16 vs. 1.32 ± 0.24,

$p < 0.001$). Regarding AST, the SCF+GM-CSF group exhibited a significantly lower level than the control group on postoperative day 1 (2970.0 ± 111.6 vs. 1724.0 ± 59.4, $p < 0.001$). ALT levels were significantly lower in the SCF+GM-CSF group than in control group on postoperative days 2 and 4 (day 2: 308.0 ± 73.6 vs. 131.2 ± 3.9, $p = 0.037$; day 4: 180.2 ± 18.9 vs. 104.0 ± 10.7, $p = 0.006$).

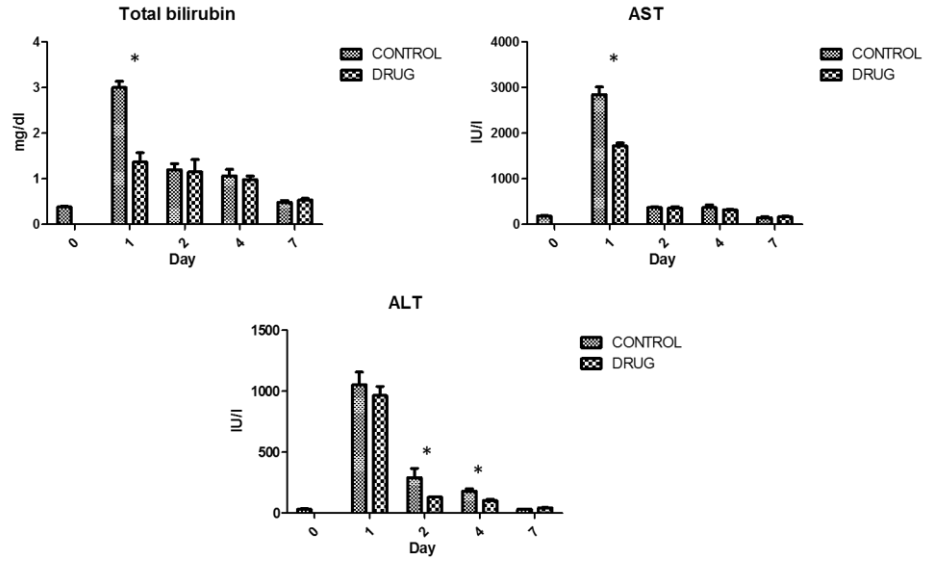

Fig. 7. The results of liver function tests between two groups
The liver function test including total bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) at preoperative status, postoperative day 1, 2, 4, and 7 between control and SCF+GM-CSF group (*: $p < 0.05$).

4) Hepatocyte proliferation according to Ki-67 immunohistochemistry and Cyclin D1 expression

A liver specimen was obtained on the day of surgery and on postoperative days 2, 4, and 7. The specimen was stained with

hematoxylin, eosin, and Ki-67. In immunohistochemistry using Ki-67, the SCF+GM-CSF group exhibited a higher expression value than the control group during the postoperative period, particularly on day 4 (343.3 ± 35.4 vs. 535.7 ± 56.4, p = 0.045, Fig. 8) The maximum proliferation of hepatocytes was observed on postoperative day 2. In western blotting, the SCF+GM-CSF group showed a higher cyclin D1 expression than the control group on postoperative days 1 and 2 (Fig. 9).

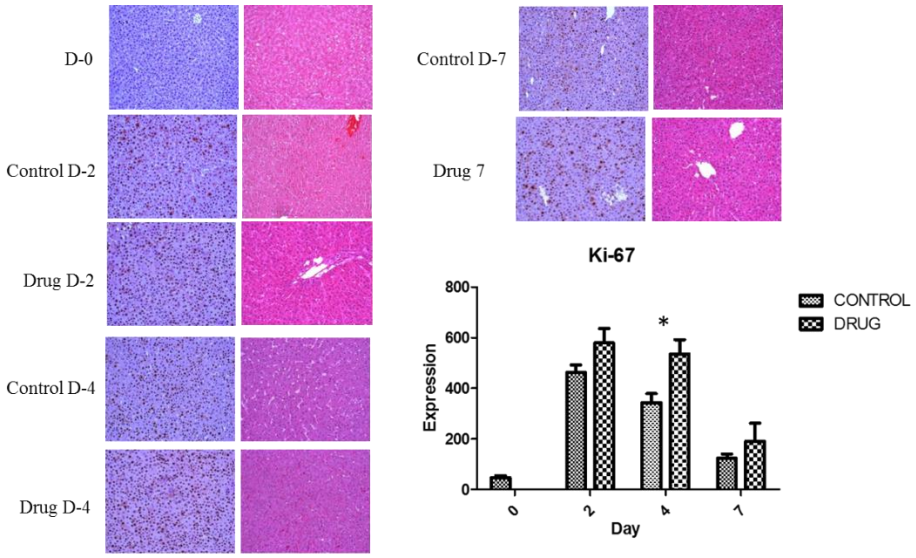

Fig. 8. The results of immunohistochemistry using Ki-67 between two groups
SCF+GM-CSF group showed significant increased Ki-67 expression at postoperative day 4 compared with control group (*: $p < 0.05$).

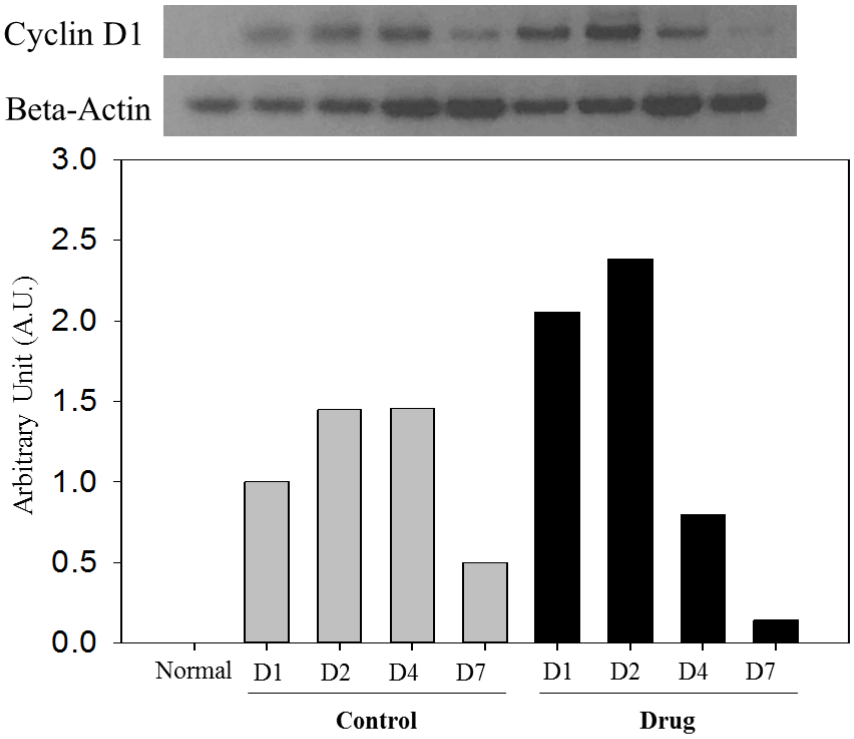

Fig. 9. Western blotting using cyclin D1 antibody
SCF+GM-CSF group showed a higher cyclin D1 expression than the control group on postoperative days 1 and 2.

5) Modulated IL-6 and TGF-β expression and CXCL12/CXCR4 signaling

IL-6 and TGF-β levels in the blood were checked preoperatively and 1, 4, 8, and 24 h after 90% PH. The expression of IL-6 and TGF-β in SCF+GM-CSF group was significantly higher than that in the control group (Fig. 10). In the case of IL-6 in particular, the control group displayed a restoration to the normal range at 24 h after surgery, but IL-6 levels were elevated at this time point in the SCF+GM-CSF

group.

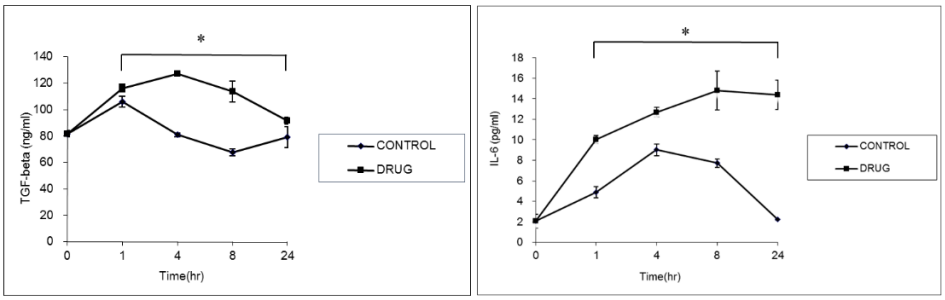

Fig. 10. The results of ELISA assays using IL-6 and TGF- β between two groups
The trend of TGF-β and IL-6 after 90% PH in control and SCF+GM-CSF group (*: $p < 0.05$).

CXCL12 and CXCR4, which are secreted by cells within injured tissue and which appear to be crucial for the migration of mesenchymal stem cells to certain damaged tissues including the liver, were checked by quantitative real-time PCR in liver tissue preoperatively and 1, 2, 4, and 7 days after surgery. In the SCF+GM-CSF group, CXCL12 expression was significantly increased on postoperative days 1 and 7 compared with that in the control group, whereas the level of CXCR4 was significantly increased on postoperative day 2 (Fig. 11).

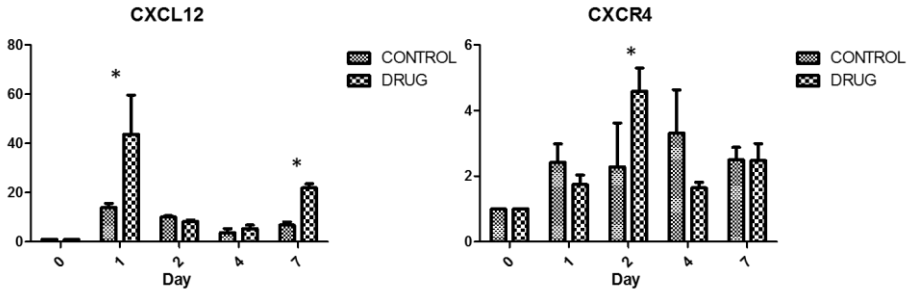

Fig. 11. The relative expression of CXCL12 and CXCR4 between two groups
The relative expression of CXCL12 and CXCR4 after 90% PH in control and SCF+GM-CSF group analyzed by quantitative PCR (*: $p < 0.05$).

6) Changes in MMP and TIMP-3 levels after the administration of SCF and GM-CSF

Liver resection triggers hepatocyte proliferation and hepatic/biliary matrix remodeling. These are important events in the regenerating liver. MMPs comprise a family of zinc-containing neutral proteinases that are involved in matrix remodeling in both normal and pathophysiological processes. To confirm the functional relevance of SCF and GM-CSF-dependent modulation of regeneration, we assessed the expression of MMPs involved in cell remodeling. Treatment with SCF and GM-CSF significantly increased MMP-2 mRNA expression in the liver tissue on postoperative day 1 and 4 and MMP-9 expression on postoperative days 2 and 7 compared with the control levels (Fig. 12). However, levels of the 3 -UTR of TIMP-3 were decreased on postoperative day 2 compared to those in the control group. TIMP-3 has unique domains that interact with extracellular matrix components, and unlike the other TIMPs, is mainly bound to tissue matrix. Our findings provide evidence of a link between SCF and GM-CSF with expression of mediators of cell remodeling in liver cells.

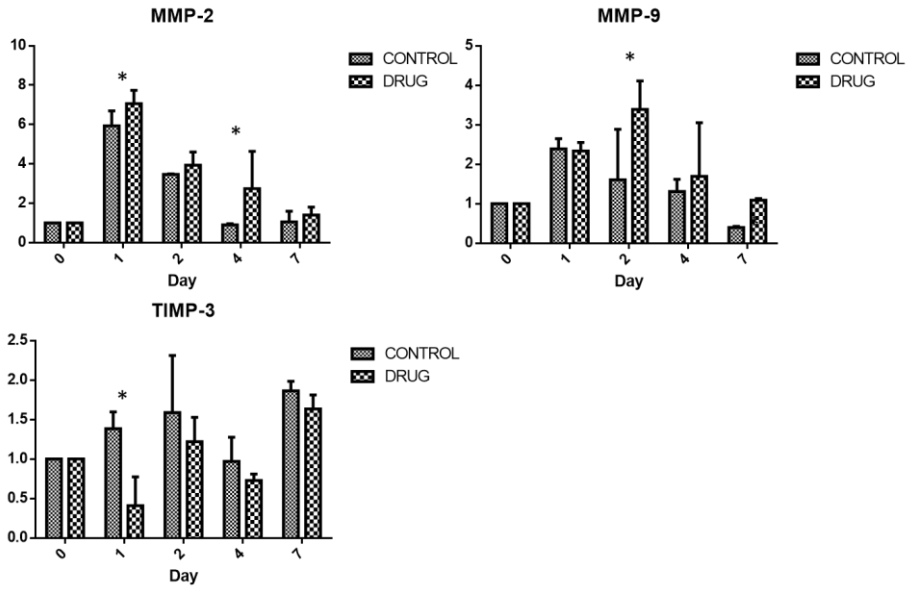

Fig. 12. The relative expression of MMP-2, MMP-9, and TIMP-3 between two groups
The relative expression of MMP-2, MMP-9, and TIMP-3 after 90% PH in control and SCF+GM-CSF group analyzed by quantitative PCR (*: $p < 0.05$).

Discussion

Although various cytokines have been proposed to be involved in liver regeneration, specific drugs for promoting liver regeneration through *in vivo* administration in extreme 90% major hepatectomy models have not been established. In this study, we suggested that combined SCF and GM-CSF administration immediately after rat major hepatectomy promoted liver regeneration successfully. Furthermore, we attempted a new technique for bile duct sparing portal pedicle ligation under microscopy in extreme 90% major hepatectomy and achieved good survival rates of up to 95% after surgery. The combined SCF and GM-CSF administration modulated the upregulation of the CXCL12/CXCR4 pathway and MMPs in remained liver tissue. In addition, in the short term, these drugs were associated with increased levels of IL-6 and TGF-β, two known promoters of hepatic regeneration. An encouraging finding was that the combined intravenous administration of SCF and GM-CSF accelerated liver regeneration concerning the hepatic volume and liver function test results. In particular, the

Ki-67 proliferation index was significantly increased in the SCF+GM-CSF group compared with that in the control group.

Regarding surgery involving the rat liver, it has been not easy to obtain a successful survival rate for performing research. In particular, extreme major hepatectomy, such as 90% or 95% PH, requires greater skill and experience for animal surgery. After trial and error, less bleeding, a shorter operation time, and avoiding compromise of the inferior vena cava were found to be extremely important for obtaining optimal survival rates after major hepatectomy. Through microscopy, we started to dissect the hilum and divide the bile duct and portal vein/hepatic artery. We hypothesized that sparing the bile duct resulted in a more natural flow of the remnant liver and permitted liver cell proliferation. Furthermore, this strategy may have avoided biloma formation or extrahepatic biliary strictures, which could occur during total portal pedicle ligation. In addition, controlling the hilar area is more effective for obtaining an exact portion during hepatectomy and results in less bleeding during lobe ligation.(7) However, time-consuming efforts and fine surgical experience were needed to perform this technique.(8) To perform *in vivo* drug administration for liver regeneration, we

required an extreme major hepatectomy method such as 90% or 95% to identify statistical differences compared with the control group. In this study, we uncovered meaningful differences and results between the control and drug administration groups using this new modified ligation technique.

Inadequate liver regeneration remains an unsolved problem in major liver resection and living donor liver transplantation. Various studies have implicated the usage of cytokines as the exogenous stimulators of liver generation in animal models of liver resection and liver transplantation.(27) SCF and GM-CSF have been demonstrated to affect cellular differentiation and proliferation in various types of cells as well as hepatocytes and cholangiocytes. SCF enhances growth and differentiation when combined with other cytokines.(28) Specifically, SCF and GM-CSF induce synergistic proliferation and differentiation in myeloid progenitor cells and cholangiocytes.(26, 29) This synergistic effect is biologically important, because hematopoietic stem cells and early progenitor cells require growth factors in combination for self-renewal and differentiation. In hepatic remodeling, the combination of SCF and GM-CSF supports the possibility of TGF-β-dependent

mechanisms contributing to synergistic effects.(26) In this study, we observed an elevation of IL-6 and TGF-β expression within 24 h after surgery in the SCF+GM-CSF group (Fig. 10). This result supported the activation of IL-6- and TGF-β-dependent mechanisms for promoting liver regeneration. TGF-β has been associated with intracellular matrix deposition and hepatic/biliary tissue repair/damage. This cytokine stimulates mesenchymal proliferation, inhibits epithelial growth, and facilitates organogenesis. In particular, TGF-β is produced in PH. According to our data, the combination of SCF and GM-CSF induced TGF-β and enhanced the proliferation of liver cells in the early period after surgery.

The importance of these cytokines associated with HSCs for liver regeneration has been demonstrated in several studies revealing that HSCs can differentiate into hepatocytes.(30, 31) The mechanism of HSC hepatic regeneration remains unresolved. However, a therapeutic role of HSCs in liver injury has been described in rodents, albeit with varying contributions of transdifferentiation and fusion.(32, 33) Murine and human studies illustrated that CXCL12 (SDF-1) and its receptor, CXCR4, are involved in recruiting inflammatory cells to injured

livers as well as inducing the proliferation of endogenous hepatic oval cells.(34, 35) CXCL12/CXCR4 interactions participate in the mobilization of HSCs from bone marrow to the liver during injury.(36, 37) Recent research demonstrated the key role that CXCL12/CXCR4-mediated signaling plays in the migration of human progenitors to the murine liver. Neutralization of the CXCR4 receptor with an anti-CXCR4 antibody significantly inhibited the homing of human cord blood to the liver of irradiated NOD/SCID mice.(15) Moreover, injection of human CXCL12 into the murine liver parenchyma enhanced the hepatic migration of human stem cells. In the present study, we assessed changes of CXCL12/CXCR4 expression after 90% rat PH. In the SCF+GM-CSF group, the expression of CXCL12/CXCR4 was higher than that in the control group (Fig. 11). With this result, we could suggest that SCF+GM-CSF enhanced the CXCL12/CXCR4 pathway and promote HSC homing to the liver.

In the early phase of liver regeneration, activation of MMP-9 is observed within 30 min after PH.(38) Studies of wound healing and tumor biology indicated that matrix remodeling causes signaling through integrins and this signaling is associated with release of locally bound growth factors and peptides that have

signaling capabilities.(39) Although there is little proteinaceous matrix in the liver visible under the microscope, there is a great abundance of heavily glycosylated proteins in the pericellular space surrounding hepatocytes. The overall regulation of the extracellular matrix during liver regeneration is an extremely complex process involving MMPs and TIMPs.(40) Hepatic extracellular matrix binds many growth factors. Prominent among matrix binding growth factors in the liver is HGF.(41) MMP and TIMP levels are important in the regulation of HGF release and its availability for activation during regulation. In this study, we found a correlation between SCF+GM-CSF treatment and MMP expression (Fig. 12). MMP-2 and MMP-9 were significantly upregulated in the drug group compared with their expression in the control group. In contrast, the level of TIMP-3 was lower in the drug group than in the control group. This finding indicated that SCF and GM-CSF regulated matrix remodeling in the liver after PH and enhanced liver regeneration through the upregulation of MMPs.

In addition to the molecular change after SCF+GM-CSF administration, clinical parameters including liver regeneration indices and liver enzyme levels were improved in this study. The

velocity of liver regeneration to a normal volume is an important maker of postoperative liver failure in human liver resection and transplantation.(2, 42, 43) In the present study, the SCF+GM-CSF group achieved restoration to the original volume on postoperative day 4 (Fig. 6). However, restoration in the control group was delayed until postoperative day 7. Faster liver regeneration in the SCF+GM-CSF group was also identified on immunohistochemistry using Ki-67 (Fig. 8). In particular, the levels of total bilirubin, AST, and ALT were significantly decreased in the early period after major hepatectomy (Fig. 7). In the clinical setting, total bilirubin is a significant prognostic factor for predicting liver failure.(44) Therefore, therapeutic strategies for administering SCF+GM-CSF may be potentially useful for rebuilding the hepatobiliary system after liver resection.

There are some limitations to this study. We did not investigate the use of SCF or GM-CSF alone. Therefore, the exact synergistic effect of the *in vivo* administration of SCF and GM-CSF could not be proven in this study. However, with data supporting the combined effect of SCF and GM-CSF, we could confirm the clinical and molecular effects of these cytokines after

major hepatectomy in a rodent model. In addition, we did not demonstrate the effect of the possibility of cancer proliferation after the administration of these cytokines. Regenerating factors could influence tumor growth and metastasis. Therefore, further studies are needed to prove the effect of SCF and GM-CSF on tumor growth using rat or mouse liver cancer.

Conclusion

In conclusion, our data suggest that the administration of SCF and GM-CSF can enhance liver regeneration immediately after major hepatectomy by modulating IL-6/TGF-β signaling, the CXCL12/CXCR4 pathway, and matrix remodeling in the critical period. We have identified the possibility of therapeutic treatment using the combination of SCF and GM-CSF after human major hepatectomy to promote liver regeneration.

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요약 (국문초록)

새로운 절제 방법으로 시행한 90% 부분 흰쥐 간절제 모델에서 간재생에 영향을 주는 stem cell factor와 granulocyte macrophage colony-stimulating factor 동시 투여의 효과

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배경: 간절제와 간이식후에 간재생 능력에 관한 문제는 수술 후 간기능 부전을 예방하는 중요한 요소로 간주되어 왔다. 최근 Stem cell factor (SCF)와 Granulocyte macrophage colony-stimulating factor (GM-CSF)가 간재생에 상호보완적으로 중요한 역할을 하는

것이 in-vitro 연구에서 밝혀졌다. 본 연구의 목적은 90% 부분 간절제를 시행한 흰쥐에서 SCF와 GM-CSF를 동시에 투여하여 간재생의 효과를 알아보는 것이다.

방법: Sprague Dawley 흰쥐를 현미경 하에서 담도를 살리고 문맥만 결찰하는 새로운 방법으로 90% 부분 간절제를 시행하였다. 수술 후 두 그룹으로 나누어 그룹 1은 수술 직후 하대정맥을 통하여 phosphate-buffered saline만 투여하고, 그룹2는 SCF와 GM-CSF를 각각 25mcg/kg씩 동시에 투여하였다. 간재생 능력과 조혈모세포의 이동에 관여하는 물질에 대한 연구가 수술 후 1, 2, 4, 7일째 이루어 졌다. 통계적 유의성을 확보하기 위해 각 단계별로 5마리의 흰쥐를 희생하여 샘플을 채취하였다.

결과: 새롭게 개발된 방법으로 시행한 90% 흰쥐 부분 간절제에서 생존율이 기존의 결찰법으로 시행한 55%보다 95%로 향상을 보였다. (p = 0.004) 그룹2는 그룹1에 비해서 간재생지수가 수술 후 2일과 4일째 유의하게 증가하였다. (287.5 ± 19.6 vs. 513.9 ± 67.1, p = 0.025 at day 2; 647.6 ± 108.8 vs. 941.7 ± 53.9, p = 0.046) 혈액내 total bilirubin, aspartate aminotransferase, alanin aminotransferase 수치가 그룹2에서 수술 후 1일째 그룹1에 비해서 유의하게 낮았다. Ki-67을 이용한 면역염색에서 그룹 2에서 그룹1보다 수술 후 4일째 유의하게 더 높은 발현을 보였다. (343.3 ± 35.4 vs. 535.7 ± 56.4, p = 0.045) 그룹2는 IL-6와 TGF-β

수치가 간절제 시행 후 24시간 이내에 그룹1에 비해서 더 높게 유지되는 양상을 보였으며, quantitative real-time PCR을 이용한 간내 CXCL12/CXCR4과 MMP-2,9 수치도 수술 후 더 높게 유지되는 결과를 보였다.

결론: 본 연구 결과에서 90% 대량 간절제에서 SCF와 GM-CSF를 동시에 투여하는 것이 IL-6/TGF-β와 CXCL12/CXCR4 및 matrix remodeling을 조절하여 간재생을 강화하는 것을 확인하였다. 이 결과를 토대로 임상에서 간재생을 촉진하기 위해 SCF와 GM-CSF 병용투여가 후보 치료약제로 가능성을 보였다.

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중심 단어: 간재생, SCF, GM-CSF, 부분 간절제, 흰쥐

학번: 2010-30536