

Sympathetic Nervous System Inhibition Increases Hepatic Progenitors and Reduces Liver Injury

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Recovery from liver damage might be enhanced by encouraging repopulation of the liver by endogenous hepatic progenitor cells. Oval cells are resident hepatic stem cells that promote liver regeneration and repair. Little is known about the mediators that regulate the accumulation of these cells in the liver. Parasympathetic nervous system inhibition reduces the number of oval cells in injured livers. The effect of sympathetic nervous system (SNS) inhibition on oval cell number is not known. Adrenergic inhibition mobilizes hematopoietic precursors into the circulation and has also been shown to promote liver regeneration. Thus, we hypothesized that SNS inhibition would promote hepatic accumulation of oval cells and reduce liver damage in mice fed antioxidant-depleted diets to induce liver injury. Our results confirm this hypothesis. Compared with control mice that were fed only the antioxidant-depleted diets, mice fed the same diets with prazosin (PRZ, an α -1 adrenoceptor antagonist) or 6-hydroxydopamine (6-OHDA, an agent that induces chemical sympathectomy) had significantly increased numbers of oval cells. Increased oval cell accumulation was accompanied by less hepatic necrosis and steatosis, lower serum aminotransferases, and greater liver and whole body weights. Neither PRZ nor 6-OHDA affected the expression of cytokines, growth factors, or growth factor receptors that are known to regulate progenitor cells. In conclusion, stress-related sympathetic activity modulates progenitor cell accumulation in damaged livers and SNS blockade with α -adrenoceptor antagonists enhances hepatic progenitor cell accumulation. (HEPATOLOGY 2003;38:664-673.)

The liver's progenitor cell compartment is activated if mature hepatocytes reach a critically low number, such as after severe hepatic injury, or if the mature hepatocytes are prevented from dividing by hepatotoxic drugs.¹ One hepatic progenitor cell (HPC) com-

partment, the oval cells, is resident within the liver's canals of Herring, the terminal branches of the biliary tree. The source of oval cells is debated. Because transplanted bone marrow cells can rescue experimental animals from liver failure by reconstituting lethally damaged livers^{2,3} and oval cells express hematopoietic markers,⁴⁻⁶ some have argued that oval cells may be derived from pluripotent progenitors that reside in the bone marrow.^{2,3} It is possible, however, that oval cells are a truly unique population of HPC, and oval cell expression of hematopoietic markers⁴⁻⁶ may not be indicative of their lineage. In any case, the factors involved in expanding HPC populations within the liver are not well understood. The identification of such factors is an important goal because they may be useful to support patients with liver failure until a suitable organ is found for transplantation. Indeed, if successful, targeted expansion of endogenous HPC may even obviate the need for orthotopic liver transplantation.

The autonomic nervous system (ANS) may regulate the accumulation of HPC in the liver. The parasympathetic nervous system promotes this process because vagotomy reduces HPC in rats with drug-induced hepatitis. Similarly, after transplantation (which surgically denervates the liver), human livers that develop hepatitis have fewer HPC than native, fully innervated livers with simi-

Abbreviations: HPC, hepatic progenitor cell; ANS, autonomic nervous system; SNS, sympathetic nervous system; PRZ, prazosin; 6-OHDA; 6-hydroxydopamine; NK, natural killer; HMCDE, half methionine-choline deficient plus ethionine; CMCD, control methionine choline diet; IP, intraperitoneal; ALT, alanine aminotransferase; RPA, ribonuclease protection assay; SCF, stem cell factor; HGF, hepatocyte growth factor; IL, interleukin; LIF, leukemia inhibitory factor; GM-CSF, granulocyte-macrophage colony stimulating factor; M-CSF, macrophage colony stimulating factor; G-CSF, granulocyte colony stimulating factor; VEGF, vascular endothelial growth factor; PH, partial hepatectomy; NE, norepinephrine; TNF- α , tumor necrosis factor α .

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Received March 4, 2003; accepted June 4, 2003.

Supported by NIH NIAAA RO1-10154 (to A.M.D.), NIAAA RO1-12059 (to A.M.D.), and F.W.O. Vlaanderen G.0139.00N (to T.R.).

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0270-9139/03/3803-0017\$30.00/0

doi:10.1053/jhep.2003.50371

lar degrees of injury.⁷ This may alter the graft's regenerative response because the rate of fibrosis is often accelerated in liver transplant recipients with chronic hepatitis.⁸

Although the sympathetic nervous system (SNS) is known to modulate both liver regeneration⁹ and hepatic fibrogenesis,^{10,11} it is not known whether these effects reflect its ability to influence HPC accumulation. Thus, the present study tests the hypothesis that the SNS regulates the expansion of HPC. We used established models of HPC accumulation involving administration of antioxidant-depleted diets plus ethionine to cause liver injury and inhibit mature hepatocyte replication.¹² We then inhibited the SNS by adrenoceptor antagonism with Prazosin (PRZ) or chemical sympathectomy with 6-hydroxydopamine (6-OHDA) and used flow cytometry and immunohistochemistry to compare HPC numbers in control and SNS-inhibited livers.^{4,7} Because the SNS is known to promote the hepatic accumulation of natural killer (NK) T cells,¹³ liver NK T cells were evaluated concurrently to monitor the physiologic efficacy of SNS inhibition. Our results demonstrate that SNS inhibition significantly enhances the accumulation of HPC and reduces net liver damage induced by chronic hepatotoxin exposure.

Materials and Methods

Animals. C57BL/6 mice, 10 to 18 weeks old, were from Jackson Laboratory (Bar Harbor, ME).

Diets and Drugs. The diet was a commercial, half-choline-deficient diet (ICN, Aurora, OH) also 50% deficient in methionine, administered with ethionine (0.15%) in drinking water, to enhance oxidative injury to the liver and cause hepatic accumulation of oval cells within 2 weeks.¹² The combination treatment is hereafter referred to as half methionine choline-deficient diet plus ethionine (HMCDE). The control methionine choline diet (CMCD) was also from ICN. PRZ and DL-ethionine (E) were from Sigma (St. Louis, MO).

Chemical Sympathectomy. Chemical sympathectomy was achieved by intraperitoneal (IP) injection of 6-OHDA 100 mg/kg for 5 consecutive days.¹⁴ Thereafter, 6-OHDA was administered at 100 mg/kg IP, 3 times per week to ensure continued sympathectomy.¹¹ This regimen of 6-OHDA treatment depletes norepinephrine in rodent tissues.^{11,14}

Experimental Design. Mice were divided into 4 groups (10 to 12 mice/group): control diet, HMCDE plus saline IP, HMCDE plus PRZ in drinking water, and HMCDE plus 6-OHDA IP. Experiments were per-

formed on 2 separate occasions. Therefore, final results are derived from ~100 mice (10-12 mice/group/experiment \times 2 experiments).

All mice were weighed at the beginning of the feeding period and weekly thereafter. At death, sera were collected from all animals and liver tissue from half the animals in each group. These livers were fixed in buffered formalin, preserved in OCT compound (Sakura, Torrance, CA), and processed for histology or snap frozen in liquid nitrogen and stored at -80°C until RNA was isolated. Livers from the remaining animals were prepared for flow cytometry as described below. All experiments satisfied NIH and our institutional guidelines for humane animal care.

Histology. Wedges of liver were prepared for histology and immunochemistry as described.^{7,15,16} Coded, hematoxylin-eosin-stained sections were examined by an experienced liver pathologist blinded to treatment groups. Hepatocellular fat accumulation was scored as the following: no fat = 0, focal fat accumulation in $<1\%$ of the hepatocytes = F, fat in 1% to 30% of the hepatocytes = 1+, fat in 31% to 60% of the hepatocytes = 2+, and fat in 61% to 100% of the hepatocytes = 3+. To evaluate the amount of hepatocyte necrosis, the number of necrotic hepatocytes was counted in 10 randomly selected fields with a $20\times$ lens.

Immunohistochemistry. Immunohistochemical analysis of HPC was performed with a mouse monoclonal OV6-type antibody (a gift from Dr. Stewart Sell, Albany Medical College, Albany, NY) reacting with cytokeratins 14 and 19, a rabbit polyclonal antibody against 56 and 64 kd human callus cytokeratins (Dako, Denmark), and a rat monoclonal antibody to cytokeratin 19.^{7,15,17}

Details of the staining procedures are as we have detailed.^{7,15} Incubation with the primary antibodies was performed at room temperature for 30 minutes. Mouse monoclonal OV6 antibody and rat anti-cytokeratin 19 were detected using the Dako Animal Research Kit peroxidase (Dako, Denmark). The rabbit polyclonal antibody against 56 and 64 kd human callus cytokeratins was detected by anti-rabbit Envision (Dako) as described.¹⁵

Oval cells were defined as small cells with an oval nucleus and little cytoplasm. These cells occur either singularly or organized in arborizing, ductular structures. They have strong reactivity for liver type cytokeratins, OV6, and bile duct type cytokeratin 19.^{7,15,17}

To evaluate the effect of treatments on the HPC compartment, coded samples were examined by an experienced liver pathologist blinded to treatment groups. For each liver section, the number of oval cells in 5, randomly selected, nonoverlapping, high-power ($40\times$ objective) fields was counted. Interlobular bile ducts were defined as

bile ducts with a lumen, associated with a branch of the hepatic artery. Interlobular bile ducts were not considered progenitor cells and, thus, not counted as such.

The presence of α -1 adrenergic receptors on oval cells was detected on frozen sections using a rabbit polyclonal anti- α -1 adrenergic receptor antibody (sc10721, Santa Cruz Biotech, Santa Cruz, CA, dilution 1:20), followed by undiluted anti-rabbit Envision (Dako). For immunofluorescence studies, the anti- α -1 adrenergic receptor antibody was combined with a polyclonal antibody against 56 and 64 kd human callus cytokeratins (Dako; dilution 1:100). The primary antibodies were applied sequentially and detected with swine-anti-rabbit FITC or TRITC conjugates. In control sections, primary antibodies were omitted. All stainings were performed on 4 representative sections.

Serum Markers of Liver Injury. Sera were analyzed for alanine aminotransferase (ALT) activity by the Johns Hopkins Clinical Chemistry Laboratory.

RNA Isolation and Ribonuclease Protection Assay. Total RNA was isolated from frozen liver samples as described.¹⁶ RNA concentration was determined by optical density and quality assessed by agarose gel electrophoresis and ethidium bromide staining. Ribonuclease protection assay (RPA) kits with probes for murine cytokines (PharMingen, San Diego, CA) were used to evaluate factors that might be involved in the recruitment and expansion of HPC after liver injury.¹⁶ The factors studied were stem cell factor (SCF), hepatocyte growth factor (HGF), interleukin 6 (IL-6), IL-7, IL-11, leukemia inhibitory factor (LIF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), and vascular endothelial growth factor (VEGF) and its receptors, VEGFR1 and VEGFR3.

Flow Cytometry. The hepatic nonparenchymal cell fraction, containing the oval cell and NK T cell populations, were isolated by described techniques.^{4,18} Purified mononuclear cells were incubated with normal mouse serum (Sigma, St Louis, MO) and Fc-receptor block (anti-CD16/CD32) to prevent nonspecific binding, plus APC-conjugated anti-mouse Thy-1.2 (the C57BL/6 form of the Thy-1 antibody) and antibodies directed against hematopoietic lineage markers (LIN; a mix of anti-mouse CD4, CD8, CD3, CD45, CD19, Mac-1, Gr-1, Ter119). For NK T-cell labeling, the mononuclear cells were incubated with FITC-conjugated anti-mouse NK-1.1 and PE-conjugated anti-mouse CD3. Antibodies were from Pharmingen except Ter119 (Cedarline Lab, Canada). After incubation, washed pellets were fixed with 2% formaldehyde and evaluated by FACS (Becton Dickinson). As described,^{2,4} LIN^{-ve}/Thy-1^{+ve} cells were clas-

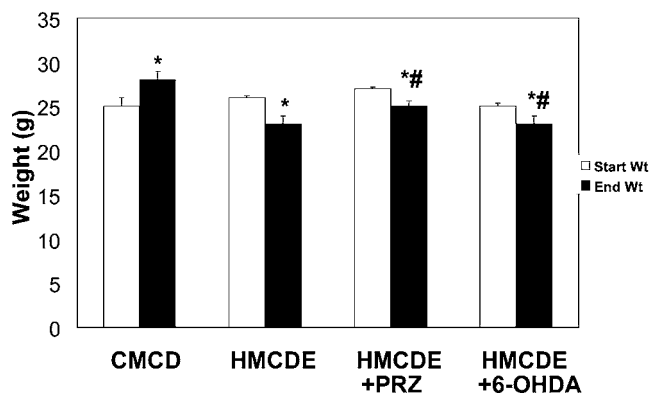


Fig. 1. Effect of control and antioxidant-depleted diets on body weight. Mean \pm SEM body weights of mice before and after 4 weeks of feeding. Only mice fed the control diet (CMCD) gained weight ($*P = .04$ vs. baseline); all groups that were fed half methionine choline-deficient diets (HMCDE) lost weight ($*P = .001$ for post- vs. pre-HMCDE, $P = .008$ for post- vs. pre-HMCDE + PRZ, $P = .03$ for post- vs. pre-HMCDE + 6-OHDA). However, HMCDE + PRZ and HMCDE + 6-OHDA groups lost less weight than the HMCDE group ($\#P = .05$).

sified as oval cells. Data were analyzed by Cell Quest software (Becton Dickinson).

Statistical Analysis. All values are expressed as mean \pm SEM. Group means were compared by unpaired t test or ANOVA using Graphpad Prism 3.03 (San Diego, CA).

Results

To determine the gross effects of the diets, the weights of the animals at the start and end of the experiments were compared. Mice fed the control diet gained a mean of 3 g (12% of starting body weight) during the study (Fig. 1). In contrast, mice fed the HMCDE diet lost a mean of 3 g (12% of starting body weight). Mice fed the HMCDE diet in the presence of PRZ or 6-OHDA, however, only lost a mean of 2 g (7% and 8% of starting body weight). Therefore, SNS inhibition slightly, but significantly, attenuates the weight loss that occurs during consumption of antioxidant-depleted diets.

The treatments also influenced liver mass (Fig. 2A and B). In mice with an intact SNS, as well as in those treated with SNS inhibitors, the HMCDE diet caused an increase in liver mass (Fig. 2A), as well as liver/body mass ratio (Fig. 2B) above that of the control diet. Increases in both parameters tended to be greater in mice treated with SNS inhibitors, but the differences in liver mass achieved statistical significance only for the HMCDE + PRZ-treated group. Thus, although SNS inhibition reduced diet-related loss of body mass, it tended to enhance diet-induced hepatomegaly.

Liver histology confirms that, as expected, HMCDE diets caused hepatic steatosis and necrosis (Fig. 3A-C).

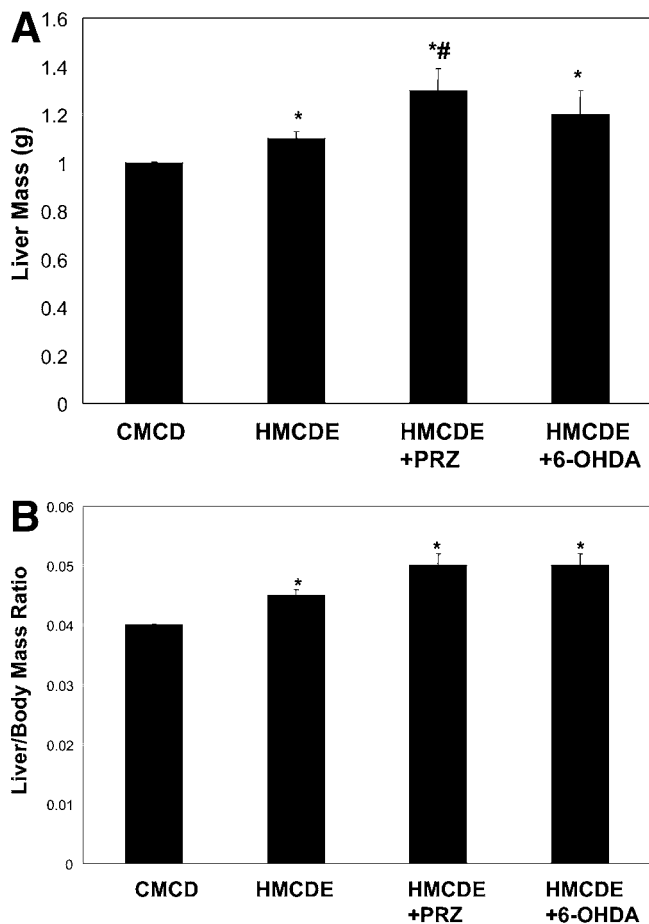


Fig. 2. Effect of SNS inhibition on liver mass in mice with diet-induced liver damage. (A) Compared with mice fed control diets (CMCD), absolute liver mass was greater in all groups fed HMCDE diets ($*P = .01$). Absolute liver mass in the HMCDE + PRZ group was greater than the group fed HMCDE alone ($\#P = .04$). (B) Liver/body weight ratios also increased on HMCDE diets ($*P = .02$ for all groups vs. CMCD) and tended to be greater in HMCDE-treated mice that received SNS inhibitors, although the difference between these groups and those fed HMCDE diets alone did not achieve statistical significance.

Histologic evidence of liver injury was accompanied by significant increases in serum ALT values (Fig. 3D). Treatment with 6-OHDA, but not PRZ, significantly reduced the fat score (Fig. 3B). However, both SNS inhibitors significantly reduced hepatic necrosis (Fig. 3C) and serum ALT values (Fig. 3D). These findings demonstrate that PRZ and 6-OHDA-related increases in liver mass occurred despite improvements in hepatic steatosis (6-OHDA) and/or necrosis (PRZ and 6-OHDA). Diet-induced liver injury itself elicits the accumulation of oval cells in control mice that were fed the HMCDE diet. The increased HPC were demonstrated immunohistochemically by an increase in the numbers of bile duct type cytokeratin-positive oval cells (Fig. 4A and B) and by flow cytometry quantification of bone marrow lineage marker negative (LIN 8-) cells that expressed Thy 1.2 (Fig. 4C).

SNS inhibition with either PRZ or 6-OHDA significantly augments diet-induced oval cell expansion by both assays (Fig. 4A-C). The hepatic accumulation of oval cells is a fairly specific consequence of SNS inhibition because, as expected,¹³ the numbers of NK T cells in the livers of HMCDE-treated mice ($8\% \pm 1\%$ liver mononuclear cells) decrease significantly after treatment with either PRZ ($3.5\% \pm 0.5\%$, $P = .05$) or 6-OHDA ($3.6\% \pm 0.6\%$, $P = .05$). Given that SNS inhibition also reduces HMCDE-induced liver injury (Fig. 3) and stabilizes body weight (Fig. 1), the net effect of SNS inhibition is beneficial in this model of chronic liver injury. Diverse mechanisms may contribute to the hepatoprotective actions of SNS inhibitors.

Other groups have shown that the hepatocyte mitogen, HGF, induces oval cell proliferation, promotes liver regeneration, and protects the liver from hepatotoxicity.¹⁹ Given the similarities between the effects of SNS inhibition and HGF, it was important to determine whether SNS inhibition increased hepatic HGF expression. Consistent with other reports that liver injury induces compensatory expression of HGF and other factors that promote regeneration,²⁰ we found that treatment with HMCDE increased the hepatic expression of HGF about 2-fold above control (Table 1). However, SNS inhibition with PRZ or 6-OHDA did not augment this response. Therefore, the hepatoprotective effects of SNS inhibition are not easily explained by HGF induction.

Like certain hematopoietic progenitor cells, oval cells express c-kit, the receptor for SCF, and are responsive to this growth factor.^{4,5,21} Other cytokines, such as IL-7 and LIF, may also promote progenitor cell accumulation in injured tissues because, after cardiac injury, these factors help to recruit bone marrow-derived stem cells to the injured heart.²² IL-6 is expressed by bone marrow-derived cells in regenerating livers,²³ and this cytokine has an important hepatoprotective effect because mice that are genetically deficient in IL-6 exhibit inhibited liver regeneration after partial hepatectomy (PH).²⁴ Other cytokines, such as G-CSF, that signal through gp-130 receptors may be able to compensate for IL-6 deficiency and promote regeneration when the latter cytokine is deficient.²⁵ VEGF may also play some role in the expansion of HPC because it is a growth factor for hematopoietic stem cells, which express VEGF receptors.²⁶ To begin to clarify the mechanisms by which SNS inhibition enhances HPC accumulation in injured livers, we evaluated the effects of SNS inhibition on the hepatic expression of G-CSF, GM-CSF, M-CSF, IL-6, IL-7, IL-11, LIF, SCF, and VEGF and its receptors VEGFR1 and 3. RPA of whole liver RNA was used to compare the expression of these factors in control (CMCD) mice and mice treated with HM-

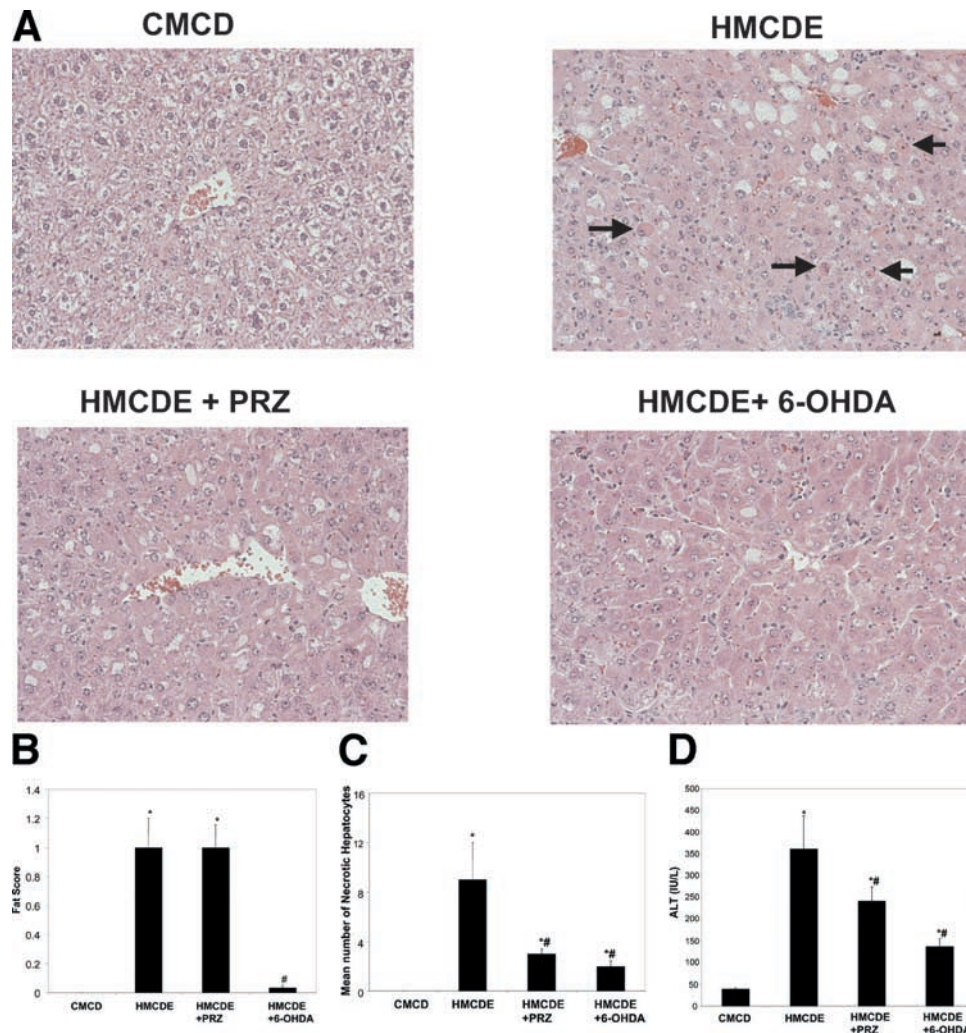


Fig. 3. Effect of SNS inhibition on diet-induced liver injury. (A) Liver Histology. Images were captured with a 25 \times lens. Hematoxylin and eosin-stained sections of representative mice that were fed control diet (CMCD) (**top left panel**) showed no fat accumulation or necrosis. A section from a representative HMCDE fed animal showed 2+ fat accumulation and areas of hepatocyte death (**arrows, top right panel**), whereas one from an HMCDE + PRZ-fed mouse showed 1+ fat accumulation and reduced liver cell death (**bottom left panel**). The liver section from a representative HMCDE + 6-OHDA-fed animal showed focal (F+) fat accumulation and minimal necrosis (**bottom right panel**). (B) Fat score. Compared with mice fed control diets (CMCD), the HMCDE and HMCDE + PRZ groups had more fat ($*P = .0004$). The HMCDE + 6OHDA treated group had significantly less fat than the HMCDE alone group ($\#P = .0001$). (C) Necrosis score. Compared with controls (CMCD), all HMCDE-fed groups had more necrotic hepatocytes ($*P = .01$), but, compared with mice that were fed the HMCDE diet alone, the numbers of necrotic hepatocytes were reduced in HMCDE + PRZ ($\#P = .05$) or HMCDE + 6-OHDA ($\#P = .05$). (D) Serum ALT. Serum levels of ALT, a marker of liver injury, were increased in all HMCDE-fed groups compared with CMCD controls ($*P = .01$). Compared with HMCDE-fed mice, mice treated with HMCDE + PRZ or HMCDE + 6-OHDA had lower ALT levels ($\#P = .03$).

CDE plus or minus SNS inhibitors. No appreciable GM-CSF, M-CSF, IL-6, IL-7, IL-11, SCF, or LIF expression could be demonstrated by this assay (Fig. 5). HMCDE-treatment, however, increased G-CSF expression about 2-fold, regardless of SNS inhibition (Table 1). VEGF and its receptors were expressed in both control and all HMCDE-treated mice, but SNS inhibition did not alter the expression of these factors (Table 1).

To determine whether the effects of SNS inhibition on the HPC compartment might be mediated via direct interaction between norepinephrine (NE) and adrenoceptors on HPC, we used immunohistochemistry to

determine whether oval cells express α -1 adrenoceptors. Our results show that bile duct type cytochrome-positive oval cells do express α -1 adrenoceptors (Fig. 6A and B). Therefore, direct regulation of HPC by NE is plausible.

Discussion

Shortages of donor livers for orthotopic liver transplantation have become a major limiting factor in efforts to reduce mortality of patients with end-stage liver disease.²⁷ Therefore, alternative strategies to replace severely damaged livers must be developed. Studies in mice with mas-

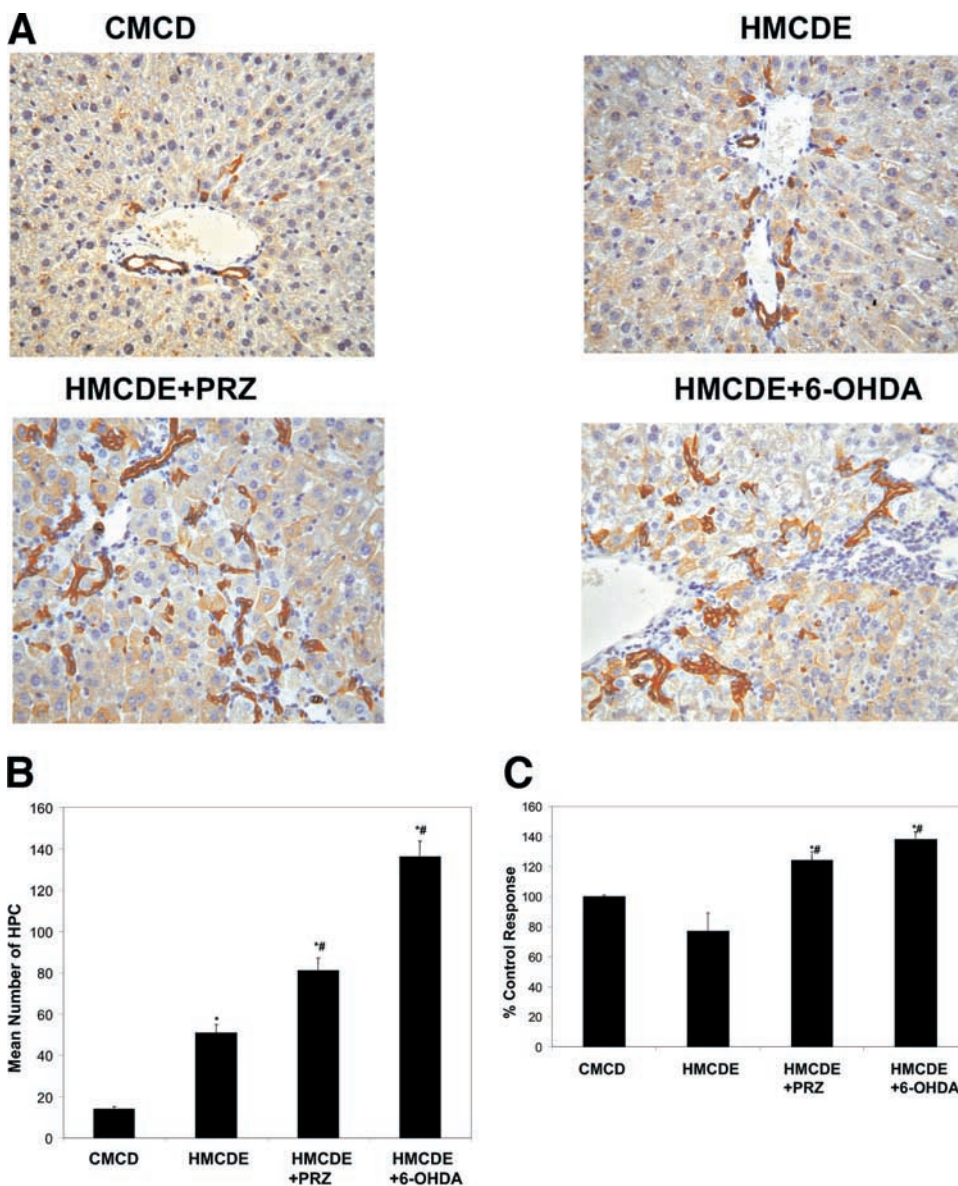


Fig. 4. Effect of SNS inhibition on the numbers of hepatic progenitors in livers with diet-induced damage. (A) Immunohistochemistry for oval cells, in representative mice that were fed control diet (CMCD) (top left panel), HMCDE (top right panel), HMCDE diet + PRZ (bottom left panel), or HMCDE + 6-OHDA (bottom right panel). Oval cells are stained brown. (B) The numbers of oval cells were increased in all HMCDE-fed groups compared with CMCD controls ($*P = .0001$). Both groups treated with SNS inhibitors had more oval cells than mice that were fed HMCDE diets alone ($\#P = .001$). (C) When putative bone marrow-derived hepatic progenitors (*i.e.*, LIN^{-ve}/Thy-1^{+ve}) are quantified by flow cytometry, livers from groups treated with HMCDE + PRZ or HMCDE + 6-OHDA contain more of these cells than CMCD controls ($*P = .01$), although HMCDE feeding alone did not expand this compartment. Compared with mice fed HMCDE diets alone, mice fed HMCDE + PRZ or HMCDE + 6-OHDA had more LIN^{-ve}/Thy-1^{+ve} cells ($\#P = .03$ and $P = .05$, respectively).

sive toxin-induced liver injury have demonstrated that liver cell transplantation can effectively regenerate the liver (reviewed in Forbes et al.²⁸). Hence, many groups are working to optimize cell transplantation strategies. An alternative, but complementary, approach that might be used to improve the outcome of liver injury involves treatment to encourage repopulation of the liver by endogenous hepatic progenitors. The general feasibility of this strategy is supported by recent evidence that the administration of cytokine mixtures to mobilize native, bone marrow-derived progenitor cells heals experimentally induced myocardial infarcts in mice.²² Although transplanted bone marrow cells can also reconstitute lethally damaged livers,^{2,3} the relative importance of native bone marrow-derived progenitors, or resident hepatic progenitors (*i.e.*, oval cells) and mature hepatocytes, for liver

regeneration remains uncertain.²⁸ Moreover, even if certain progenitor cell populations do contribute to recovery from liver injury, little is known about the mediators that regulate their accumulation within the liver. Therefore, the identification of these factors is an important first step in the development of treatments that seek to expand hepatic progenitor cell populations.

Presumably, endogenously produced factors that induce the hepatic accumulation of liver progenitors are increased, to some extent, during liver damage because this response is evident in most injured livers.²⁰ However, unless the compensatory increase in proliferative activity of mature hepatocytes or their progenitors can keep pace with liver cell death, recovery is incomplete and damage persists. Therefore, when factors that increase during injury inhibit both mature hepatocyte proliferation and

Table 1. Total Liver RNA Obtained From 4 Mice per Treatment Group and Analyzed by RPA

Treatment	Gene Expression	Statistical Significance
<i>HGF</i>		
CMCD	1.0 ± 0.1	
HMCDE	1.7 ± 0.24	<i>P</i> = .05 vs. CMCD
HMCDE + PRZ	2.1 ± 0.30	<i>P</i> = .05 vs. CMCD; NS vs. HMCDE
HMCDE + 6-OHDA	2.4 ± 0.75	<i>P</i> = .05 vs. CMCD; NS vs. HMCDE
<i>G-CSF</i>		
CMCD	0.5 ± 0.04	
HMCDE	0.8 ± 0.1	<i>P</i> = .05 vs. CMCD
HMCDE + PRZ	1.0 ± 0.3	<i>P</i> = .05 vs. CMCD; NS vs. HMCDE
HMCDE + 6-OHDA	1.4 ± 0.3	<i>P</i> = .05 vs. CMCD; NS vs. HMCDE
<i>VEGF</i>		
CMCD	1.7 ± 0.2	
HMCDE	1.2 ± 0.2	NS vs. CMCD
HMCDE + PRZ	1.4 ± 0.4	NS vs. HMCDE
HMCDE + 6-OHDA	2.3 ± 0.5	NS vs. HMCDE
<i>VEGFR1</i>		
CMCD	0.3 ± 0.04	
HMCDE	0.3 ± 0.5	NS vs. CMCD
HMCDE + PRZ	0.2 ± 0.2	NS vs. HMCDE
HMCDE + 6-OHDA	0.4 ± 0.1	NS vs. HMCDE
<i>VEGFR3</i>		
CMCD	1.1 ± 0.1	
HMCDE	1.4 ± 0.4	NS vs. CMCD
HMCDE + PRZ	1.7 ± 0.4	NS vs. HMCDE
HMCDE + 6-OHDA	2.1 ± 1.0	NS vs. HMCDE

NOTE. Twenty μg RNA sample from each mouse was evaluated. Results are normalized to concurrently assessed expression of GAPDH in the same RNA samples. Data shown are the mean \pm SEM results of 4 mice per treatment group. Similar results were obtained in a second experiment.

Abbreviations: NS, not statistically significant; *P* > .05.

progenitor cell expansion, reconstruction of a healthy organ becomes compromised. One way to enhance recovery in this situation might be to neutralize the actions of endogenous factors that limit the expansion of native HPC populations. To explore the validity of this concept, we studied mice that were treated with HMCDE because

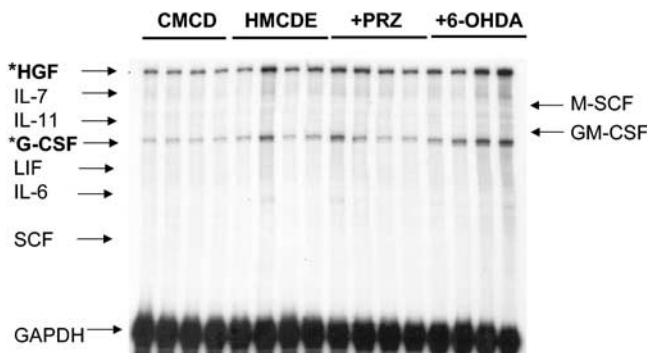


Fig. 5. Effect of SNS inhibition on hepatic expression of growth-regulatory factors. Total liver RNA (20 μg per mouse per lane) was evaluated by RPA. Results from 4 mice per treatment group are demonstrated on this representative phosphoimage. Similar findings were obtained in a duplicate experiment. Ingestion of the hepatotoxic diet (HMCDE) increased the expression of HGF and G-CSF relative to that of mice fed the control diet (CMCD). These differences are detailed in Table 1.

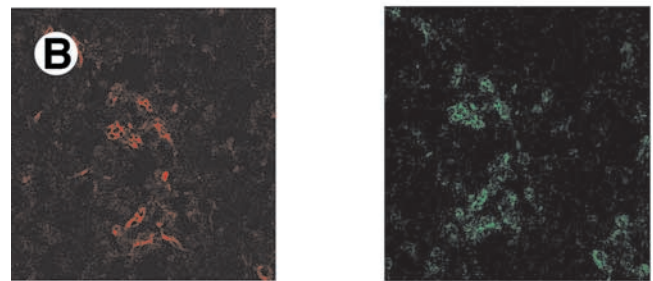
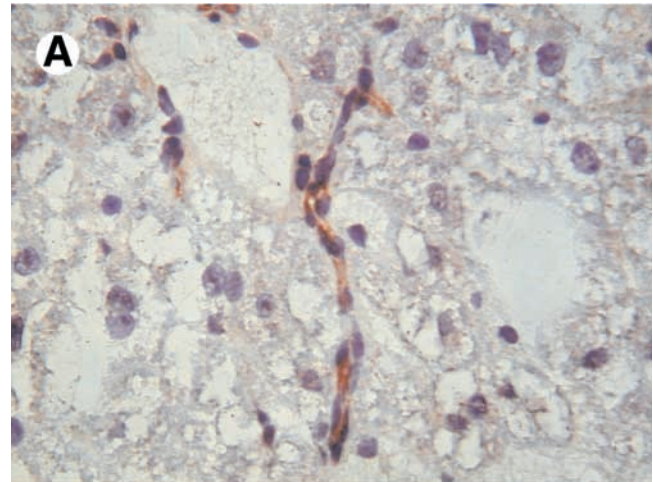


Fig. 6. Oval cells express α -1 adrenoceptors. (A) Immunohistochemistry for α -1 adrenoceptors on bile duct type cytokeratin-positive oval cells in a liver section from representative mice fed HMCDE. Oval cells expressing α -1 adrenoceptors are stained **brown**. (B) Immunofluorescence studies confirm the colocalization of α -1 adrenoceptors on bile duct type cytokeratin-positive oval cells. Without the primary antibodies, binding of the secondary antibodies was negligible (not shown). α -1 Adrenoceptors (**red**), cytokeratins (**green**), colocalization, (**yellow**).

this model of liver injury is known to inhibit replication in mature hepatocytes¹ and increase hepatic oval cells.¹² Our results show that stress-related SNS activity is one of the endogenous factors that limits HPC accumulation in HMCDE-damaged livers because inhibiting SNS activity magnifies the compensatory expansion of oval cell populations that normally occurs in this model. However, the mechanisms for this remain uncertain.

In rats pretreated with PRZ immediately before PH, the subsequent, compensatory induction of hepatocyte DNA synthesis is inhibited.²⁹ Because liver regeneration after PH results from the replication of mature hepato-

cytes,³⁰ this raises the possibility that SNS inhibitors may have compounded the antiproliferative effects of ethionine and further suppressed mature hepatocyte replication in our model of liver injury. If so, then SNS inhibition might have promoted oval cell accumulation by amplifying poorly understood signals that trigger expansion of HPC when the replication of mature hepatocytes is inhibited. However, other data argue against this mechanism. For example, the same group who showed that PRZ inhibits hepatocyte DNA synthesis also reported that chronic treatment with SNS inhibitors did not inhibit post-PH liver regeneration in rats.²⁹ Moreover, Kato and Shimazu found that subjecting rats to surgical sympathectomy before PH actually enhanced posthepatectomy DNA synthesis in the liver.⁹ Another group³¹ also reported that rats with reduced SNS activity because of ventral median hypothalamic lesions exhibit significantly greater hepatic DNA synthesis at 24 hours post-PH and a higher hepatic DNA content from 36 hours through 7 days following PH than sham-operated controls. Thus, the effects of SNS inhibition on the replicative activity of mature hepatocytes appear to be inconsistent. Given this, the massive oval cell expansion that accompanied SNS inhibition in our model may have been mediated by mechanisms other than those that are triggered when the replication of mature hepatocytes is blocked.

As mentioned earlier, liver injury increases the death rate of liver cells, and the latter provides a strong stimulus for liver regeneration.^{20,30} We observed many more oval cells in the livers of mice that were treated with SNS inhibitors, although these groups reproducibly exhibited less severe liver injury than controls, 4 weeks after beginning the hepatotoxic diets. We did not study the mice at earlier time points and, therefore, cannot directly exclude the possibility that SNS inhibition might have transiently exacerbated diet-induced liver injury, evoking more potent injury signals to induce compensatory hyperplasia. However, the latter possibility seems very unlikely because Dubuisson et al.¹¹ showed that liver weight, body weight, and liver weight to body weight ratios increased significantly without any associated increase in serum ALT values in healthy rats treated chronically with 6-OHDA to induce chemical sympathectomy.¹¹ In addition, several groups have demonstrated that NE exacerbates cytokine-mediated hepatotoxicity, whereas agents that block NE typically inhibit this and are hepatoprotective.^{32,33} Therefore, it is unlikely that oval cells increased to compensate for an earlier exacerbation of diet-induced liver injury in the mice that received SNS inhibitors.

HGF, IL-6, VEGF, and other factors play important roles in liver and other organ regeneration after

injury.^{19,23,34} Because SNS inhibitors enhanced HPC accumulation and improved the outcomes of mice that were exposed to hepatotoxic diets, we expected that SNS inhibitors would increase 1 or more of these factors, but we were unable to demonstrate this. However, our analysis of whole liver RNA may not have been sufficiently sensitive to detect increased expression of these molecules in small subpopulations of liver cells. Moreover, we cannot exclude the possibility that SNS inhibitors might have sensitized liver cells to the trophic actions of these or other factors. Therefore, whether or not SNS inhibitors interact with other growth factors to enhance hepatic accumulation of oval cells remains an open question.

The latter possibility merits further investigation because Knight et al. showed that tumor necrosis factor α (TNF- α) increases in mice that are fed choline-deficient diets and demonstrated that proliferating hepatic oval cells produce this cytokine.³⁵ Moreover, they found that TNF- α is required for oval cell expansion because this response is abrogated by genetic disruption of TNFR1. Their observations are particularly intriguing because TNF- α and TNFR1 are necessary for liver regeneration after PH and other types of liver injury.^{36,37} There is strong evidence for cross talk between signaling mechanisms that are activated by TNF- α and those that are modulated by sympathetic neurotransmitters, such as NE (reviewed in Elenkov et al.³⁸). In addition, NE regulates TNF production and *vice versa*.³⁸⁻⁴² Thus, SNS inhibition may promote HPC accumulation and decrease liver injury indirectly by effecting TNF- α activity. We have begun to explore this possibility by comparing hepatic expression of TNF- α mRNA in HMCDE-treated controls and mice treated with HMCDE + PRZ. No differences in TNF- α gene expression were detected in whole liver RNA samples from 3 controls and 3 PRZ-treated mice. However, before firm conclusions can be drawn, these studies must be extended to include more animals, and assays for TNF- α protein and activity will be necessary.

Finally, NE may inhibit HPC expansion by directly interacting with its receptors on oval cells or their precursors. Another SNS neurotransmitter, NPY, interacts with its receptors on neuronal progenitors to regulate their proliferation.⁴³ Although we have shown here that oval cells express α -1 adrenoceptors, it is unknown whether their precursors also express these receptors. However the bone marrow receives SNS innervation, adrenoceptors have been demonstrated on certain types of bone marrow progenitor cells,^{44,45} and treatment with PRZ or 6-OHDA mobilizes murine bone marrow-derived hematopoietic progenitors into the circulation.^{44,45} These findings suggest that injury/inflammation-related increases in NE

might normally limit accumulation of HPC. If so, then SNS inhibition would be expected to disinhibit this process, permitting expansion of HPC populations within damaged livers. The observation that treatment with PRZ or 6-OHDA increased hepatic accumulation of Thy-1 expressing cells that lack appreciable surface markers for the hematopoietic lineage is consistent with this hypothesis.

Controversy rages about the mechanisms that permit hepatic reconstitution of massively damaged livers from bone marrow progenitors, as well as the relative importance of the bone marrow compartment for hepatic regeneration under less extreme circumstances. Our studies were not designed to address either question. Nevertheless, our findings open important new areas for investigation in light of new evidence that donor bone marrow cells can fuse with residual recipient liver cells to generate functional hepatocytes.^{46,47} Bone marrow cells can also differentiate into pancreatic cells.^{5,48} Pancreatic and liver cells are derived from a common progenitor during embryogenesis,⁴⁹ and, in adult rodents, the pancreas may be a source of oval cells.⁵⁰ Whether or not SNS inhibition mobilizes bone marrow cells to the pancreas, where they give rise to progenitors that ultimately migrate into the liver and become oval cells, merits further study. Of course, because hepatic oval cells themselves express adrenoceptors, extrahepatic compartments need not be implicated at all to account for the fact that SNS inhibition increases oval cells in the liver. Adrenoceptor inhibition may directly enhance oval cell survival, and more work is also needed to delineate cellular mechanisms that might be involved.

Despite the remaining uncertainties about the mechanism(s) through which SNS inhibition promotes expansion of the endogenous HPC compartment, the observation that this process can be induced by PRZ, a widely available, relatively safe, oral agent, has potential therapeutic implications. In our study, PRZ was well tolerated, none of the PRZ-treated mice died, and most developed less cachexia as well as less liver damage overall than the liver disease controls. These findings complement those of an earlier study, which demonstrated that PRZ prevents the development of cirrhosis in carbon tetrachloride-treated rats.¹¹ Taken together, these results suggest that α adrenoceptor blockade might be an effective strategy to reduce the progression of chronic liver disease.

References

1. Evarts R, Hu Z, Omori N, Omori M, Marsden E, Thorgeirsson S. Precursor-product relationship between oval cells and hepatocytes: comparison between tritiated thymidine and bromodeoxyuridine as tracers. *Carcinogenesis* 1996;17:2143-2151.
2. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, et al. Bone marrow as a potential source of hepatic oval cells. *Science* 1999;284:1168-1170.
3. Theise ND, Badve S, Saxena R, Henegariu O, Sell S, Crawford JM, Krause DS. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. *HEPATOLOGY* 2000;31:235-240.
4. Petersen B, Goff J, Greenberger J, Michalopoulos G. Hepatic oval cells express the hematopoietic stem cell marker Thy-1 in the rat. *HEPATOLOGY* 1998;27:433-445.
5. Yang L, Li S, Hatch H, Ahrens K, Cornelius JG, Petersen BE, Peck AB. In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. *PNAS* 2002;99:8078-8083.
6. Petersen BE, Grossbard B, Hatch H, Pi L, Deng J, Scott EW. Mouse A6-positive hepatic oval cells also express several hematopoietic stem cell markers. *HEPATOLOGY* 2003;37:632-640.
7. Cassiman D, Libbrecht L, Sinelli N, Desmet V, Deneef C, Roskams T. The vagal nerve stimulates activation of the hepatic progenitor cell compartment via muscarinic acetylcholine receptor type 3. *Am J Pathol* 2002;161:521-530.
8. Berenguer M. Natural history of recurrent hepatitis C. *Liver Transpl* 2002;8:S14-S18.
9. Kato H, Shimazu T. Effect of autonomic denervation on DNA synthesis during liver regeneration after partial hepatectomy. *Eur J Biochem* 1983;134:473-478.
10. Hsu CT. The role of the autonomic nervous system in chemically induced liver damage and repair—using the essential hypertensive animal model (SHR). *J Auton Nerv Syst* 1995;51:135-142.
11. Dubuisson L, Desmouliere A, Decourt B, Evade L, Bedin C, Boussarie L, Barrier L, et al. Inhibition of rat liver fibrogenesis through noradrenergic antagonism. *HEPATOLOGY* 2002;35:325-331.
12. Akhurst B, Croager EJ, Farley-Roche CA, Ong JK, Dumble ML, Knight B, Yeoh GC. A modified choline-deficient, ethionine-supplemented diet protocol effectively induces oval cells in mouse liver. *HEPATOLOGY* 2001;34:519-522.
13. Minagawa M, Oya H, Yamamoto S, Shimizu T, Bannai M, Kawamura H, Hatakeyama K, et al. Intensive expansion of natural killer T cells in the early phase of hepatocyte regeneration after partial hepatectomy in mice and its association with sympathetic nerve activation. *HEPATOLOGY* 2000;31:907-915.
14. Tang Y, Shankar R, Gamboa M, Desai S, Gamelli R, Jones SB. Norepinephrine modulates myelopoiesis after experimental thermal injury with sepsis. *Ann Surg* 2001;233:266-275.
15. Libbrecht L, Meerman L, Kuipers F, Roskams T, Desmet V, Jansen P. Liver pathology and hepatocarcinogenesis in a long-term mouse model of erythropoietic protoporphyria. *J Pathol* 2003;199:191-200.
16. Lin HZ, Yang SQ, Kujhada F, Ronnet G, Diehl AM. Metformin reverses nonalcoholic fatty liver disease in obese leptin-deficient mice. *Nat Med* 2000;6:998-1003.
17. Braun KM, Thompson AW, Sandgren EP. Hepatic microenvironment affects oval cell localization in albumin-urokinase-type plasminogen activator transgenic mice. *Am J Pathol* 2003;162:195-202.
18. Li Z, Lin HZ, Yang SQ, Diehl AM. Murine leptin deficiency alters Kupffer cell production of cytokines that regulate the innate immune system. *Gastroenterology* 2002;123:1304-1310.
19. Shiota G, Kunisada T, Oyama K, Udagawa A, Nomi T, Tanaka K, Tsutsumi A, et al. In vivo transfer of hepatocyte growth factor gene accelerates proliferation of hepatic oval cells in a 2-acetylaminofluorene/partial hepatectomy model in rats. *FEBS Lett* 2000;470:325-330.
20. Michalopoulos GK, DeFrances MC. Liver regeneration. *Science* 1997;276:60-66.
21. Matsusaka S, Tsujimura T, Toyosaka A, Nakasho K, Sugihara A, Okamoto E, Uematsu K, et al. Role of c-kit receptor tyrosine kinase in development of oval cells in the rat 2-acetylaminofluorene/partial hepatectomy model. *HEPATOLOGY* 1999;29:670-676.
22. Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, Nadal-Ginard B, et al. Mobilized bone marrow cells repair the infarcted heart,

- improving function and survival. *Proc Natl Acad Sci U S A* 2001;98:10344-10349.
23. Aldeguer X, Debonera F, Shaked A, Krasinkas AM, Gelman AE, Que X, Zamir GA, et al. Interleukin-6 from intrahepatic cells of bone marrow origin is required for normal murine liver regeneration. *HEPATOLOGY* 2002;35:40-48.
 24. Cressman DE, Greenbaum LE, DeAngelis RA, Ciliberto G, Furth EE, Poli V, Taub R. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. *Science* 1996;274:1379-1383.
 25. Theocharis SE, Margeli AP, Kittas CN. Effect of granulocyte colony-stimulating-factor administration on tissue regeneration due to thioacetamide-induced liver injury in rats. *Dig Dis Sci* 1999;44:990-1996.
 26. Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin DJ, et al. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1+ stem cells from bone-marrow microenvironment. *Nat Med* 2002;8:841-849.
 27. Trotter JF. Expanding the donor pool for liver transplantation. *Curr Gastroenterol Rep* 2000;2:46-54.
 28. Forbes S, Vig P, Poulsom R, Thomas H, Alison M. Hepatic stem cells. *J Pathol* 2002;197:510-518.
 29. Cruise JL, Knechtle SJ, Bollinger RR, Kuhn C, Michalopoulos G. Alpha 1-adrenergic effects and liver regeneration. *HEPATOLOGY* 1987;7:1189-1194.
 30. Fausto N, Campbell JS. The role of hepatocytes and oval cells in liver regeneration and repopulation. *Mech Dev* 2003;120:117-130.
 31. Kiba T, Tanaka K, Numata K, Hoshino M, Inoue S. Facilitation of liver regeneration after partial hepatectomy by ventromedial hypothalamic lesions in rats. *Pflugers Arch* 1994;428:26-29.
 32. Yang S, Koo DJ, Zhou M, Chaudry IH, Wang P. Gut-derived norepinephrine plays a critical role in producing hepatocellular dysfunction during sepsis. *Am J Physiol* 2000;279:G1274-G1281.
 33. Collins JL, Vodovotz Y, Yoneyama T, Hatakeyama K, Green AM, Billiar TR. Catecholamines decrease nitric oxide production by cytokine-stimulated hepatocytes. *Surgery* 2001;130:256-264.
 34. Lowes KN, Croager EJ, Olynyk JK, Abraham LJ, Yeoh GCT. Oval cell-mediated liver regeneration: role of cytokines and growth factors. *J Gastroenterol Hepatol* 2003;18:4-12.
 35. Knight B, Yeoh GC, Husk KL, Ly T, Abraham LJ, Yu C, Rhim JA, et al. Impaired preneoplastic changes and liver tumor formation in tumor necrosis factor receptor type 1 knockout mice. *J Exp Med* 2000;192:1809-1818.
 36. Akerman P, Cote P, Yang SQ, McClain C, Nelson S, Bagby GJ, AM D. Antibodies to tumor necrosis factor alpha inhibit liver regeneration after partial hepatectomy. *Am J Physiol* 1992;263:G579-G585.
 37. Yamada Y, Kirillova I, Peschon JJ, Fausto N. Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. *Proc Natl Acad Sci U S A* 1997;94:1441-1446.
 38. Elenkov IJ, Chrousos GP, Wilder RL. Neuroendocrine regulation of IL-12 and TNF- α /IL-10 balance. Clinical implications. *Ann N Y Acad Sci* 2000;917:94-105.
 39. Zhou M, Yang S, Koo DJ, Ornan DA, Chaudry IH, Wang P. The role of Kupffer cell α (2)-adrenoceptors in norepinephrine-induced TNF- α production. *Biochim Biophys Acta* 2001;1537:49-57.
 40. Kalinichenko VV, Mokyr MB, Graf LH, Cohen RL, Chambers DA. Norepinephrine-mediated inhibition of antitumor cytotoxic T lymphocyte generation involves a β -adrenergic receptor mechanism and decreased TNF- α gene expression. *J Immunol* 1999;163:2492-2499.
 41. Spengler RN, Chensue SW, Giachero DA, Blenk N, Kunkel SL. Endogenous norepinephrine regulates tumor necrosis factor- α production from macrophages in vitro. *J Immunol* 1994;152:3024-3031.
 42. De Lugi A, Terreni L, Sironi M, De Simoni MG. The sympathetic nervous system tonically inhibits peripheral interleukin-1 β and interleukin-6 induction by central lipopolysaccharide. *Neuroscience* 1998;83:1245-1250.
 43. Hansel DE, Elpper BA, Ronnett GV. Neuropeptide Y functions as a neuroproliferative factor. *Nature* 2001;410:940-944.
 44. Maestroni GJM, Conti A. Modulation of hematopoiesis via α 1-adrenergic receptors on bone marrow cells. *Exp Hematol* 1994;22:313-320.
 45. Marino F, Cosentino M, Bombelli R, Ferrari M, Maestroni GJ, Conti A, Lecchini S, et al. Measurement of catecholamines in mouse bone marrow by means of HPLC with electrochemical detection. *Haematologica* 1997;82:392-394.
 46. Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz D, Nakano Y, Meyer E, et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* 2002;416:542-545.
 47. Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* 2003;422:897-901.
 48. Ianus A, Holz GG, Theise ND, Hussain MA. In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. *J Clin Invest* 2003;111:843-850.
 49. Deutsch G, Jung J, Zheng M, Lora J, Zaret K. A bipotential precursor population for pancreas and liver within the embryonic endoderm. *Development* 2001;128:871-881.
 50. Ide H, Subbarao V, Reddy JK, Rao MS. Formation of ductular structures in vitro by rat pancreatic epithelial oval cells. *Exp Cell Res* 1993;209:38-44.