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ORIGINAL PAPER

Expression of Transforming Growth Factor-α and -β in Hepatic Lobes After Hemihepatic Portal Vein Embolization

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Abstract Hemihepatic portal vein embolization (PVE) concomitantly induces atrophy in embolized and compensatory hypertrophy in nonembolized hepatic lobes. The aim of the present study was to evaluate the involvement of growth stimulatory and inhibitory factors in these hepatic lobes after PVE. Liver specimens from the embolized and nonembolized lobes of ten patients who underwent hepatectomy (8–22 days) after undergoing PVE were obtained. Proliferation and apoptosis were examined immunohistochemically using Ki-67 and the Tdt-mediated dUTP-biotin nick end-labeling method. The expression of transforming growth factor-α (TGF-α) and transforming growth factor-β (TGF-β) was also examined by immunohistochemical staining. PVE induced hepatocyte apoptosis in the embolized lobe and hepatocyte proliferation in the nonembolized lobe. TGF-α expression in the hepatocytes of the nonembolized lobe was markedly increased, whereas TGF-α was also overexpressed, albeit moderately, in the embolized lobe. In contrast, TGF-β expression in the hepatocytes of the embolized lobe was significantly increased, and TGF-β expression was also increased, although to a lesser extent, in the nonembolized lobe. The degree of volume changes of the nonembolized lobe and the embolized lobe after PVE was statistically correlated with the ratios of TGF-α and TGF-β expression in these lobes ($r = 0.886, P < .0001$). In conclusion, these findings indicate that TGF-α and TGF-β expression (assessed by immunohistochemical staining) increase in relation to hepatocyte proliferation and apoptosis, respectively, after PVE in humans and the balance of the two factors may contribute to hepatic atrophy and hypertrophy concomitantly observed in this model.

Keywords Hepatocyte proliferation · Hepatocyte apoptosis · Ki-67 · TUNEL · Immunohistochemistry

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Introduction

Although normal liver is mitotically quiescent, a loss in mass as a result of a partial hepatectomy leads to hepatocyte and nonparenchymal cell proliferation and the rapid restoration of liver parenchyma. Various mitogens, such as transforming growth factor-α (TGF-α) [1–3] and hepatocyte growth factor (HGF) [4, 5], are thought to be involved in the process of liver regeneration. Of interest, inhibitors of hepatocyte proliferation, that is, transforming growth factor-β (TGF-β) [6, 7] and activin [8], have also been reported to be upregulated during the early phase of regeneration after a partial hepatectomy. This phenomenon is postulated to be a preventative mechanism against uncontrolled cellular growth as well as acting to terminate the regeneration process. On the other hand, portacaval shunt has an opposite effect on liver size, compared to the effect produced by a partial hepatectomy. Portacaval shunt is accompanied by hepatocyte apoptosis, resulting in liver atrophy. A recent study showed that hepatic atrophy

after portacaval shunt occurred despite the upregulation of (hepatotrophic) peptides, including HGF and TGF-α [9]. This study also revealed that the expression of TGF-β was increased during the same phase of hepatotrophic factor upregulation. These observations in animals indicated that liver regenerative and atrophic processes are controlled by the coordination of both growth stimulatory and inhibitory factors.

Preoperative portal vein embolization (PVE) is an interventional procedure to induce the atrophy of the embolized lobe and the compensatory hypertrophy of the contralateral nonembolized lobe. The procedure is widely used as a preoperative adjunct to increase the safety of extensive liver resections for hilar bile duct carcinoma, metastatic liver tumors, and hepatocellular carcinoma [10–13]. PVE produces a unique situation in that liver regeneration and atrophy coexist in the same individual.

In the present study, we investigated the involvement of hepatocyte-stimulatory and -inhibitory factors in the livers of patients who underwent PVE. We first evaluated hepatocyte proliferation and apoptosis in the nonembolized and embolized lobes by measuring the number of Ki-67–positive and Tdt-mediated dUTP-biotin nick end-labeling (TUNEL)–positive hepatocytes and the volumetric changes in each lobe. Then, we determined the lobar expressions of TGF-α and TGF-β, respectively.

Patients and methods

Ten consecutive patients who gave informed consent to participate in the study were enrolled. These patients underwent PVE of the right portal vein or the left portal vein prior to an extended right hepatectomy ($n = 8$) or an extended left hepatectomy ($n = 2$), respectively.

Five of the subjects were men, and the other five were women; their average age was 64.0 ± 4 years (range, 59–71 years). The underlying liver diseases in these ten patients were hilar bile duct carcinoma ($n = 5$), metastatic liver tumors ($n = 2$), hepatocellular carcinoma ($n = 1$), cholangiocarcinoma ($n = 1$), and bile duct cystadenocarcinoma ($n = 1$). None of the patients had liver cirrhosis.

PVE was performed using a transileocolic approach according to a previously described procedure 8–22 days (mean $\pm$ SD, 17 ± 4 days) prior to hepatic resection [10]. The embolization material consisted of a mixture of 2.0 g of Gelfoam powder (Upjohn Company, Kalamazoo, MI), 10,000 U of thrombin (The Green Cross Corporation, Osaka, Japan), 20 mL of diatrizoate sodium meglumine (60% Urografin, Schering AG, Berlin, Germany), and 200 mg of amikacin. Serial transverse CT scans were obtained before and 7–14 days (mean $\pm$ SD, 11 ± 2 days) after PVE. The volume of each liver region was calculated from serial transverse CT scanning images according to a previously reported method [14]. The volume changes in the nonembolized and embolized lobes were expressed as the ratio of the volumes measured after and before PVE and were defined as the hypertrophy ratio and the atrophy ratio (volumetric ratios).

Liver biopsy specimens

Wedge biopsy specimens (nontumorous tissue) from the regions to be resected were obtained intraoperatively from the embolized and nonembolized lobes prior to liver resection. For example, in patients scheduled to undergo an extended right hepatectomy, the inferior region of segment 4 in the nonembolized lobe was resected, and a biopsy specimen of the nonembolized lobe was obtained. (To compare TGF-α and TGF-β staining in the embolized and nonembolized lobes with staining in normal liver tissue, biopsy specimens were also taken from nontumorous parts of the liver in five age-matched patients who underwent hepatectomy for metastatic liver tumors.) The specimens were fixed with 10% formaldehyde, embedded in paraffin, and 4-μm thick sections were cut from the tissue blocks, deparaffinized, and dehydrated using a graded ethanol series.

Immunohistochemistry for Ki-67

The proliferative cell activity was determined immunohistochemically using a mouse monoclonal antibody for Ki-67 (MIB-1, Immunotech SA, Marseilles, France), as described previously [15]. Briefly, after incubating the sections overnight at 4 °C with the primary antibody for Ki-67, the bound antibody was detected using the avidin–biotin–peroxidase complex method and a commercial kit, according to the manufacturer's instructions (Vectastatin ABC Elite kit, Vector Laboratories, Burlingame, CA).

In situ detection of apoptosis

Hepatocyte apoptosis was identified according to the TUNEL method, as previously described [16], using the Wako Apoptosis In Situ Detection Kit (WAKO Jun-yaku, Co. Ltd., Tokyo, Japan).

Ki-67 labeling index and apoptotic index

The proportions of hepatocytes undergoing cell proliferation or apoptosis were assessed using the following methods. Hepatocyte nuclei positive for Ki-67 and TUNEL staining were counted at high magnification (400×) in randomly chosen visual fields by observers who were unaware of the identities of the sections (K.K., H.I.). After counting the number of positive cells among 1,000–2,000 hepatocytes per section, the labeling index (LI) for Ki-67 and the apoptotic index (AI) were defined as the percentage of hepatocyte nuclei that stained positive for Ki-67 or TUNEL, respectively, out of the total number of cells counted.

Immunohistochemistry for TGF-α and TGF-β

Immunohistochemical staining was performed using an anti–TGF-α monoclonal antibody (Ab-2; Oncogene Research Products, Cambridge, MA) and an anti–TGF-β polyclonal antibody (H-112; Santa Cruz Biotechnology, Santa Cruz, CA). After boiling the sections in a 0.01 mol/L citric acid buffer for 5 min in a microwave oven, the sections were preincubated with 0.3% H_2O_2 in a methanol solution for 30 min to block endogenous peroxidase activity. After blocking nonspecific reactions with 10% goat serum, the sections were immunostained with the primary antibody dissolved in Block Ace solution (Dainihonseiyaku, Tokyo, Japan) containing 0.1% BSA for 60 min at room temperature. The antigen–antibody binding sites were visualized after reacting with peroxidase-conjugated streptavidin, affinity-purified (ab) 2 goat antimouse IgG (Nichirei, Tokyo, Japan), followed by color development with a 3-3′-diaminobenzidine tetrachloride medium. To confirm that the nonspecific staining was negligible, no primary antibody control slides were prepared for both TGF-α and TGF-β.

TGF-α and TGF-β expression in the hepatocytes was quantitated as follows: parenchymal cells positive for TGF-α and TGF-β staining were counted in the same manner as that for Ki-67 and TUNEL staining, that is, by observers (K.K., H.I.) who were unaware of the identities of the section (embolized lobe, nonembolized lobe, or normal liver tissue). The indexes of TGF-α and TGF-β expression were defined as the percentage of cells that stained positive for TGF-α and TGF-β, respectively.

Statistics

All results are expressed as the mean ± SD. The Mann-Whitney U test and the Wilcoxon test were used to analyze differences in values. Differences among the three groups (embolized lobe, nonembolized lobe, and normal liver) were analyzed using a Kruskal-Wallis ANOVA followed by Bonferroni's correction, and P values less than .0167 were considered to be statistically significant. The significance of the correlations was analyzed using Spearman's correlation analysis. P values of less than .05 were considered to be statistically significant for all other tests.

Results

Changes of liver volumes in embolized and nonembolized lobes after PVE

The volume of the embolized lobes decreased from 615 ± 283 cm^3 before PVE to 525 ± 274 cm^3 after PVE ($P <$ .01) (Fig. 1a). In contrast, the volume of the nonembolized lobes increased from 509 ± 169 cm^3 to 592 ± 160 cm^3 ($P <$ .01) (Fig. 1b). The hypertrophy ratio of the nonembolized lobe was 1.21 ± 0.16 (range, 1.04–1.60) and the atrophy ratio of the embolized lobe was 0.84 ± 0.08 (range, 0.72–0.96).

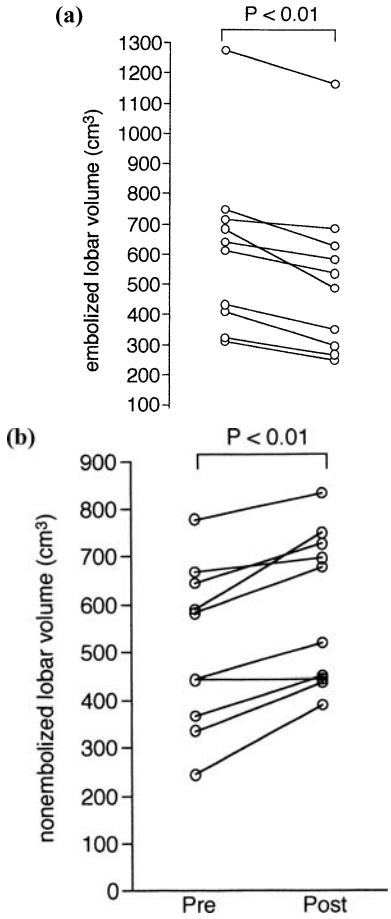

Fig. 1 Changes in (**a**) embolized and (**b**) nonembolized lobar volumes before and after PVE

Ki-67 labeling index and apoptotic index in embolized and nonembolized lobes after PVE

Immunoreactivity to the Ki-67 proliferating antigen was detected in the nuclei of the hepatocytes. Proliferating hepatocytes were distributed throughout the hepatic lobule in the embolized lobe (Fig. 2a) and in the nonembolized lobe (Fig. 2b); thus, hepatocyte nuclei positive for Ki-67 staining were counted throughout the entire hepatic lobule. The mean LI of Ki-67 positive hepatocytes in the nonembolized lobe was $4.8 \pm 5.7\%$, whereas positive hepatocytes were rarely found in the embolized lobe ($0.7 \pm 0.6\%$) (Table 1). On the other hand, although apoptotic hepatocytes identified by TUNEL staining were observed in the embolized lobe, concentrated exclusively in the pericentral area (Fig. 3a), they were rarely detected in the nonembolized lobe (Fig. 3b); Therefore,

Table 1 Percentages of cells immunohistochemically positive for apoptosis and cell proliferation in patients who underwent PVE

Immunostaining	Embolized lobe	Nonembolized lobe
AI (%)	14.0 ± 14.4 (2.5–41.8)	2.2 ± 2.9[a] (0–8.5)
LI (%)	0.7 ± 0.6 (0.2–2.2)	4.8 ± 5.7[a] (0.9–17.2)

Note. Data are expressed as the mean ± SD (range).

AI apoptotic index (of the pericentral area), *LI* labeling index for Ki-67.

[a] $P < .005$ compared with the embolized lobe.

TUNEL-positive hepatocyte nuclei were counted in the pericentral area, so the AI represented the AI of the pericentral area. The AI for the embolized lobe (14.0 ± 14.4%) was significantly higher than that in the nonembolized lobe (2.2 ± 2.9%) (Table 1).

Expressions of TGF-α and TGF-β in embolized and nonembolized lobes aftere PVE

Nonspecific staining as assessed using no primary control slides was negligible for both TGF-α and TGF-β (Figs. 4a and 5a). The TGF-α expression index in the normal liver tissues was very low (1.5 ± 1.1%). In contrast, the marked expression of TGF-α was observed in the cytoplasm of hepatocytes throughout the entire hepatic lobule in the nonembolized lobe (62.9 ± 14.9%) (Fig. 4b), and TGF-α–expressing hepatocytes were counted throughout the entire hepatic lobule. In addition, a considerable number of hepatocytes in the embolized lobe were TGF-α positive, although the number of such cells was much lower than that in the nonembolized lobe (Fig. 4c).

Although the index of TGF-β expression in the normal liver tissues was very low (1.3 ± 1.3%), a marked

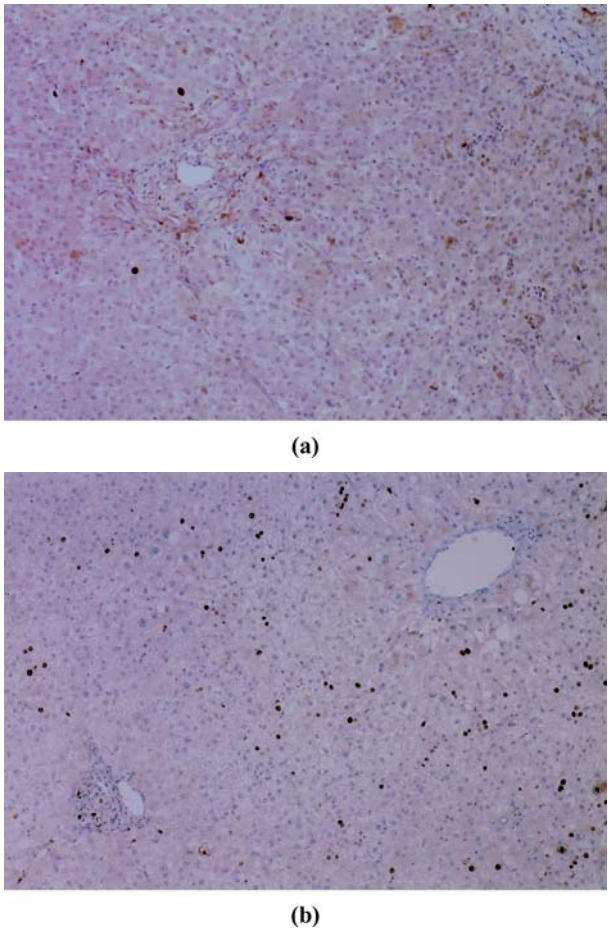

Fig. 2 Ki-67 expression detected with MIB-1 in the (**a**) embolized lobe and the (**b**) nonembolized lobe. Ki-67 expression appears as a dark-brown stain. (Original magnification, a, b, × 140)

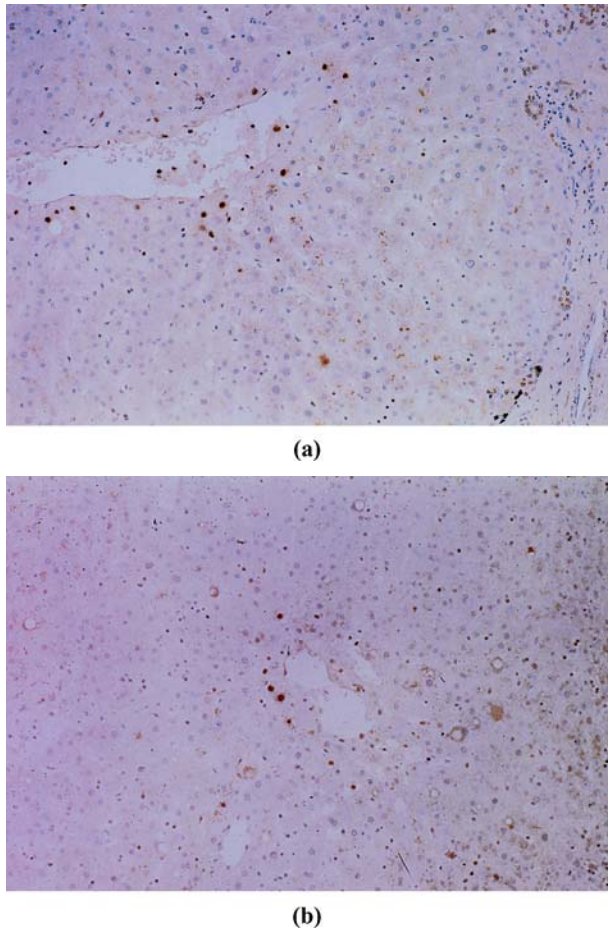

Fig. 3 Detection of apoptosis in the (**a**) embolized lobe and the (**b**) nonembolized lobe using the TUNEL method. The TUNEL-positive cells are stained dark brown. Positive hepatocyte nuclei are concentrated in the pericentral area. (Original magnification, A, B, × 140)

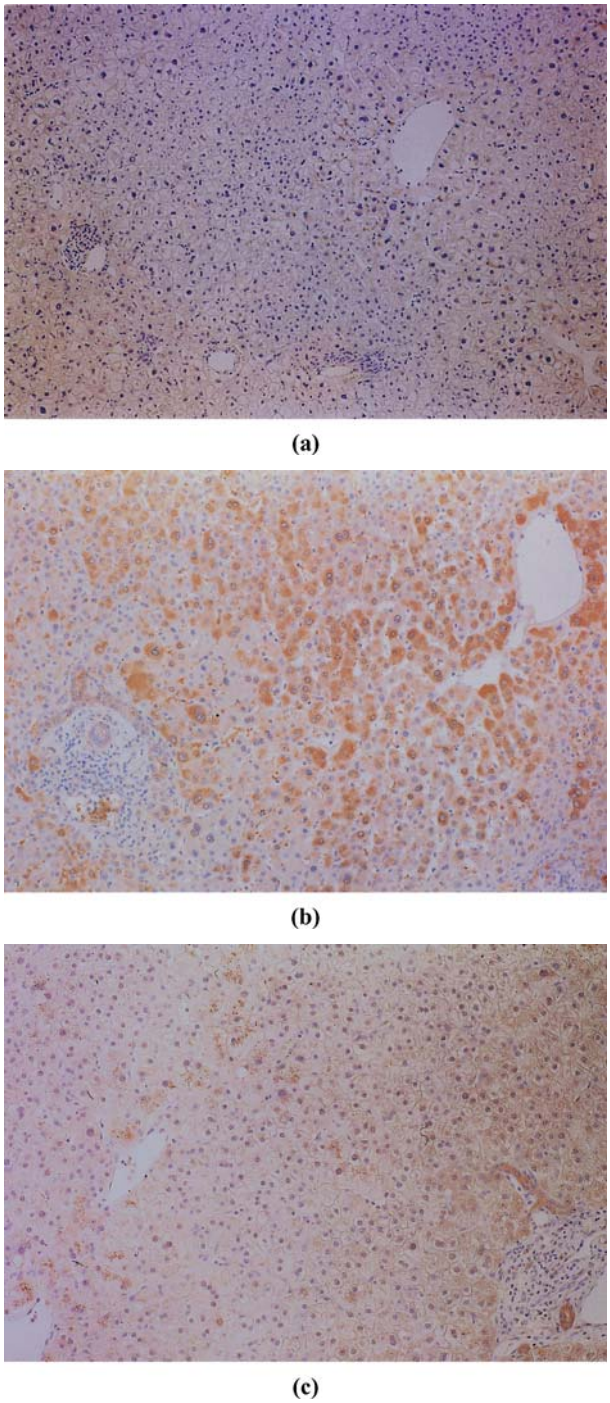

Fig. 4 Immunohistochemical staining for TGF-α. (**a**) No primary antibody control slide. (**b**) Nonembolized lobe. (**c**) Embolized lobe. (Original magnification, A, B, C $\times$140.)

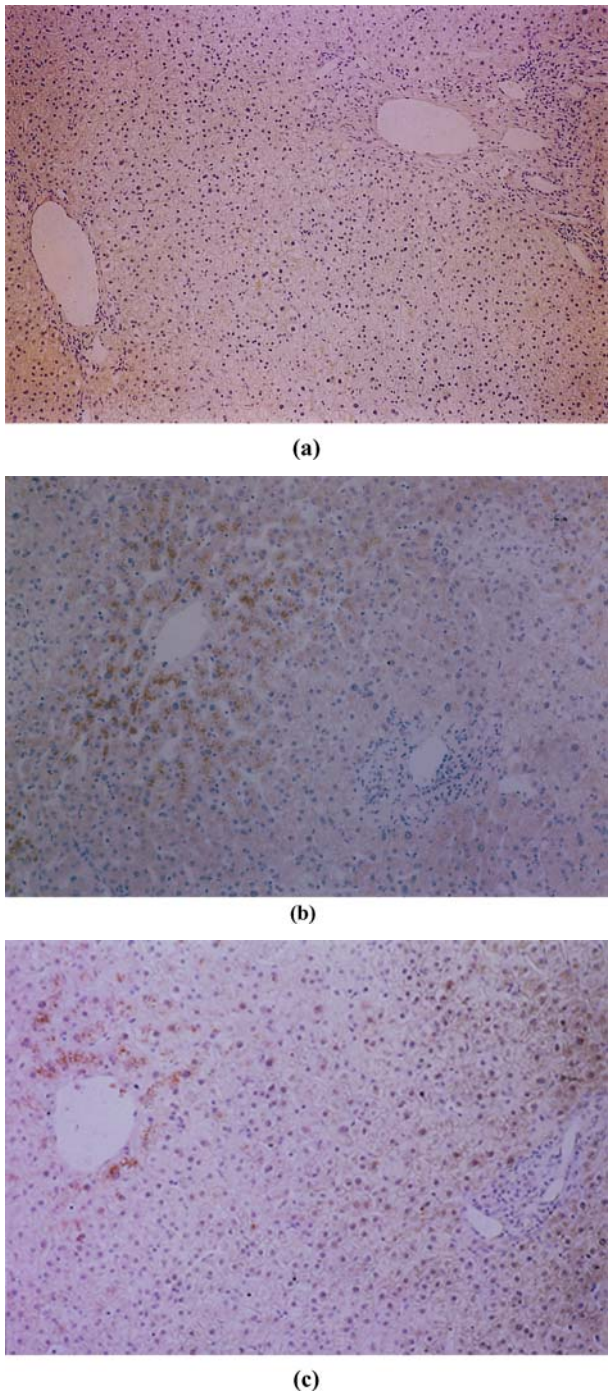

Fig. 5 Immunohistochemical staining for TGF-β. (**a**) No primary antibody control slide. (**b**) embolized lobe. (**c**) Nonembolized lobe. (Original magnification, A, B, C $\times$140.) Hepatocytes are mainly positive in the pericentral area

expression of TGF-β was observed in the cytoplasm of hepatocytes in the embolized lobe, mainly in the pericentral area ($48.1 \pm 16.1\%$) (Fig. 5b); thus, TGF-β–expressing hepatocytes were counted in the pericentral area. A substantial number of hepatocytes in the nonembolized lobe were TGF-β positive, although to a much lower extent than that in the embolized lobe (Fig. 5c). The differences in TGF-α expression between any two groups were significant (Fig. 6a), as were the differences in the expression of TGF-β between any two groups (Fig. 6b).

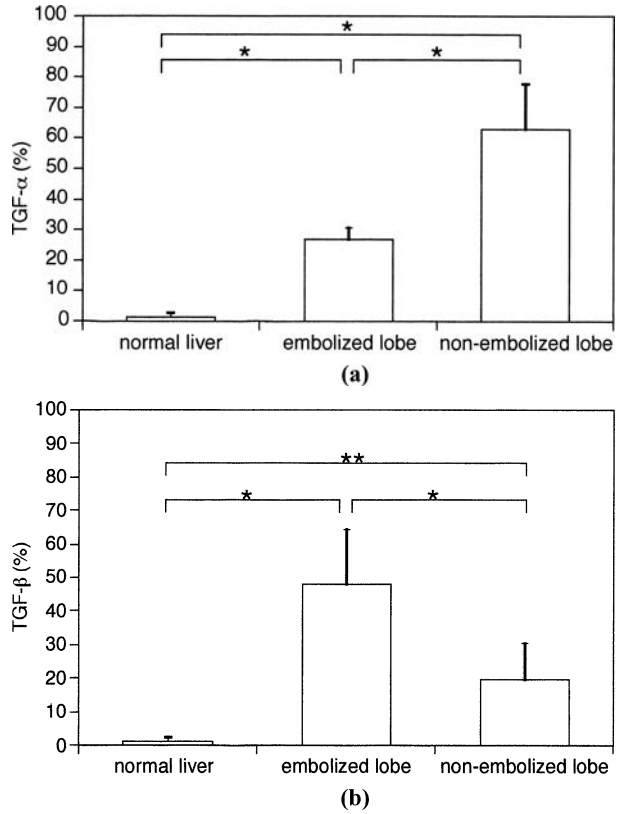

Fig. 6 (**a**) Index of TGF-α expression in the normal liver, the embolized lobe, and the nonembolized lobe. (**b**) Index of TGF-β expression in the normal liver, embolized lobe, and the nonembolized lobe. Data are expressed as the mean ± SD. $^{*}P < .0001$. $^{**}P = .01$

Relationship between the volumetric ratios and the ratios of TGF-α and TGF-β

The hypertrophy ratio of the nonembolized lobe and the atrophy ratio of the embolized lobe (the volumetric ratios) after PVE were statistically correlated with the ratios of TGF-α and TGF-β expression in the lobe ($r = 0.886$, $P < .0001$: Fig. 7).

Relationship Between Ki-67 LI and the Index of TGF-α Expression

The Ki-67 LI and the index of TGF-α expression were significantly and positively correlated in both lobes ($r = 0.846$, $P < .001$).

Relationship Between AI and the Index of TGF-β Expression

The AI and the index of TGF-β expression were significantly and positively correlated in both lobes ($r = 0.652$, $P < .01$).

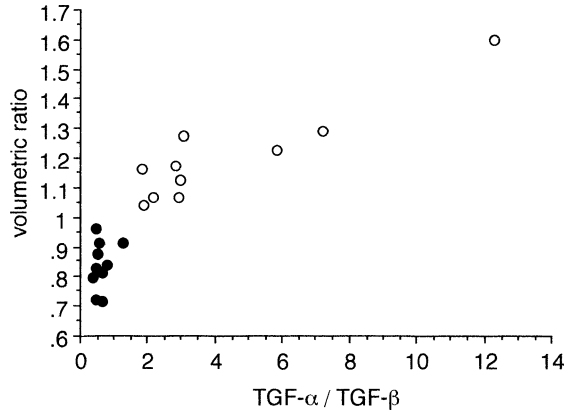

Fig. 7 Correlation between the volumetric ratio after PVE and the ratio of TGF-α and TGF-β in the lobe ($r = 0.886$, $P < .0001$). The term *volumetric ratio* refers to the hypertrophy ratio of the nonembolized lobe (○) and the atrophy ratio of the embolized lobe (●)

Discussion

The most notable finding in the present study was that the expression of both hepatic growth stimulatory and inhibitory factors was increased in both hepatic lobes undergoing hypertrophy or atrophy, although the increment in the respective factors differed in the embolized and nonembolized lobes. Furthermore, the balance between the increments in these factors was closely correlated with the extent of both lobar atrophy and compensatory hypertrophy in the liver.

PVE is a clinical application of the experimental observation that portal vein ligation leads to atrophy of the manipulated lobe and hypertrophy of the contralateral lobe [17, 18]. The contribution of cytokines, hepatotrophic factor, and the hemodynamic changes induced by portal branch occlusion has been discussed [19]. However, the mechanisms leading to liver atrophy in the embolized lobe and concomitant hypertrophy in the nonembolized lobe after PVE are poorly understood. It is likely that some regional changes in the hepatic lobes determine progression toward atrophy or hypertrophy. The present study represents the first attempt to address whether both growth stimulatory and inhibitory factors contribute to the development of hepatic atrophy and hypertrophy after human PVE.

Ki-67 is a nuclear antigen expressed during all phases of the cell division cycle, except in resting cells (G_0) [20]. The expression of Ki-67 is correlated with thymidine incorporation [21] and the expression pattern is correlated with the labeling pattern obtained by bromodeoxyuridine incorporation, thereby identifying liver cells undergoing DNA replication [22].

TGF-α is an established growth stimulatory factor, and its expression has been reported to increase in regenerating liver after a partial hepatectomy [1–3] or toxic injury [23] in rats. TGF-α levels in liver and blood parallel hepatocyte

proliferative activity after partial hepatectomy in rats [24]. In acute and chronic liver injuries in humans, the expression of TGF-α mRNA has been reported to be closely correlated with hepatocyte proliferative activity [25, 26]. On the other hand, TGF-β is the most potent growth inhibitory factor presently known to exist in the liver [7, 27, 28] and is thought to be involved in the induction of apoptosis in the rat liver [29, 30]. Because lobar hypertrophy and atrophy following PVE have been reported to be attributable to liver cell proliferation and apoptosis [31], we assessed the expression of TGF-α and TGF-β and evaluated their activities as a hepatocyte growth stimulatory factor and inducer of hepatocyte apoptosis, respectively. In this investigation, we assessed the expression of TGF-α and TGF-β using immunohistochemical staining to identify the existence of these proteins. To study the expressions of these growth factors more quantitatively, the mRNA and proteins levels in the tissues should be determined using an ELISA. However, the production of TGF-β is reportedly regulated by transcriptional as well as post-transcriptional mechanisms. In addition, platelets contain and release TGF-β. Because this study used clinical materials, it was impossible to wash the blood out of the specimens in situ. Thus, we used an immunohistochemical method; other reports on TGF-β expression in clinical samples have also used immunohistochemical methods [32, 33].

A high level of hepatocyte proliferation, as evidenced by an increase in Ki-67–positive cells, was observed in the nonembolized lobe. In contrast, substantial numbers of apoptotic hepatocytes, identified using the TUNEL method, were detected in the embolized lobe. The respective changes in lobe volume appeared to closely reflect the difference in these cell kinetic parameters. These results agree with those of former reports [31].

In normal liver tissues, the expression of both TGF-α and TGF-β was low. In the nonembolized lobe undergoing hypertrophy, however, the expression of TGF-α was markedly elevated. TGF-α expression was observed throughout the hepatic lobule, showing a similar distribution pattern to that of the Ki-67–positive cells. This finding suggests that TGF-α participates in hepatocyte proliferation in the nonembolized lobe, as it does in other models of liver regeneration like partial liver resection [1–3] or toxic injury [23] in rats. On the other hand, in the embolized lobe undergoing atrophy the expression of TGF-β was remarkably elevated, particularly in the pericentral hepatocytes. The sublobular distribution of TGF-β expression coincided well with that of apoptotic hepatocytes. A recent study suggested that TGF-β mRNA is detectable in hepatocytes and nonparenchymal cells after a partial hepatectomy [34]. Using immunohistochemistry, the expression of TGF-β was shown to be elevated in hepatocytes after choline deficiency to induce liver apoptosis [35] and in liver regeneration after a partial hepatectomy [36]. The increase in TGF-β expression in hepatocytes in the embolized and nonembolized lobes in the present study is compatible with the previous reports. TGF-β has been reported to be a strong inducer of hepatocyte apoptosis in vitro [27] and in vivo [37], and the present results indicate that the induction of apoptosis and resulting atrophy in the embolized lobe may be attributable, at least in part, to TGF-β.

TGF-α expression was also elevated in the embolized lobe where the atrophying process was in progress, although the extent of its expression was much smaller than that in the nonembolized lobe. Furthermore, the expression of TGF-β was also elevated in the nonembolized hypertrophying lobe, although to a lesser degree compared with that in the embolized lobe. The upregulation of growth stimulatory factors, including TGF-α, has also been reported in a rat model of portacaval shunt, in which the apoptotic effect of TGF-β was postulated to overwhelm the significant increase in growth promoters [9]. A similar increase in TGF-β during hepatocyte replication has been reported in regenerating livers after partial hepatectomy in rats [6, 7]; this increase was hypothesized to prevent uncontrolled growth during liver regeneration. The present findings in humans seem to agree with these observations in animals.

The extent of hepatic hypertrophy and atrophy, as reflected in the volumetric ratio after PVE, appeared to be closely correlated with the ratio of TGF-α to TGF-β (Fig. 7). This finding suggests that the cessation of unilateral portal flow causes both hepatic lobes to change from a quiescent state to a state where the levels of both growth stimulators and inhibitors are increasing; whether the respective liver lobes undergo hypertrophy or atrophy and the extent to which these processes occur may depend on the balance between these factors.

The major limitation of the present study in humans lies in the fact that liver growth factor expression—TGF-α and TGF-β—was assessed only at a single time point after PVE (mean of 17 days). Because liver regenerative signals after liver resection are known to emerge in a time-dependent manner, a more comprehensive study, either in humans or in animal models, evaluating the chronological expression of growth factors after PVE is needed to confirm the present findings. In addition, although we focused on TGF-α and TGF-β as hepatocyte growth stimulatory factors and as inducers of hepatocyte apoptosis in the present study, we must bear in mind that the observed lobar hypertrophy and atrophy complex might also be a function of other liver growth factors, such as HGF and/or the epidermal growth factor receptor, a target of TGF-α and other potent hepatocyte mitogens including epidermal growth factor.

In conclusion, these findings indicate that TGF-α and TGF-β expression increase in relation to hepatocyte proliferation and apoptosis, respectively, after PVE in humans. The balance of the two factors may contribute to hepatic atrophy and hypertrophy concomitantly observed in this model.

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