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• *BASIC RESEARCH* •

Vascular endothelial growth factor and angiopoietins regulate sinusoidal regeneration and remodeling after partial hepatectomy in rats

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Abstract

AIM: To study the regulatory mechanisms of sinusoidal regeneration after partial hepatectomy.

METHODS: We investigated the expression of angiopoietin (Ang)-1, Ang-2, Tie-2, and vascular endothelial growth factor (VEGF) in regenerating liver tissue by quantitative reverse-transcription polymerase chain reaction (RT-PCR) using a LightCycler (Roche Diagnostics) and also immunohistochemical staining after 70% hepatectomy in rats. In the next step, we isolated liver cells (hepatocytes, sinusoidal endothelial cell (SEC), Kupffer cell, and hepatic stellate cells (HSC)) from regenerating liver tissue by *in situ* collagenase perfusion and counterflow elutriation, to determine potential cellular sources of these angiogenic factors after hepatectomy. Proliferation and apoptosis of SECs were also evaluated by proliferating cell nuclear antigen (PCNA) staining and the terminal deoxynucleotidyl transferase d-uridine triphosphate nick end labeling (TUNEL) assay, respectively.

RESULTS: VEGF mRNA expression increased with a peak at 72 h after hepatectomy, decreasing thereafter. The expression of Ang-1 mRNA was present at detectable levels before hepatectomy and increased slowly with a peak at 96 h. Meanwhile, Ang-2 mRNA was hardly detected before hepatectomy, but was remarkably induced at 120 and 144 h. In isolated cells, VEGF mRNA expression was found mainly in the hepatocyte fraction. Meanwhile, mRNA for Ang-1 and Ang-2 was found in the SEC and HSC fractions, but was more prominent in the latter. The PCNA labeling index of SECs increased slowly, reaching a peak at 72 h, whereas apoptotic SECs were detected between 120 h and 144 h.

CONCLUSION: Ang-Tie system, together with VEGF, plays a critical role in regulating balance between SEC proliferation and apoptosis during sinusoidal regeneration after hepatectomy. However, the VEGF system plays a more important role in the early phase of sinusoidal regeneration than angiopoietin/Tie system.

Key words: Vascular endothelial cell growth factor; Angiopoietin; Sinusoidal endothelial cell; Hepatectomy; Liver regeneration

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INTRODUCTION

The regenerative capacity of the liver is typically triggered by hepatic injuries, including partial hepatectomy or hepatocyte loss caused by viral or chemical injury[1-3]. Liver regeneration is a highly complex and organized process, but extensive studies have revealed that a number of cytokines and growth factors are involved in the regulation of hepatocyte proliferation[4-6]. Nonparenchymal cells (Kupffer cells, sinusoidal endothelial cells (SECs)) have both stimulatory and inhibitory influences on hepatocyte replication though a paracrine manner after hepatectomy[7]. As a consequence of hepatocyte proliferation, remodeling of the sinusoids has to be carried out for the supply of blood flow to the newly replicating hepatocytes in the process of liver regeneration, most probably with penetration of SECs into hepatocyte clusters. Presumably, SECs proliferate and assemble into tubes with cell-cell connections, followed by recruitment of hepatic stellate cells (HSCs) and Kupffer cells, to restore normal sinusoidal architecture. Lindahl *et al*.[8] reported that during vascular development and remodeling, endothelial cells initially proliferate, and recruit mural cell (pericytes or smooth muscle cells) precursors via secretion of platelet-derived growth factor-BB, and endothelial cell-mural cell contact is essential for vessel stabilization[9]. On the other hand, Martinez-Hernandez *et al*.[10] suggested that during liver regeneration, the laminin-containing Ito cells initially extend cell processes into the

hepatocyte clusters, followed by SECs, to form new sinusoids after hepatectomy, but at present the precise steps involved in sinusoidal regeneration remain poorly understood. However, recent studies including ours showed that vascular endothelial growth factor (VEGF) greatly contributes to the proliferation of SECs via up-regulated VEGF receptors during liver regeneration[11-15]. Moreover, we have clearly shown that newly replicated hepatocytes that are devoid of sinusoids are the major source of VEGF after hepatectomy[13]. However, the detail mechanisms of sinusoidal regeneration and remodeling are still open to discussion.

Recently, the angiopoietin (Ang)-Tie system has been identified as a second family of endothelial cell-specific growth factors[16-18], and has been reported to act in a complementary and coordinated fashion with VEGF, thus playing an important role in vascular maturation and remodeling[17,18]. The modulatory effect of angiopoietins on VEGF-induced angiogenesis is distinct[19-21], although angiopoietins alone are not mitogenic for endothelial cells *in vitro*[18], and do not promote neovascularization *in vivo*[19]. Angiopoietins include both receptor activators (Ang-1) and receptor antagonists (Ang-2)[18]. Binding of Ang-1 causes auto-phosphorylation of tyrosine kinase Tie-2 receptor, whereas Ang-2 binding conversely suppresses auto-phosphorylation of Tie-2. The Tie-2 signal is, therefore, defined by quantitative balance between Ang-1 and Ang-2 activities[19,20]. The Ang-1/Tie-2 pathway in the presence of VEGF is thought to mediate the vital functions of vascular stabilization and vascular maturation, via integration of peri-endothelial cells into the vascular wall. In contrast to Ang-1, Ang-2 induces vascular regression in the absence of VEGF but increases vascular sprouting in its presence[19-21]. Accordingly, it seems to be reasonable to assume that the Ang-Tie system and the VEGF system, participate in the process of sinusoidal regeneration and remodeling after partial hepatectomy.

In this study, we investigated the expression of Ang-1, Ang-2, Tie-2, and VEGF in regenerating liver tissue by quantitative reverse-transcription polymerase chain reaction (RT-PCR) using a LightCycler (Roche Diagnostics, Mannheim, Germany) and immunohistochemical staining after 70% hepatectomy in rats. In the next step, we isolated liver cells (hepatocyte, SEC, Kupffer cell, and HSC) from regenerating liver tissue by *in situ* collagenase perfusion and counterflow elutriation, to determine potential cellular sources of these angiogenic factors after hepatectomy. Proliferation and apoptosis of SECs were also evaluated by proliferating cell nuclear antigen (PCNA) staining and the terminal deoxynucleotidyl transferase d-uridine triphoshate nick end labeling (TUNEL) assay, respectively, to determine the putative role of VEGF and angiopoietins, especially their complementary and coordinated effects during sinusoidal regeneration after hepatectomy.

MATERIALS AND METHODS

Animals

Male Wistar rats, weighing 250 to 300 g, were used with the approval of the Chiba Animal Care Committee. The animals were housed in a temperature- and humidity-controlled environment with a 12-h light dark cycle. The study followed our institution's criteria for care and use of laboratory animals in research, which conform to the National Institutes of Health guidelines. Under ether anesthesia, 70% of the liver was resected as previously described[22]. The operative procedure was carried out by means of a clean, but not sterile technique. For the assessment of the Ang-1, Ang-2, Tie-2, and VEGF expressions, the right inferior lobe of the liver was carefully excised before and 12, 24, 48, 72, 96, 120, 144, and 240 h (n = 6 at each time point) after hepatectomy.

Table 1 Primer sequences

Gene		Primer sequence	T (℃)
VEGF	Sense	GCA CTG GAC CCT GGC TTT AC	56
	Antisense	CTG CAG GAA GCT CAT CTC TC	
Ang-1	Sense	GTG GCT GGA AAA ACT TGA GA	52
	Antisense	TGG ATT TCA AGA CGG GAT GT	
Ang-2	Sense	GAC CAG TGG GCA TCG CTA CG	56
	Antisense	CTG GTT GGC TGA TGC TAC TG	
Tie-2	Sense	TGC CAC CAT CAC TCA ATA CC	54
	Antisense	AAA CGC CAA TAG CAC GGT GA	
GAPDH	Sense	GGC ATG GAC TGT GGT CAT GAG	56
	Antisense	TGC ACC ACC AAC TGC TTA GC	

T : annealing temperature.

Quantitative RT-PCR analysis of VEGF, angiopoietins, and Tie-2 mRNA expressions

Total RNA was extracted from liver tissues or freshly isolated liver cells by the acid guanidium-thiocyanate/phenol/chloroform method, and 1 mg of extracted total RNA was subjected to a reverse transcription reaction, using Ready To Go™ T-primed 1st strand cDNA synthesis kit (Amersham Pharmacia Biotech, Buckinghamshire, England). The cDNA from 33 ng of total RNA was used as a template. VEGF, Ang-1, Ang-2, and Tie-2 mRNA levels were quantified by a LightCycler (Roche Diagnostics, Mannheim, Germany), using the double-strand-specific dye SYBE Green I. Details of the primers used in this study are summarized in Table 1. The PCR conditions were as follows: initial denaturation at 95 ℃ for 10 min, followed by 45 cycles of denaturation at 95 ℃ for 15 s, annealing for 10 s, and extension at 72 ℃ for 20 s. The expression level of each angiogenic factor was adjusted by the level of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA, and expressed as a ratio to GAPDH mRNA. To confirm the specific amplification from the target mRNAs, some of the PCR products of each primer set were directly sequenced.

Liver cell isolation from regenerating liver tissue

In the next step, liver cells were isolated from the regenerating liver tissue at the peak periods of each angiogenic factor expression (at 48 and 72 h for VEGF, at 72 and 96 h for Ang-1, at 120 and 144 h for Ang-2, and at 72 and 96 h for Tie-2) after hepatectomy, to determine potential cellular sources of VEGF, Ang-1, Ang-2, and

Tie-2 mRNA. Rat hepatocytes were isolated according to the methods of Gumucio *et al*.[23] Briefly, the rat liver was portally perfused first with Mg^{2+}/Ca^{2+}-free Hanks' buffer at 37 ℃, and then with Eagle's minimal essential medium (EMEM) containing 0.05% collagenase (Type I, Sigma, St Louis, MO, USA). The liver was excised and placed in a petri dish containing fresh EMEM with 0.05% collagenase. Glisson's capsule was stripped, and cells were released by gentle manipulation. The resulting crude preparation was filtered though 250-mm and 100-μm nylon meshes. Hepatocytes were then separated by differential centrifugation. Cell viability and purity were >95%. The SECs, Kupffer cells, HSCs were also isolated by *in situ* collagenase perfusion and counterflow elutriation, as described by Knook *et al*.[24] with minor modifications. A JE-5.0 elutriator rotor (Beckman Instruments, Palo Alto, CA, USA) was used in a J6-MI Beckman centrifuge. The separation process was started by adding the nonparenchymal cell suspension to a sample-mixing chamber. The HSCs were eluted at a flow rate of 16 to 18 mL/min, and at a speed of 3 200 r/min. The SECs were then eluted at a flow rate of 23 to 26 mL/min, and Kupffer cells at a flow rate of 36 to 39 mL/min, and at a speed of 2 500 r/min. The purity of HSCs was >92%, as assessed 24 h after seeding by their typical light-microscopic appearance and their positive immunofluorescent staining for desmin[25]. The purity of SECs and Kupffer cells was >95%, >90%, respectively, as assessed 24 h after seeding by diacetyl-low-density lipoprotein incorporation[26], and positive immunofluorescent staining for ED-1[27]. To evaluate the expression of Ang-1, Ang-2, Tie-2, and VEGF mRNA in each cell fraction, freshly isolated cells were used for total RNA extraction.

Immunohistochemical examination of Ang-1, Ang-2 and Tie-2

For immunohistochemical examination of Ang-1, Ang-2, and Tie-2, the liver tissues excised after hepatectomy were fixed overnight in 2% paraformaldehyde solution at 4 ℃, embedded in tissue-tek compound (Miles Laboratories, Elkhart, IN, USA), and frozen in liquid nitrogen for preparation of cryostat sections. Sections (6 μm) were stained with Ang-1, Ang-2, and Tie-2 antibodies (dilution: 1:100, respectively, Santa Cruz Biotechnology Inc. Delaware, CA, USA) and incubated overnight at 4 ℃. Immunostained cells were detected out using LSAB kit (Dako, Copenhagen, Denmark) according to the manufacturer' s instructions. All sections were then lightly counterstained with 0.1% hematoxylin.

PCNA labeling index

Immunohistochemical staining for PCNA was performed on formalin-fixed and paraffin-embedded liver tissue with anti-PCNA antibody as previously described[28, 29]. The three-step immunoperoxidase method using streptavidin biotin complex (Dako, Copenhagen, Denmark) was performed, according to the procedure described by Hall *et al*.[29]. PC-10 monoclonal antibody (Dako, Copenhagen, Denmark) was used at a dilution of 1:100 and incubated overnight at 4 ℃. PC-10 immunostaining was evaluated based on the percentage of positive nuclei of 100 SECs at high power (400x), and expressed as a PCNA labeling index. In this study, the spindle-shaped sinusoid-lining cells in the open sinusoids at high power (400x) were regarded as SECs[30].

TUNEL assay

Regenerating liver tissue was evaluated using the TUNEL assay to identify SECs undergoing apoptosis. Portions of the liver were fixed with 4% paraformaldehyde and embedded in paraffin. Six micrometer thick sections were prepared, and stained by the TUNEL method using an *in situ* apoptosis detection kit (Takara Biomedicals Co., Osaka, Japan) according to the manufacturer' s instructions. Each stained section was examined at high power fields (200 x), and TUNNEL-positive cells in sinusoids were evaluated.

Statistical analysis

The results were expressed as mean±SD. Statistical analysis was made by the Mann-Whitney test for unpaired data. $P<0.05$ was considered significant.

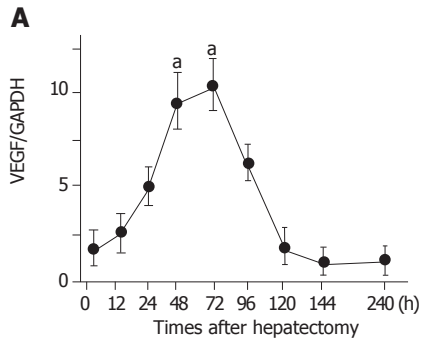
RESULTS

Changes expression of VEGF, angiopoietin, and Tie-2 mRNA in regenerating liver tissue

The expression of VEGF mRNA was at very low levels before hepatectomy. However, VEGF mRNA in the regenerating liver tissue started to increase 24 h after hepatectomy, with a peak at 72 h. A significant increase was found at 48 and 72 h when compared to the prehepatectomy value ($P<0.05$, respectively). Thereafter, VEGF mRNA returned to baseline levels at 120 h (Figure 1A). The changes in Ang-1, Ang-2, and Tie-2 mRNA expressions are shown in Figure 1B. Ang-1 mRNA was present at detectable levels even before hepatectomy, and increased after 48 h of hepatectomy, with a peak at 96 h. A significant increase was seen at 72 and 96 h ($P<0.05$ *vs* 0 h, respectively). Meanwhile, Ang-2 mRNA was hardly detectable before hepatectomy, but was significantly up-regulated at 120 and 144 h ($P<0.03$ *vs* 0 h, respectively). The expression of Tie-2 mRNA was low before hepatectomy, but tended to increase between 72 h and 120 h after hepatectomy.

VEGF, angiopoietin, and Tie-2 mRNA in specific cell populations isolated from regenerating liver tissue

To determine the cellular sources of VEGF, Ang-1, Ang-2, and Tie-2 mRNA in the regenerating liver tissue, four types of liver cells were isolated at key time points: at 48 and 72 h for VEGF, at 72 and 96 h for Ang-1, at 120 and 144 h for Ang-2, at 72 and 96 h for Tie-2, because the expression of each was strongly induced at the indicated time points after hepatectomy (Figure 1). The results are shown in Figure 2. In cell isolates, the mRNA for VEGF was found mainly in the hepatocyte fraction, but in other



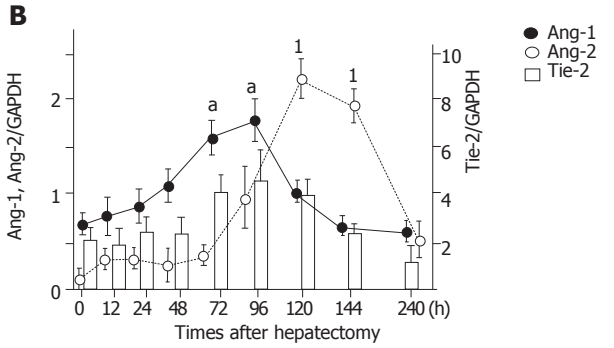


Figure 1 Changes in vascular endothelial growth factor (VEGF), angiopoietin (Ang) and Tie-2 mRNA expressions after 70% hepatectomy. **A:** Quantification of VEGF mRNA levels after hepatectomy by RT-PCR using a LightCycler (Roche Diagnostics). [a]*P*<0.05 *vs* 0 h; **B:** quantification of Ang-1 (closed circle), Ang-2 (open circle), and Tie-2 (open bar) mRNA levels after hepatectomy by RT-PCR using a LightCycler. [a]*P*<0.05 *vs* 0 h, [1]*P*<0.03 *vs* 0 h, respectively.

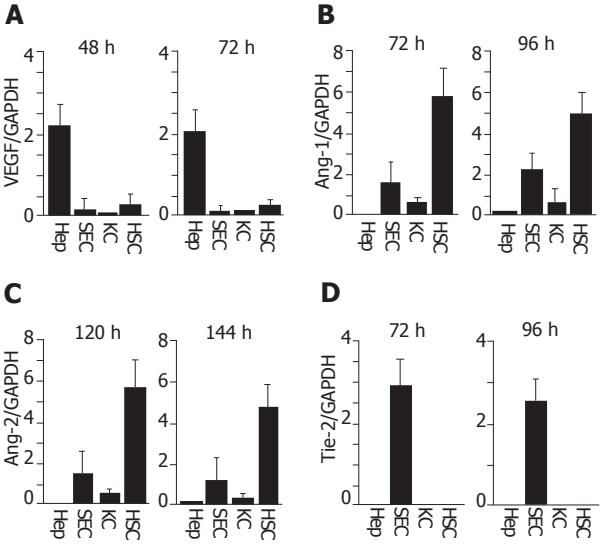


Figure 2 Expressions of VEGF (**A**), Ang-1 (**B**), Ang-2 (**C**) and Tie-2 mRNA (**D**) in isolated liver cells at different time points.

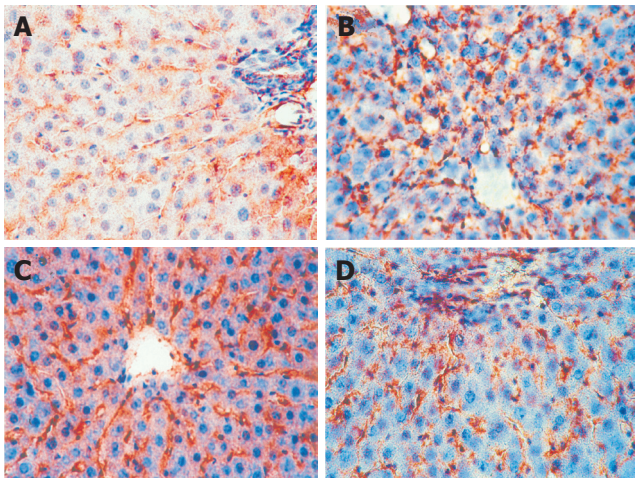


Figure 3 Immunohistochemical staining for angiopoietins (Ang) and Tie-2 protein. Ang-1 protein before hepatectomy (**A**), 96 h after hepatectomy (**B**), Ang-2 protein induction 120 h after hepatectomy (**C**), Tie-2 protein before hepatectomy (**D**) (original magnification x 200).

cell fractions, VEGF mRNA was hardly expressed. On the other hand, the mRNA for Ang-1 and Ang-2 was found mainly in the SEC and HSC fractions, but Ang-2 mRNA expression was more prominent in the latter. The mRNA for Tie-2 was found to be in the SEC fraction alone.

Immunohistochemical staining for angiopoietins and Tie-2

Ang-1 protein was detectable along sinusoids even before hepatectomy (Figure 3A), and was upregulated at 96 h after hepatectomy (Figure 3B). Meanwhile, Ang-2 protein was not detected before hepatectomy, but was remarkably up-regulated along sinusoids at 120 h after hepatectomy (Figure 3C). The Tie-2 protein was observed along sinusoids even before hepatectomy (Figure 3D).

PCNA labeling index and TUNEL staining

The PCNA labeling index was less than 5% in SECs before hepatectomy. However, a slow increase in the PCNA labeling index of SECs was observed after 24 h

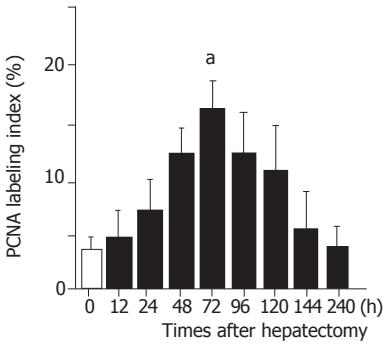


Figure 4 PCNA labeling index of sinusoidal endothelial cells (SECs) after 70 % hepatectomy in rats. [a]*P*<0.05 *vs* 0 h.

of hepatectomy, reaching a peak of 16 % at 72 h (*P*< 0.05 *vs* 0 h) after hepatectomy (Figure 4). Figure 5 shows TUNEL staining of the regenerating liver at 120 h after hepatectomy. Before hepatectomy, apoptotic cells in

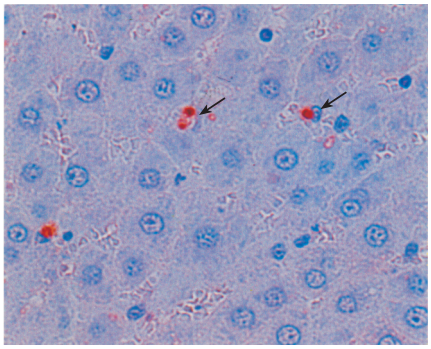


Figure 5 TUNEL staining of regenerating liver tissue at 120 h after hepatectomy. Arrows indicate TUNEL stained-positive sinusoidal cells.

sinusoids were uncommon in the liver, and even fewer such cells were detected within 72 h of hepatectomy. However, a few sinusoidal cells were found to be TUNEL stain-positive between 120 h and 144 h after hepatectomy.

DISCUSSION

In the present study, we showed that VEGF expression in the remnant liver started to increase 24 h after hepatectomy, with a peak at 72 h, which coincided with SEC proliferation, as assessed by PCNA staining. Moreover, in cell isolates from the regenerating liver tissue, VEGF mRNA expression was found mainly in the hepatocyte fraction, suggesting that VEGF secreted from hepatocytes may greatly contribute to SEC proliferation in a paracrine manner. Thereafter, Ang-1 and Ang-2 mRNA expression in the remnant liver increased, but the Ang-2 peak was much later than the Ang-1 peak. In isolated cells, both Ang-1 mRNA and Ang-2 mRNA were found to be abundant in nonparenchymal cells, especially in HSCs. Furthermore, apoptosis of SECs was only detected at 120 and 144 h after hepatectomy, which overlapped with elevated levels of Ang-2. Viewed from the standpoint of Ang-1 function as previously reported in angiogenesis, increased expression of Ang-1 might be related with maturation of sinusoids, presumably characterized by cell-cell contact between SECs and surrounding cells. Meanwhile, elevated Ang-2 in the absence of VEGF induction might be associated with the apoptotic changes in superfluous SECs for cessation of the regenerative process. On the other hand, their receptor, Tie-2 was constitutively expressed on SECs, and tended to increase in the later phase of regeneration, synchronized with up-regulation of Ang-1 and Ang-2. Altogether, these results strongly suggest that angiopoietins released from nonparenchymal cells may largely contribute to the later phase of sinusoidal regeneration and remodeling via Tie-2 receptor after hepatectomy.

In the present study, Ang-1 was constitutively expressed in the liver at the mRNA level and also at the protein level, but VEGF mRNA and Ang-2 mRNA were hardly detectable before hepatectomy. These results might be associated with the maintenance of stability and the maturation of existing sinusoids in the steady state of the liver. Meanwhile, Ang-2 mRNA expression was largely induced in the late phase of regeneration after hepatectomy. Furthermore, apoptotic SECs were detected between 120 h and 144 h, suggesting that SEC apoptosis might be associated with elevated levels of Ang-2 in the absence of VEGF induction. Since apoptosis is known to be a strategic biologic process of eliminating unnecessary cells under certain physiological conditions[31], the SEC apoptosis observed in this study might be related to the presence of superfluous SECs in the late phase of regeneration. Greene *et al.*[32] recently demonstrated that SECs play an important role in the regulation of regenerating hepatic mass by means of proliferation and apoptosis. Therefore, apoptosis of SECs in the late phase of regeneration in this study might be associated with cessation of the regenerative process of the liver.

In cell isolates from the regenerating liver tissue, Ang-2-produced cells were found to be nonparenchymal cells, mainly HSCs. HSCs are known to be located in the space of Disse, below the sinusoidal endothelial cell lining, in close to and partially intercalated between hepatocytes, with their long processes extending along sinusoids [33]. Under physiological conditions, HSCs embrace the sinusoids as "liver-specific pericytes"[34]. At present, the factors that initiate HSC activation are not yet fully understood, although platelet-derived growth factor and transforming growth factor-b (TGF-b) may play a key role in HSC activation and proliferation[35]. On the other hand, TGF-b, a potent inhibitor of hepatocyte proliferation has been reported to increase significantly in the later phase of regeneration, and is known to be mainly released from sinusoidal cells after 70% hepatectomy in rats[36-38]. That is, it would be reasonable to expect activation and proliferation of HSCs in a late phase of regeneration, and subsequent release of several growth factors, including angiopoietins, as well as induction of extracellular matrix, after hepatectomy.

In this study, we focused on the role of angiopoietins and VEGF, especially their complementary and coordinated role in the process of sinusoidal regeneration after hepatectomy, and clearly demonstrated that the Ang-Tie system, together with VEGF, played a critical role in regulating the balance between SEC proliferation and apoptosis during sinusoidal regeneration and remodeling after partial hepatectomy. Accordingly, it is clear that production of VEGF and angiopoietins in the liver must be coordinated both quantitatively and temporally to ensure appropriate sinusoidal regeneration after hepatectomy. However, the VEGF system plays a more important role in the early phase of sinusoidal regeneration than the angiopoietin/Tie system. In addition, close communications between different types of liver cells are clearly important in proceeding through these complex steps of regeneration. However, more detail studies are required to elucidate the regulatory mechanisms of sinusoidal regeneration and remodeling, which are only understood at a descriptive level at present, and

also to provide clues for treatment of impaired hepatic regeneration after major hepatectomy, from this point of view.

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