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Granulocyte Colony-stimulating Factor Supports Liver Regeneration in a Small-for-size Liver Remnant Mouse Model

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Abstract Experimental partial hepatectomy of more than 80% of the liver weight bears an increased mortality in rodents, due to impaired hepatic regeneration in small-for-size liver remnants. Granulocyte colony-stimulating factor (G-CSF) promotes progenitor cell expansion and mobilization and also has immunomodulatory properties. The aim of this study was to determine the effect of systemically administered G-CSF on liver regeneration and animal survival in a small-for-size liver remnant mouse model. Mice were preconditioned daily for 5 days with subcutaneous injections of 5 μg G-CSF or aqua ad injectabile. Subsequently, 83% partial hepatectomy was performed by resecting the median, the left, the caudate, and the right inferior hepatic lobes in all animals. Daily sham or G-CSF injection was continued. Survival was significantly better in G-CSF-treated animals ($P<0.0001$). At 36 and 48 h after microsurgical hepatic resection, markers of hepatic proliferation (Ki67, BrdU) were elevated in G-CSF-treated mice compared to sham injected control animals ($P<0.0001$) and dry liver weight was increased ($P<0.05$). G-CSF conditioning might prove to be useful in patients with small-for-size liver remnants after extended hepatic resections due to primary or secondary liver tumors or in the setting of split liver transplantation.

Keywords Liver regeneration · Granulocyte colony-stimulating factor · *In vivo* study · Rodent

Introduction

Intense regeneration and almost 100% survival follows partial hepatectomy (PH) of 70% of liver mass in rodents.[1–3] More extensive resections of 70 to 85% PH bear increased mortality due to impaired liver regeneration and the development of acute hepatic failure.[4–7]

Similarly, the human liver regenerates after hepatic resection. The size of the remaining liver tissue after resection is crucial for successful restoration of liver mass. In humans, a liver remnant of 45%, corresponding to at least 1.2% of body weight, results in excellent regeneration and uncomplicated recovery.[8] More extensive resections (i.e., 50 to 70% resections) with smaller liver remnants can cause impaired regeneration and subsequent hepatic failure. Therefore, 0.8% of body weight is currently considered the minimal weight of the liver remnant in patients undergoing hepatic resection.[8–11]

While liver regeneration after partial hepatectomy is a well-characterized phenomenon, the reasons for impaired regeneration in small-for-size liver remnants (i.e., <0.8% of body weight) are far from being understood.[3,12,13] Recently identified bone marrow-derived adult liver progenitor cells might play an important role in the pathophysiology of impaired liver regeneration.[14,15]

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Granulocyte colony-stimulating factor (G-CSF) promotes proliferation and mobilization of the bone marrow progenitor cell population.[16–18] Furthermore, G-CSF has antiinflammatory and antiinfectious effects.[16,19] Clinical data in humans indicate that G-CSF administration provides hepatic support during acute liver failure and is also beneficial after major surgical interventions.[19,20]

In rodent models of toxic liver injury, G-CSF accelerated recovery and improved animal survival.[21–23] From a surgical point of view, the support of hepatic recovery after extended liver resection is of crucial importance.[10,24] We hypothesized that G-CSF could provide such support in an experimental setting. Using a microsurgical small-for-size liver remnant mouse model (remnant liver weight below 0.8% of mouse body weight), we determined the effects of G-CSF on animal survival, on the number of nucleated bone marrow cells, and on hepatic regeneration.

Material and Methods

Adult male BalbC mice (n=102, 20–25 g, 6–8 weeks) were kept under standard conditions. All animal experimentation was approved by the local committee for animal welfare in accordance with the European Convention on Animal Care. Surgeries were performed as previously described.[25]

Experimental Groups

Animals were stratified in a G-CSF (n=53) and a sham-conditioned group (n=49). G-CSF animals received a daily subcutaneous injection of 5 μg G-CSF in 100 μl of aqua ad injectabile (Granocyte®, Aventis Pharma AG, Zurich, Switzerland) for 5 days preoperatively and daily after liver resection until the end of the experiment. Sham controls were injected daily with 100 μl of aqua ad injectabile.

Surgical Procedures

From a microsurgical point of view, the mouse liver consists of five lobes. For male adult BalbC mice, the relative weight of each liver lobe as a percent of the whole is known: the left lobe=34%, the median lobe=26%, the right superior lobe=17%, the right inferior lobe=15%, and the caudate lobe=8%.[25] For the 83% PH, the lesser omentum was incised and the caudate lobe resected. After incision of the left triangular and the falciform ligament, the left and the median lobes were resected. The pedicle of the right inferior lobe was exposed by incision of the ligament between the vena cava posterior and the anterior liver capsule. The pedicle of the right inferior lobe was then carefully ligated and the right inferior lobe excised.[7] The resected liver tissue and the entire mouse were weighed. Animals were kept under a warming lamp for 24 h postoperatively. To prevent postoperative hypoglycemia, 1.0 ml of 5% glucose (Bioren SA, Couvet, Switzerland) was injected subcutaneously.[6,26] Daily subcutaneous G-CSF and sham conditioning were continued.

Tissue Harvest

The regenerating liver was examined 36 and 48 h after 83% PH in 20 animals. 5-bromo-2'-deoxyuridine (BrdU, 50 mg/kg body weight, Fluka Biochemica, Buchs, Switzerland) was injected intraperitoneally 2 h before liver harvesting. Under inhalation anesthesia, animals were then killed and the remnant right superior liver lobe excised, weighed, and fixed in 4% formalin (Sigma, Buchs, Switzerland). Dry liver weight was determined 72 h after 65°C heat exposure. Tissue from the duodenum and testis served as positive controls for BrdU incorporation.

Immunohistochemistry (Ki67 Expression, BrdU Incorporation)

To measure hepatic proliferation, the expression of Ki67 and BrdU incorporation were determined in the right superior liver lobe at 36 and 48 h after 83% PH on paraffin-embedded tissue sections as described.[7] Briefly, before Ki67 staining, 2–3 μm paraffin-embedded sections were dewaxed, rehydrated, and pretreated by boiling in 10 mM citrate buffer, pH 6.0, in a pressure cooker. Sections were then washed in Tris-buffered saline (TBS) and incubated with a rat anti-mouse Ki67 antibody (clone TEC-3; Dako, Glostrup, Denmark) diluted 1:200 in TBS with 0.5% casein and 5% normal goat serum for 60 min at room temperature. Next, a 1:300 dilution of a biotinylated goat anti-mouse immunoglobulin antiserum (DakoCytomation, Glostrup, Denmark) was applied for 45 min. Thereafter, sections were incubated with an avidin–biotin-complex/horseradish peroxidase system (1:100 in TBS, Vector, Burlingame CA, USA) for 45 min. Finally, sections were developed in 0.1% 3,3′-diaminobenzidine (Sigma, St. Louis MO, USA) with 0.03% H_2O_2, counterstained with hematoxylin, and mounted. Ki67 positive and negative nuclei were counted in 10 high-power field microscopy images by two independent researchers, and the Ki67 labeling index was calculated from the data obtained.[27]

After BrdU staining,[7] BrdU positive cells in duodenal crypts and testis demonstrated systemic BrdU uptake and nuclear incorporation. Liver samples not treated with the primary anti-BrdU antibody served as negative controls. BrdU positive and negative cells were counted and the BrdU labeling index was calculated as described.[7]

Cell Isolation from the Adult Mouse Bone Marrow and Magnetic Cell Sorting of β_2-Microglobulin Negative/ Thy-1 Positive Progenitor Cells

Femoral bone marrow cells were harvested by aspiration through a 23-gauge needle (Venflon, Becton Dickinson, Fraga, Spain) as described,[28] filtered through a 30 μm filter (Nr. 130-041-407, Myltenyi Biotech, Bergisch Gladbach, Germany), and counted. To determine the amount of adult bone marrow-derived liver progenitor cells, β_2-microglobulin negative/Thy-1 positive cells were isolated according to a recently developed magnetic cell-sorting protocol and counted using a Neubauer counting chamber in a blinded fashion.[15,29]

Statistical Analysis

Results are expressed as mean ± standard deviation. Cumulative survival was analyzed according to Kaplan–Meier and survival curves compared by the use of the log-rank test. For normally distributed data, Student's *t* test was applied (Jandel Scientific 1.0, San Rafael, CA, USA), and for nonnormally distributed data the Mann–Whitney rank sum test was used. The significance level was set at $P<0.05$.

Results

The Small-for-size Liver Remnant Model (83% PH) in the Mouse

The amount of liver tissue resected corresponded to 3.8± 0.4% of mouse body weight in the sham-conditioned group and was not different in the G-CSF-conditioned group (3.9±0.3% of body weight, *t* test: $P=0.54$).

Cumulative Survival

The cumulative survival was determined according to Kaplan–Meier in G-CSF-conditioned ($n=35$) and in sham-conditioned animals ($n=33$). The survival curve is depicted in Fig. 1. By postoperative day 3 all animals of the sham-conditioned group were dead. G-CSF-conditioned animals survived significantly better (25.7% on day 7 and thereafter, log rank test: $P<0.0001$). A total of nine G-CSF-conditioned animals were censored 14 days after 83% PH.

Dry Liver Weight

Dry liver weight was significantly increased in G-CSF-conditioned mice (0.475±0.050% of body weight) when compared to sham-treated control animals (0.325±0.096% of body weight, *t* test: $P<0.05$) 36 h after 83% partial hepatectomy.

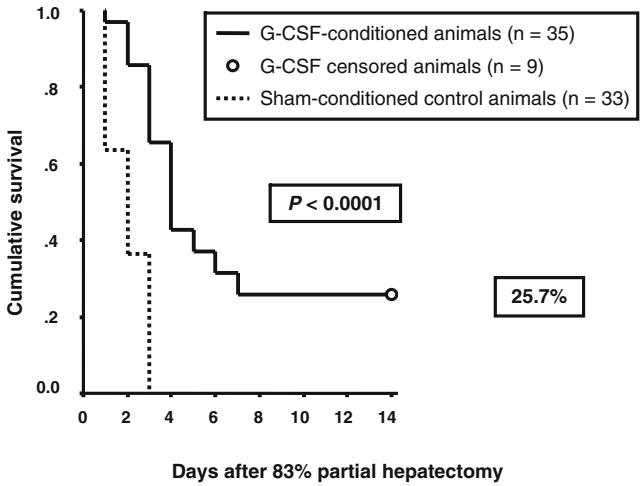

Figure 1 Cumulative survival (according to Kaplan–Meier) in G-CSF-conditioned mice and sham-treated control animals after 83% partial hepatectomy. Survival after extended 83% hepatic resection in mice was significantly better with granulocyte colony stimulating factor (G-CSF) conditioning. No sham-conditioned animals survived longer than 72 h after 83% partial hepatectomy, while 25.7% survival was observed in the G-CSF-conditioned group.

Markers of Liver Regeneration (Ki67 Expression and BrdU Incorporation)

Ki67 expression in hepatocytes was elevated in the G-CSF group at 36 h (2.8±2.6 vs 0.03±0.2%, rank sum test: $P<0.0001$) and at 48 h (45.1±34.6 vs 0.7±1.0%, rank sum test: $P<0.0001$) after 83% PH. BrdU labeling of hepatocytes at 48 h was 0.1±0.3% in the sham and 35.2±34.2% in the G-CSF group (rank sum test: $P<0.0001$; Fig. 2). No zone-specific BrdU-positive cell clusters were seen.

Isolation of Nucleated Cells from the Adult Bone Marrow

The total nucleated cell count of the adult femoral mouse bone marrow was $9.5 \pm 0.8 \times 10^6$ cells ($n=3$ for each experimental group and each time point) in sham-conditioned animals before hepatic resection, and significantly lower at $6.9 \pm 0.1 \times 10^6$ cells 24 h after 83% PH ($P<0.05$).

After 5 days of G-CSF preconditioning, $8.5 \pm 1.7 \times 10^6$ nucleated cells were present in the bone marrow (P=ns when compared to sham-conditioned control animals). At 24 h after 83% resection, the total number of nucleated cells rose significantly during hepatic regeneration in G-CSF-conditioned animals, to $13.4 \pm 1.4 \times 10^6$ cells ($P<0.05$ when compared to preoperative values, and $P<0.05$ when compared to bone marrow cell numbers in sham-conditioned mice 24 h after 83% PH).

Adult liver progenitor cells were purified by β_2-microglobulin negative and Thy-1 positive magnetic cell sorting. In sham-conditioned animals, 6.3±0.8% were identified before and 7.5±5.8% after 83% PH as β_2-microglobulin

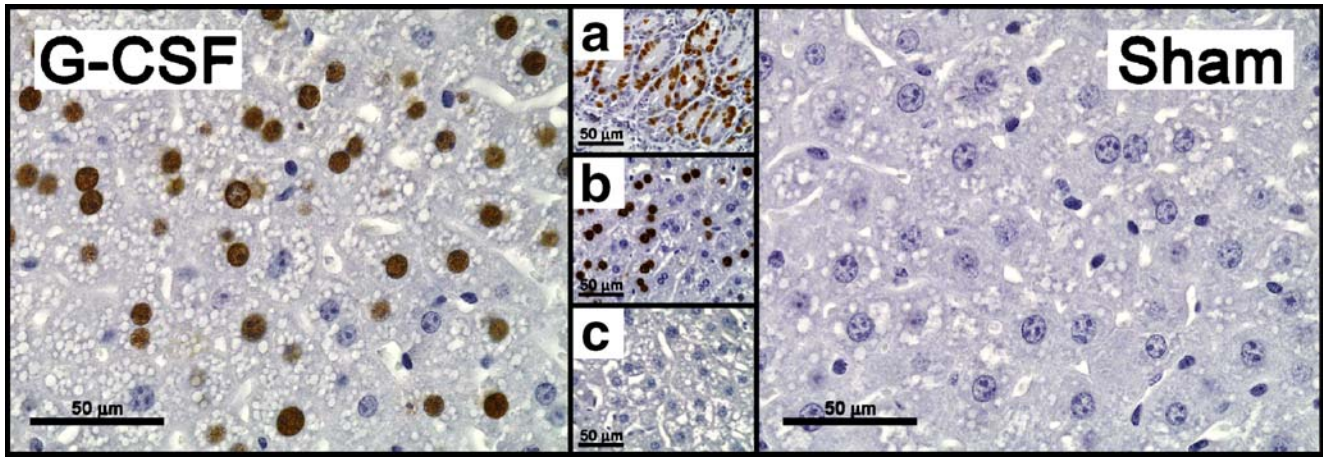

Figure 2 BrdU staining 48 h after 83% partial hepatectomy. While no BrdU positive cells were detectable in the sham-conditioned group, active liver regeneration, and positive BrdU staining were seen in G-CSF-treated animals. Duodenal tissue (a) served as an internal control to ascertain adequate BrdU uptake and incorporation. Liver samples after 70% partial hepatectomy served as positive (b) and negative (no primary antibody, c) controls.

negative/Thy-1 positive (P=ns). In G-CSF-conditioned animals, 5.4±4.3% of bone marrow cells were β_2-microglobulin negative/Thy-1 positive before resection and 5.8±2.1% were β_2-microglobulin negative/Thy-1 positive after 83% PH (P=ns).

Discussion

The microsurgical 83% PH mouse model is suitable for testing hepatic supportive regimens in the experimental setting of small-for-size liver remnants. Control mice showed impaired liver regeneration, hepatic encephalopathy, and consequent death within 3 days after 83% PH, as expected.[6,13,26]

In contrast, 25.7% of G-CSF-conditioned mice survived. Dry liver weight was significantly increased, and expression of the immunohistochemically measured markers of proliferation was significantly higher in the G-CSF group.

For clinical use, the described systemic G-CSF conditioning could under certain conditions allow more radical resections for primary or secondary liver tumors and support the small-for-size liver remnant during hepatic regeneration. This support could also be helpful in the setting of living related liver donation. Currently, a right hemihepatectomy is performed for adult living related liver donation and consequently around 65% of the liver is grafted.[30] The remaining 35% of the left liver should allow safe and uncomplicated hepatic regeneration for the donor. However, due to technical difficulties when performing right hemihepatectomies, including multiple anatomic variants of the portal triad and the hepatic veins or due to impaired hepatic regeneration, a 0.5% mortality is reported after living related liver donation in the donor population.[30] Due to limitations in the ratio of graft liver weight to recipient body weight, living donor liver transplantation of the left lateral segments II and III is so far mainly established in pediatric recipients. From the surgical point of view, this procedure is significantly safer than a right hemihepatectomy for the donor.[31] When the regenerative capacity of small split liver grafts could be augmented (i.e., by the use of G-CSF preconditioning), segmental liver transplantation from both cadaveric and living donors could be safely proposed for adult recipients as well.

On the other hand, the administration of growth factors to patients suffering from carcinomatous disease has to be critically assessed. Fortunately, 15 years of clinical experience have provided no convincing evidence that G-CSF causes malignant transformation or worsens the course of malignant disease.[32,33]

A distinct progenitor cell population was recently successfully isolated from adult rodent bone marrow by our group.[15,28,29] We expected that G-CSF might expand, activate, and mobilize the described β_2-microglobulin negative/Thy-1 positive bone marrow progenitor cells during regeneration of the small-for-size liver remnant. Nucleated bone marrow cells were therefore monitored before and after 83% PH. As expected, the total nucleated cell count was significantly elevated after 6 days of G-CSF conditioning.[18] But to our surprise, no alteration of the β_2-microglobulin negative/Thy-1 positive progenitor cell pool was detectable after 83% PH in either experimental group by the magnetic cell sorting procedure used. Furthermore, a typical pattern of progenitor cell support was not seen in the

G-CSF-conditioned regenerating liver samples and homogeneously distributed BrdU as well as Ki67 positive hepatic nuclei were found in the entire mouse liver lobes.[15,34] It is, however, possible that bone marrow progenitor cells supported liver regeneration by direct fusion, as described.[35] On the other hand, the observed G-CSF effect might be directly related to its recognized immunomodulatory properties and possibly improved neutrophil function,[19,20] thereby preventing typical systemic septic complications during the clinical course after extended liver resection.[8]

Conclusion

G-CSF supports liver regeneration and promotes survival in a small-for-size liver remnant mouse model. Additional human studies might prove that systemic G-CSF conditioning could be clinically valuable for the treatment of patients after major hepatic resections or in the setting of split liver transplantation.

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