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Increased Expressions of Vascular Endothelial Growth Factor and Its Receptors, *flt*-1 and *KDR*/*flk*-1, in Regenerating Rat Liver

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Sinusoidal endothelial cells proliferate following hepatocyte regeneration in the liver after partial resection. The expressions of vascular endothelial growth factor (VEGF) and its receptors, *flt*-1 and *KDR*/*flk*-1, were studied by Northern blotting in isolated rat liver cells and 70% resected rat liver. VEGF was expressed in hepatocytes immediately after isolation, and both *flt*-1 and *KDR*/*flk*-1 were expressed in non-parenchymal cells including sinusoidal endothelial cells. The VEGF expression in hepatocytes decreased during primary culture for 48 hr. This expression was maintained at 48 hr of culture by addition of EGF to the medium at 24 hr, increasing thereafter. VEGF, *flt*-1, and *KDR*/*flk*-1 were also expressed in normal rat liver. In 70% resected rat liver, VEGF expression increased with a peak at 72 hr after the operation, followed by expressions of *flt*-1 and *KDR*/*flk*-1 increasing between 72 and 168 hr. These results suggest that VEGF expression increases in regenerating hepatocytes, which may contribute to proliferation of sinusoidal endothelial cells of rat liver following partial resection, probably through *flt*-1 and *KDR*/*flk*-1 receptors upregulated on sinusoidal endothelial cells.

In the process of liver regeneration, non-parenchymal cells as well as hepatocytes increase in number. Among non-parenchymal cells, sinusoidal endothelial cells are assumed to be the most important, as the cells play a role in the supply of blood flow to hepatocytes (1). However, the regulatory mechanisms of proliferation of sinusoidal endothelial cells during liver regeneration are to be elucidated, because of lack of appropriate tools for investigation.

Sinusoidal endothelial cells are unique in response to growth factors. It is unlikely that platelet derived growth factor (PDGF) and fibroblast growth factor (FGF), powerful angiogenetic factors, are involved in the process of sinusoidal endothelial cell proliferation during liver regeneration, since fetal calf serum (FCS) and acidic and basic FGFs seem to be ineffective for proliferation of these cells in primary culture (2). Recently, vascular endothelial growth factor (VEGF), vascular permeability factor (VPF) originally isolated by Senger *et al.* (3), has been shown to increase the number of endothelial cells of any type including those in the hepatic sinusoids in primary culture (2,4), but not of epithelial and mesenchymal cells (5). Moreover, VEGF was shown to be expressed in epithelial cells including hepatocytes as well as hepatocellular carcinoma cells (2,4,6), and its receptors in sinusoidal endothelial cells (2,4). These observations may suggest the presence of a communication system between hepatocytes and sinusoidal endothelial cells through VEGF during liver regeneration.

In adult rats after 70% hepatectomy, mitosis of sinusoidal endothelial cells is known to follow mitosis of hepatocytes (7,8). Thus, we postulated that VEGF might induce proliferation

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Abbreviations: EGF: epidermal growth factor, FCS: fetal calf serum, FGF: fibroblast growth factor, HBSS: Hank's balanced salt solution, PDGF: platelet derived growth factor, VEGF: vascular endothelial growth factor, VPF: vascular permeability factor.

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of sinusoidal endothelial cells in a paracrine manner if its expression is increased in regenerating hepatocytes. In the present paper, we measured the expressions of VEGF as well as 2 molecules belonging to Flt tyrosine receptor family, *flt*-1 and *KDR*/*flk*-1 which are associated with VEGF signal transduction in proliferation of endothelial cells (2,9), in 2 rat models of liver regeneration, hepatocytes in primary culture stimulated by epidermal growth factor (EGF) and partially resected liver.

MATERIALS AND METHODS

Experimental Designs

Male Fisher rats (Charles River Japan, Atsugi, Japan) weighing 170 to 190 g were maintained on a commercial pelleted diet and water *ad libitum* in a room at 22 ± 2°C under normal laboratory lighting conditions. All animal study protocols conformed to National Research Council criteria for humane care.

Experiment I. Hepatocytes were isolated by Seglen's method (10), and non-parenchymal cells by gradient centrifugation in metrizamide (Sigma Chemical Company, St. Louis, Missouri) solution following digestion of the liver with collagenase (type I: Worthington Biochemical Corp., Freehold, New Jersey) and pronase E (Kaken Pharmaceutical Co. Ltd., Urayasu, Japan) (11). The viability and purity of both cells were constantly higher than 90% and 99% as tested by Trypan blue exclusion and by light microscopic examination, respectively. Pellets of 1×10^7 cells were immediately subjected to Northern blot analysis. The remaining hepatocytes were suspended in Williams' medium E (Flow Laboratories, Irvine, Scotland) containing 10% FCS (Gibco Laboratories Ltd., Life Technologies Inc., Grand Island, New York), which was heat-inactivated at 56°C for 30 min, 10^{-6} M insulin (Sigma Chemical Company) and 10^{-5} M dexamethasone (Sigma Chemical Company), and seeded in Falcon 3003 dishes (Becton Dickinson Labware, Lincoln Park, New Jersey) at a density of 5×10^4 cells/cm^2. They were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 2 hr. Following removal of non-adherent cells by washing with Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS(−); Gibco Laboratory Ltd.), adherent hepatocytes were further cultured in Williams' medium E containing 10% heat-inactivated FCS with or without addition of 50 ng/mL of EGF (Toyobo Co. Ltd., Osaka, Japan) 24 hr after plating. Adherent hepatocytes were subjected to Northern blot analysis at 2, 12, 24, 48 and 72 hr.

Experiment II. Under light anesthesia with ether, rats underwent 70% hepatectomy according to the method of Higgins & Anderson (12). They were sacrificed at 0, 12, 24, 48, 72 and 168 hr after the operation under ether anesthesia. Excised liver was immediately frozen in liquid nitrogen, and stored at −80°C.

Northern Blot Analysis

Total RNA was extracted from the pellets of isolated liver cells, cultured hepatocytes on plastic dishes and frozen sections of the liver by homogenation in guanidine thiocyanate and centrifugation through phenol/chloroform (13). Ten μg of total RNA was subjected to electrophoresis on 1% agarose-formaldehyde gel, blotted to nylon filters (Hybond-N; Amersham International Plc., Little Chalfont, England) by upward capillary transfer, and fixed to filters using UV crosslinker. cDNA fragments of rat VEGF (14), *flt*-1 (9) and *KDR*/*flk*-1 (2) were radiolabeled with [α-P^{32}]dCTP (DuPont-New England Nuclear, Boston, MA) using Megaprime DNA labeling system (Amersham International Plc.). Prehybridized filters were hybridized at 42°C for 16 hr with ^{32}P-labeled cDNA fragments, then washed under high-stringency conditions and exposed to XAR film (Eastman Kodak Company, Rochester, NY).

RESULTS & DISCUSSION

As shown in Figure 1, hepatocytes immediately after isolation expressed VEGF mRNA. In these cells, *flt*-1 and *KDR*/*flk*-1 expressions were not seen. In contrast, isolated non-parenchymal cells expressed both *flt*-1 and *KDR*/*flk*-1 mRNAs, but faintly VEGF mRNA (Figure 2). These results are in line with the observations by Yamane *et al.* (2) except for slight expression of VEGF mRNA in non-parenchymal cells. Recently, VEGF has been reported to be produced by activated macrophages *in vitro* (15). Non-parenchymal cells in the present experiments included Kupffer cells and hepatic stellate cells as well. The contribution of VEGF expression in Kupffer cells would be possible.

When isolated hepatocytes were cultured on plastic dishes, VEGF expression increased with a peak between 2 and 12 hr of culture, but decreased after 24 hr and returned at 48 hr to the level for hepatocytes immediately after isolation. In these cells, VEGF expression was slightly increased at 72 hr (Figure 1). This might reflect the previous observation that a transient small

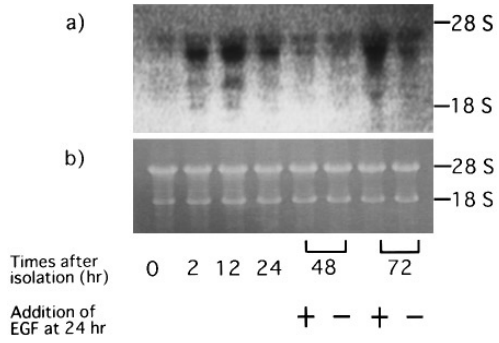

FIG. 1. VEGF mRNA expression in isolated rat hepatocytes. (a) Northern blotting. (b) Ethydium bromide staining.

peak of DNA synthesis occurred between 8 and 16 hr and at 45 hr after isolation in hepatocytes similarly cultured (16). When EGF was added to the culture medium at 24 hr, this decrease in VEGF expression at 48 hr was less, and the expression was markedly increased at 72 hr compared to no EGF addition (Figure 1). In these cells, active DNA synthesis occurs later than 12 hr of EGF addition at this concentration (16). Thus, VEGF expression seems to increase in cultured hepatocytes in association with DNA synthesis.

As shown in Figure 3, normal rat liver expressed *flt*-1 and *KDR*/*flk*-1 mRNAs as well as VEGF mRNA. In 70% resected rat liver where hepatocyte mitosis peaks 36 hr after the operation (8), VEGF expression significantly increased at 72 hr compared to normal rat liver (Figure 3a). These observations are comparable with those obtained from the experiments using hepatocytes in primary culture (Figure 1), suggesting that proliferating hepatocytes expressed the increased VEGF mRNA *in vivo* as well as *in vitro.* In the partially resected liver, *flt*-1 expression significantly increased later than 12hr after the operation, and *KDR*/*flk*-1 expression between 72 and 168 hr compared to the normal liver (Figures 3b and 3c). During this period, sinusoidal endothelial cells are shown to increase mitosis with a peak at 96 hr (7). The upregulation of VEGF receptor may be involved in such proliferation of sinusoidal endothelial cells.

In conclusion, VEGF expression increases in regenerating hepatocytes. This increase may contribute to proliferation of sinusoidal endothelial cells of rat liver after partial resection, probably through *flt*-1 and *KDR*/*flk*-1 receptors upregulated on sinusoidal endothelial cells.

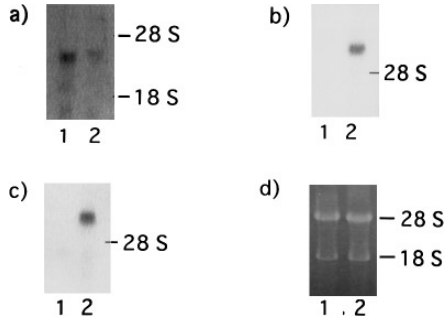

FIG. 2. VEGF, *flt*-1, and *KDR*/*flk*-1 mRNA expressions in isolated rat liver cells. (a) Northern blotting of VEGF mRNA. (b) Northern blotting of *flt*-1 mRNA. (c) Northern blotting of *KDR*/*flk*-1 mRNA. (d) Ethydium bromide staining. Lane 1: hepatocytes cultured for 12 hr on plastic dishes. Lane 2: non-parenchymal cells immediately after isolation.

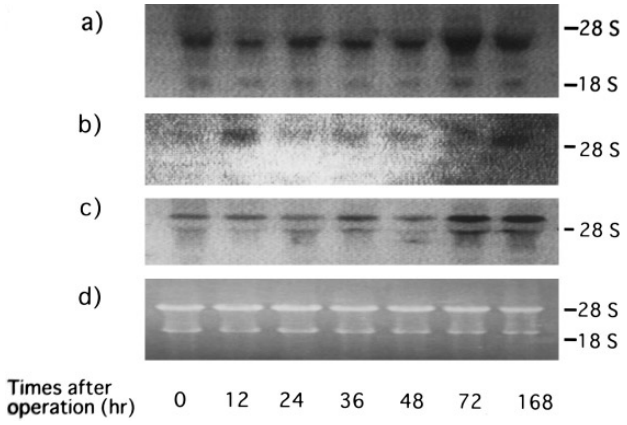

FIG. 3. VEGF, *flt*-1, and *KDR*/*flk*-1 mRNA expressions in rat liver following 70% resection. (a) Northern blotting of VEGF mRNA. (b) Northern blotting of *flt*-1 mRNA. (c) Northern blotting of *KDR*/*flk*-1 mRNA. (d) Ethydium bromide staining.

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