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Vascular Endothelial Growth Factor and Angiopoietin in Liver Regeneration

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Liver architecture remodeling following partial hepatectomy (PHx) involves the formation of a complex network of liver sinusoids through which the blood flows. The present study examines the involvement of vascular endothelial growth factor (VEGF) and angiopoietin-1 (ang-1) during liver regeneration. Following PHx, VEGF and ang-1 mRNA levels increase, followed by gradual return to baseline levels. RT-PCR analysis of VEGF mRNA reveals three isoforms, VEGF120, VEGF164 and VEGF188. Of the three, VEGF188 is the predominant isoform, VEGF120 being the less abundant. Although VEGF mRNA fluctuates following PHx, the relative expression of each isoform remains the same throughout the recovery process. The level of neuropilin-1, an accessory receptor of VEGF to main receptor corresponds with that of VEGF and ang-1. We have previously demonstrated the capacity of exogenous VEGF165 to stimulate liver cell proliferation following PHx. We now report similar effect using VEGF121, further demonstrating the benefit of manipulating growth factors where such an intervention is required.

Key Words: **VEGF; angiopoietin; neuropilin; partial hepatectomy; liver regeneration.**

The liver's capacity to reestablish optimal mass following cell loss is accompanied by the expression of a large number of genes (1). Among these are immediate early genes, delayed genes, cell cycle genes and genes involved in DNA replication and mitosis (2–7). Regardless of the large body of evidence documenting the time frame expression of each group and their role during the recovery process, little is known about the involvement of angiogenic factors during liver regeneration. Liver blood supply is carried through branches of the portal vein and hepatic artery. The blood proceeds through sinusoids and drains into the central venules. Remodeling liver architecture following liver resection thus involves the formation of a complex network of blood vessels.

Information gathered during recent years points to the association of angiogenic factors with liver regeneration. Increased mRNA expression of vascular endothelial growth factor (VEGF) and respective receptors VEGFR-1 and VEGFR-2 was evident following partial hepatectomy (PHx). In primary cultures, hepatocytes expressed VEGF mRNA while non-parenchymal cells expressed both VEGFR-1 and VEGFR-2 mRNA (8). Such a differential expression may point to a possible cross-talk between the two cell populations.

VEGF represents a number of factors all of which are the result of alternative splicing of the same gene. The gene is organized in eight exons, separated by seven introns. The amino acids encoded by exons 6 and 7 grant VEGF heparin and heparan sulfate binding capacity. In man, five different isoforms having 121, 145, 165, 189, and 206 amino acids, respectively, were identified (9, 10). While most tissues express VEGF121, VEGF165 and VEGF189, VEGF145 and VEGF206 are rare (11). The differential expression and bioavailability of VEGF isoforms may hint to different biological roles. Indeed, the transfection of MCF-7 breast cancer cells for example, with VEGF121, 165 and 189 proved VEGF121 to be more angiogenic and tumorigenic than other isoforms (12). In patients with osteosarcoma expressing VEGF165, an increased vascularity was noted compared to patients positive only to VEGF121 (13). Until recently VEGF was the only growth factor proven to be specific for blood vessel formation. Powerful genetic approaches unraveled an additional family, the angiopoietins, which consists of four members and at least one member of the ephrin family (14). Unlike

Abbreviations used: VEGF, vascular endothelial growth factor; ang-1, angiopoietin-1; PHx, partial hepatectomy; PCNA, proliferating cell nuclear antigen.

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VEGF, mice lacking angiopoietin-1 (ang-1) or its receptor Tie-2, develop normal vasculature (14–18), yet this fails to undergo normal remodeling. These experiments led to the suggestion that ang-1 helps vascular remodeling by optimizing the manner by which endothelial cells integrate with supporting cells (18). The four members of the angiopoietin family, ang-1–4 primarily bind to the Tie-2 receptor. Ligand to a second receptor, Tie-1, has yet to be identified, though it is possible that the known angiopoietins engage with Tie-1 under certain conditions (19–21).

The present study was undertaken to examine the role of angiogenesis-associated genes, VEGF and angiopoietin during liver regeneration in a rat model.

MATERIAL AND METHODS

Animals. Adult male Sprague–Dawley rats (140–200 g) were maintained on rat chow and water under standard conditions of light and dark hours. Thirty and seventy percent PHx were performed according to Higgins and Anderson (22) under light anesthesia by removing the median and median and left lateral lobes, respectively. Animals (4–5 per group) were sacrificed under ether anesthesia at different time intervals postoperatively. Excised liver was weighed and 0.5-g samples were treated with 4% formaldehyde for immunostaining or frozen in liquid nitrogen and further used for RNA extraction or Western blotting. Animals received humane care according to criteria outlined in the "Guide for Care and Use of Laboratory Animals."

PCNA expression. PCNA immunostaining was performed on liver sections fixed in 4% formaldehyde/PBS and dehydrated in graded ethanol solution. After blocking endogenous peroxidase the slices were incubated with anti-PCNA antibody (PC-10, DAKO, Carpinteria, CA). Immunohistochemical staining was performed using biotinylated anti mouse antibodies and streptavidin-peroxidase. Diaminobenzidine (Sigma) was used as chromogen substrate (23, 24).

Northern blotting. Total liver RNA was isolated using the TRI-reagent (Sigma). 20 μg of each RNA sample were resolved on formaldehyde agarose gel, transferred to nylon membranes (MCI) and ultraviolet cross-linked. Complementary DNA probes for VEGF exons 5, 7, and 8, neuropilin or ang-1 were labeled with (α-^{32}P)dCTP by the random primer extension method. Prehybridization and hybridization were performed at 65°C in buffer containing 7%SDS, 10% PEG 8000, 1.5× SSPE and salmon sperm DNA. Following extensive washes and autoradiography, the membranes were stripped for rehybridization with albumin specific probe used as internal control (25). Optical density was evaluated using BioCapt and Bio-PROFIL Bio-1D software (the same densitometry procedure was applied for RT-PCR).

RT-PCR. For RT-PCR, 8 μg of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI). PCR was performed in 50 μl volume containing PCR buffer, 10 mM dNTPs, 200 ng each primer and 2.5 U *Taq* polymerase. VEGF was amplified using VEGF primers matching exons 2 and 8; 5′GGAGGGCAGAATCATCACGAAGTG3′ and 3′ACACTGTTCGGCTCCGCCACT5′. Cycling consisted of 1-min steps at 94, 58, and 72°C. Ethidium bromide staining visualized amplified DNA product. Amount of DNA used for PCR was standardized based on albumin (25).

VEGF purification. Recombinant baculovirus for human VEGF121 was used to infect SF-9 cells (26). Supernatant was collected and loaded on a CH-Sepharose 4B anti VEGF antibody column and separated at low pH. The purity of the proteins was determined using SDS–PAGE. Such a procedure yields >90% purity as detected by silver staining. An *in vitro* proliferation assay using endothelial cells confirmed the mitogenic activity of the purified protein as measured by thymidine incorporation.

ELISA. Anti VEGF antibodies were prepared by immunizing rabbits with VEGF165 previously purified by Heparin-Sepharose column chromatography and resolved on SDS polyacrylamide gel. Monoclonal antibodies to VEGF were prepared following BALB/c mice vaccination with pcDNA3 plasmid harboring the VEGF165 gene and boosting with purified VEGF165 protein. Hybridomas secreting monoclonal antibodies to VEGF were identified using ELISA. Sandwich ELISA based on chemiluminescence rather then spectrophotometry was employed to determine the level of plasma VEGF. Black ELISA plates (Corning, Acton, MA) were coated with rabbit anti VEGF IgG purified on protein G column and blocked with 0.1% gelatin. Samples to be tested were added, followed by anti VEGF monoclonal antibody. Biotinylated rabbit anti mouse IgG1 and streptavidin horseradish-peroxidase conjugate were then used and the presence of horseradish peroxidase was visualized by luminol substrate (Super Signal, Pierce, Rockford, IL). Photons released were read in a microplate luminometer, expressed as k-counts and translated to pg/ml using purified VEGF as a standard.

RESULTS

PCNA staining following PHx. To establish liver regeneration following PHx, proliferating cell nuclear antigen (PCNA) staining was performed (Fig. 1). Immunostaining of normal liver sections was minimal with only few if any cells dividing. Twenty-four hours postsurgery hepatocyte nuclei were positively stained reaching a maximum at 48 h. Four days postsurgery PCNA staining decreased reaching close to normal level at 240 h.

VEGF, neuropilin-1 and ang1 expression during liver regeneration. Angiogenic remodeling as a rule is based on the supply of a number of factors working in perfect harmony and a coordinated manner. Accordingly we have examined the two key angiogenic factors, VEGF and angiopoietin following 70% PHx. Figure 2 denotes mRNA levels of the two factors as well as of neuropilin-1. VEGF was detected as early as 12 h posthepatectomy, peaking at 36 h and gradually decreasing thereby to baseline level at 240 h. Ang-1 mRNA peaks at 48 h followed by gradual decrease to almost normal levels at 240 h postsurgery. Neuropilin-1, a receptor for collapsins/semaphorins and for VEGF165 and VEGF145, mRNA increased gradually following PHx, tapering down thereafter.

Differential expression of VEGF during liver regeneration. As growing amount of evidence suggests differential expression of VEGF isoforms in different organs and different pathological manifestations, we next examined the level of VEGF isoforms using RT-PCR. To assess the capacity of the assay to detect each of the isoforms, cDNA was prepared from HeLa cells, rat liver, kidney, lung and spleen. Primers matching exons number 2 and 8 allowed detecting three VEGF isoforms in all samples, VEGF120, VEGF164,

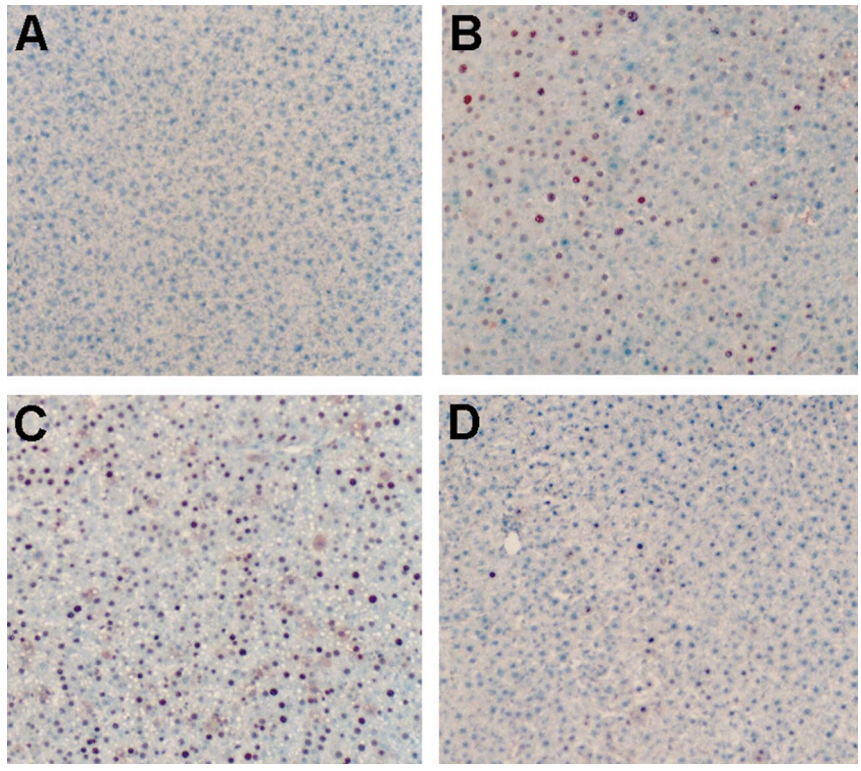

FIG. 1. Immunohistochemical staining for PCNA. Fixed liver sections from intact liver (A, time 0) or liver remnants at time intervals following 70% PHx (B 24 h; C, 48 h; D, 240 h) were stained with monoclonal anti-PCNA antibody followed by biotin-conjugated anti-mouse immunoglobulin and avidin peroxidase. Diaminobenzidine was used as a chromogen substrate.

VEGF188, yet the relative expression of each one varied. In HeLa cells, VEGF121 (42%) and VEGF165 (38%) were prominent while in the spleen and kidney the principal isoform is VEGF164, 50 and 44%, respectively. In the lung VEGF188 (37%) was the predominant isoform (Fig. 3A). In quiescent liver and throughout the recovery process VEGF188 (58%) was the predominant isoform. The level of mRNA of the two other isoforms VEGF164 (24%) and VEGF120 (18%) was considerably less pronounced. These differences did not significantly change as liver regeneration approached final stages of recovery regardless of the changes in total VEGF mRNA (Fig. 3B). The removal of 30% of the liver was shown to result in a moderate cell proliferation yet a similar trend of VEGF isoform expression was evident, VEGF188 being the predominant isoform (data not shown).

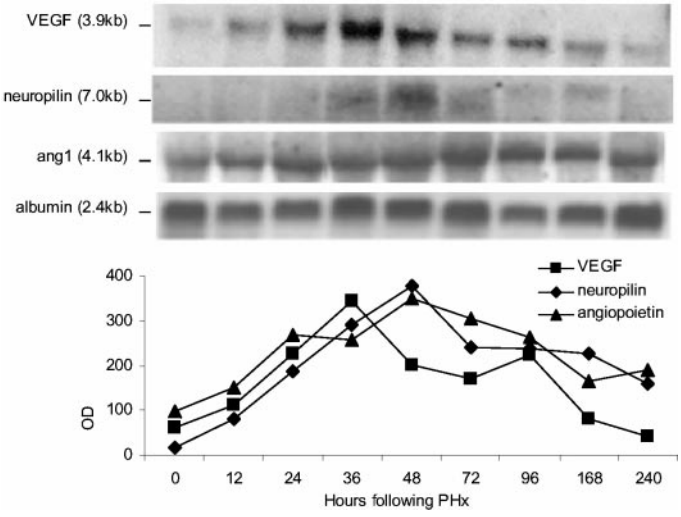

FIG. 2. Northern blot analysis of VEGF, neuropilin, and angiopoietin-1. Total RNA was isolated from intact liver (time 0) or liver remnants at time intervals specified following 70% PHx. Northern blot analysis was performed using VEGF, neuropilin, and angiopoietin-1 complementary DNA probes. Quantification of the specific signal was performed after normalization with albumin.

Effect of exogenous VEGF121 administration on liver cell proliferation following 30% PHx. The stimulatory effect of exogenous VEGF165 on liver cell proliferation following 30% PHx was previously demonstrated (27). In view of the structural differences between the isoforms and their bioavailability we set out to examine whether VEGF121 exerts similar effect. Accordingly, VEGF121 was purified and injected intravenously at the time of surgery and 6 and 24 h post hepatectomy. Saline administered rats served as a control. Figure 4 illustrates PCNA labeling index at different time interval following hepatectomy. A remarkable accelera-

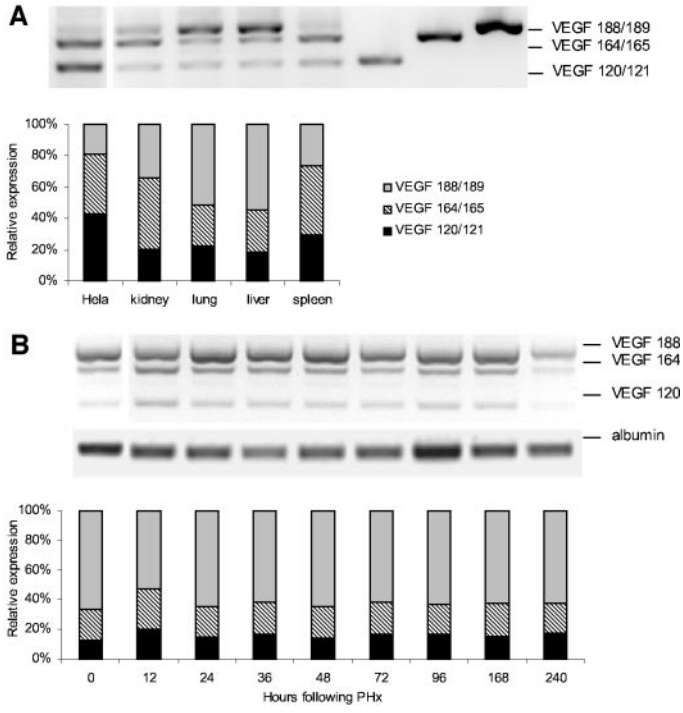

FIG. 3. VEGF RT-PCR. Total RNA was isolated from intact rat liver, kidney, spleen, and HeLa cells (A), (time 0) or liver remnants at time intervals specified following 70% PHx (B). RT-PCR was performed using primers matching VEGF exons 2 and 8. Plasmids containing DNA coding for VEGF121, VEGF165, or VEGF189 were used to mark the range of the respective isoforms. VEGF expression was normalized using albumin. Optical density is given as the ratio between VEGF isoforms.

tion of liver cell proliferation was evident as early as 12 h postsurgery. Elevated values were noted at all time points tested but 96 h postsurgery. At 7 days PCNA labeling index returned to normal in both the control and saline injected animals.

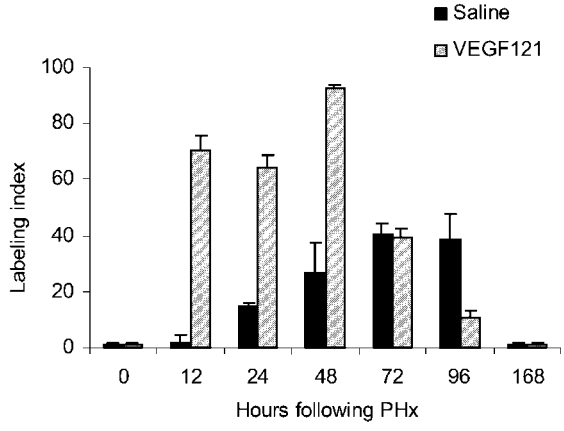

FIG. 4. PCNA labeling index. Rats were administered VEGF at the time of surgery, and 6 and 24 h posthepatectomy. Fixed liver sections were stained with monoclonal anti-PCNA antibody followed by biotin conjugated anti-mouse immunoglobulin and avidin peroxidase. Diaminobenzidine was used as a chromogen substrate.

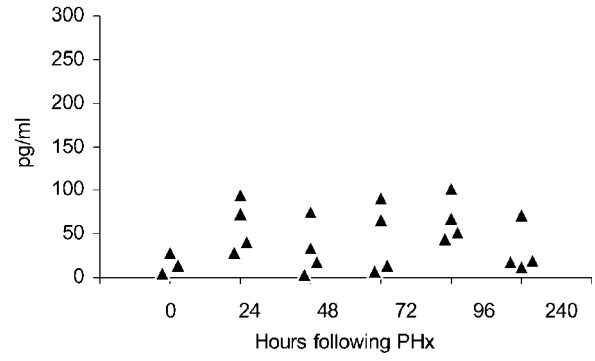

FIG. 5. VEGF plasma levels. VEGF levels were determined in plasma of rats undergoing 70% PHx by ELISA. Black ELISA plates were coated with polyclonal rabbit anti-VEGF antibodies, and blocked with gelatin. VEGF in samples was detected by monoclonal anti-VEGF antibody followed by biotinylated anti-mouse IgG1 antibodies and streptavidin-conjugated peroxidase. Luminol was used as substrate and the plates were read using Lucy luminometer.

VEGF plasma level during liver regeneration. Some of the factors participating in liver regeneration are recruited from distant organs. EGF is continually made available to the liver by the Brunner's gland of the duodenum through the portal circulation (2). Following PHx, its plasma level rises. To determine whether VEGF is supplied through an endocrine/paracrine system, a sensitive chemiluminescence-based ELISA was established. All plasma samples tested revealed a similar concentration of VEGF pointing to a paracrine supply (Fig. 5).

DISCUSSION

Liver remodeling following partial hepatectomy comprises a complex set of events including the activation and secretion of numerous factors, cell proliferation and finally reestablishing liver architecture. Rebuilding the network of blood supply is part of liver regeneration. The data presented hereby demonstrate the association of VEGF and angiopoietin, the two central angiogenic members, during liver regeneration. Northern blotting of liver RNA samples following 70% PHx demonstrates an increase in VEGF and ang1 mRNA.

VEGF acts through at least three receptors, VEGFR-1 (Flt-10), VEGFR-2 (Flk-1) and VEGFR-3 (Flt-4). Of the three, VEGFR-2 seems to be the principle mediator of growth and permeability. In the present study we have looked for neuropilin-1, a receptor reported to be associated with the binding of VEGF (28). Neuropilin-1 and neuropilin-2 are receptors for axon guidance factors belonging to the class 3 semaphorins. Recently it was shown that both bind VEGF165 but not VEGF121. Yet unlike neuropilin-1, neuropilin-2 binds VEGF145 as well (29, 30). During liver regeneration the level of neuropilin-1 mRNA increased, further demonstrating the complexity and in-

volvement of VEGF receptors in the recovery process following liver injury.

As a large body of evidence demonstrates differential organ expression of VEGF isoforms, we set to examine the involvement of each one during liver regeneration. The VEGF-A family includes 5 isoforms. The five are distinguished by their heparin and heparan-sulfate binding ability. The shortest form, VEGF121 lacks exon 6 and 7 and does not bind to heparin or extra cellular matrix. VEGF145 has an additional 21 amino acids encoded by exon 6a, which contains a heparin-binding domain. A similar stretch of amino acids is shared by VEGF189. The 44 amino acids encoded by exon 7 confer heparin-binding capacity to both VEGF165 and 189. The latter displays a higher affinity to heparin and heparan sulfate and thus is sequestered on the cell surface and extracellular matrix (10, 31). ECM association of one or more of the VEGF isoforms may have a significant importance as such binding can release factors like bFGF which are stored on heparan sulfates (32). Differential expression of VEGF isoforms in a variety of organs was reported (33). The three major forms, VEGF188, 164 and 120 were observed in the lung, kidney, brain, spleen and heart yet their relative abundance differed. VEGF188 was the predominant isoform in the heart and lung but less abundant in the others. VEGF164 mRNA levels were lower in the heart and lung but predominant in the brain and kidney. Equimolar amounts of VEGF120 and 164 were reported in the spleen. Of the five VEGF isoforms, we have been able to identify in quiescent and regenerating liver VEGF120, VEGF164 and VEGF188 using an RT-PCR assay. The assay used is based on primers matching sequences from exons 2 and 8. It allowed the identification of three VEGF isoforms, VEGF121/120, 165/164/and 189/188 in HeLa cells, rat liver, kidney, lung and spleen. Following 70% PHx total VEGF mRNA levels increased, yet the same isoform ratio was kept with VEGF188 being the predominant and VEGF120 the less abundant. A similar ratio was also evident using the 30% PHx model, which represents somewhat different regenerative response (34). The differential expression of VEGF isoforms does not necessarily point to the importance of one over the other. Their different bioavailability and distinct association with VEGF receptors determines their biological activity. In a previous study we have demonstrated the stimulatory effect of exogenous administered VEGF165 following 30% PHx (27). To demonstrate a role for VEGF121, rats were administered purified VEGF121 and subjected to 30% PHx. Following VEGF administration, both VEGF121 and VEGF165, PCNA positive cells were recorded as early as 12 h posthepatectomy. In a recent back-to-back papers Sato and colleagues and Shimizu and colleagues have demonstrated an increase of both VEGF, angiopoietins (ang1 and ang2) and respective receptors during liver regeneration (35, 36). They have also characterized an optimum proliferation time for hepatocytes and sinusoidal endothelial cells as 24 and 72 h following 70% hepatectomy. The early response in PCNA expression noted following VEGF121 and VEGF165 (data not shown) administration may reflect endothelial mitogenic activity leading to endothelial cell proliferation and growth factors secretion some of which triggers an early response of hepatocytes. As activated stellate cells (37) have been shown to express VEGF receptors as well, it is likely that VEGF culminate a wider stimulatory effect leading to an early liver cell proliferation. The 30% PHx model rather than the 70% was preferred assuming that moderate responsive response would better allow perceiving the effect of exogenous VEGF on liver cell proliferation.

The involvement of angiogenic factors during liver regeneration was noted in the past. Indeed, bFGF has been shown to influence the regenerative capacity of liver cells following PHx (38). The present study confirms the recent data published by Sato *et al.* Northern blot analysis reveals a gradual increase of ang-1 mRNA followed by a continuing decline to normal levels as liver regeneration is accomplished.

Growth factors like HGF stimulate hepatocyte replication by paracrine or endocrine mechanisms, yet others such as TGFα are produced by hepatocytes and act through autocrine mechanisms (1, 39). In the present study we have tested whether VEGF plasma levels following PHx increases. Normal VEGF concentration in rats, as detected by sandwich ELISA, ranges between 15–120 pg/ml. This level was not significantly changed following PHx, suggesting a paracrine supply. Our observation disagrees with that reported by Sato and colleagues who indicate an increase in serum VEGF levels at 72 h posthepatectomy.

Although VEGF was originally characterized to promote vascular endothelial cell proliferation (40, 41) its permeability-inducing effect may have an important role. Multiple factors and signals interact to control compensatory hepatic growth. These signals consist of molecules that circulate in the blood, primarily hormones, growth factors and growth inhibitors, or locally released molecules that can act in a paracrine or autocrine fashion. (42). It is likely that individual factors like VEGF influence hepatocytes by improving nutrient availability or through vascular permeability (40). Such an activity may contribute to acute inflammatory changes that facilitate the passage of different cytokines. In our study, ascites was not documented, suggesting that increased permeability of blood vessels is probably not part of the mechanism induced by VEGF.

Surgical hepatic resection injures the liver and thus may induce local cytokine release. Furthermore, after PHx, portal blood flow to the liver increases significantly (1, 43). Therefore, it is likely that hepatic reticuloendothelial cells are exposed to a large amount of

gut-derived endotoxins, which in turn may stimulate hepatic nonparenchymal cells to release VEGF locally. Indeed we were unable to detect appreciable VEGF in the plasma post-PHx.

The realization that multiple angiogenic factors are likely to be required following liver injury or liver resection has broad implications. A better understanding of the role of each of the factors and respective receptors in normal development and injured liver will aid in manipulating these growth factors for therapeutic benefit especially so in patients with impaired regenerative capacity where the postoperative liver failure after hepatic resection remains high.

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