Vol. 198, No. 1, 1994
January 14, 1994

HEPARIN-BINDING EGF-LIKE GROWTH FACTOR IS A POTENT MITOGEN FOR RAT HEPATOCYTES

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Received December 2, 1993

We examined the hepatotrophic activity of heparin-binding EGF-like growth factor (HB-EGF), a recently identified potent mitogen for vascular smooth muscle cells and fibroblasts. HB-EGF stimulated DNA synthesis of rat hepatocytes in primary culture in a dose-dependent manner up to 30 ng/ml. The maximal stimulation by HB-EGF represented more than 80 % of that induced by HGF. In normal rat liver, the transcript of HB-EGF gene was detected in the non-parenchymal cells and very low level in the hepatocytes. In the regenerating liver on the 3rd day after 70% hepatectomy, the HB-EGF mRNA increased in the non-parenchymal cells, suggesting that HB-EGF may contribute to liver regeneration through a paracrine mechanism.

A recently identified heparin-binding EGF-like growth factor (HB-EGF), the activity of which was originally found in the conditioned medium of human macrophages, is a potent mitogen for vascular smooth muscle cells and fibroblasts (1, 2). HB-EGF is a member of the epidermal growth factor (EGF) family with a molecular weight of 22 kDa, sharing about 35% homology with EGF. Sequence analysis of a human HB-EGF cDNA clone predicted a primary translation product of 208 amino acids. This putative molecule is likely to be proteolytically processed to form a mature HB-EGF that contains an EGF-like domain and a heparin-binding domain. In addition to the heparin-binding property, the mature HB-EGF can bind to EGF receptors in competition with EGF (2) suggesting that HB-EGF can stimulate the growth of epithelial-type cells, including hepatocytes, which have EGF receptors on their surface.

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0006-291X/94 $5.00

Although adult rat hepatocytes rarely divide, they proliferate aggressively in response to tissue injury or loss of portions of the liver (3, 4). The rat liver restores its size within 2 weeks after 70% partial hepatectomy. Several humoral factors have been reported to be hepatotrophic factors *in vitro* including EGF (5, 6), transforming growth factor-α (TGF-α) (7, 8), acidic fibroblast growth factor (aFGF) (9), basic FGF (bFGF) (10), hepatocyte growth factor (HGF) (11-14) and recently keratinocyte growth factor (KGF) (15). *In vivo*, several steps would be involved in the process of liver regeneration, such as initiation, sustainment and termination of DNA synthesis of hepatocytes. Transforming growth factor-β (TGF-β) is a possible candidate for the terminator of DNA synthesis in liver regeneration (16).

Some *in vitro* hepatotrophic factors are likely to play important roles in initiating or sustaining DNA synthesis in the process of liver regeneration. However, the precise mechanism for liver regeneration is not fully understood. This led us to focus our attention on the role of HB-EGF in liver regeneration. Here, we reported the hepatotrophic activity of HB-EGF and show the possibility of its contributing to liver regeneration.

Methods

Primary Culture of Hepatocytes. The liver of an adult male Sprague-Dawley rat weighing 200 g was perfused via the portal vein with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salts solution containing EGTA followed by perfusion with 0.05% type IV collagenase (Sigma) in Ca^{2+}-containing medium (Hepes buffer) at 37 °C (17). Hepatocytes and non-parenchymal cells were separated by the method of Shimaoka *et al.* (18). Both cell fractions were over 95% in purity and 90% in viability, respectively, according to trypan blue staining.

Analysis of DNA Synthesis. Hepatocytes were seeded at the cell density of 1 x 10^5/ml in a collagen-coated 96-well plastic dish and incubated for 24 hr with Williams medium E (WE) containing 100 μM insulin (Sigma) and 100 μM dexamethasone (Sigma), supplemented with 10% fetal calf serum. Cells were washed once with WE, followed by incubation for 20 hr with the indicated dose of HGF (a generous gift from Dr. T. Nakamura, Division of Biochemistry, Biomed. Res. Center, Osaka Univ. Med. Sch.) or HB-EGF (2) in the serum-free WE containing 100 μM insulin and 5 U/ml aprotinin (Sigma). Thereafter, 37 kBq/well of [^{3}H]thymidine (Amersham Japan) was pulsed, and the cells were incubated a further 5 hr. After washing with phosphate-buffered saline three times, the hepatocytes were harvested by trypsinization and the [^{3}H]thymidine in the DNA was counted with a Betaplate system (Pharmacia). The values were expressed as means of quadruplicates.

Northern Blot Analysis. For Northern blot analysis, total RNA was extracted from cells according to the methods of Chomczynski and Sacchi (19). The Northern blotted membrane was probed with [^{32}P]dCTP (Amersham) labeled rat HB-EGF cDNA fragment (20).

HB-EGF Gene Expression After Partial Hepatectomy. To better assess the role of HB-EGF for liver regeneration, the modulation of HB-EGF gene expression in the remnant liver after partial hepatectomy was investigated. The rat was anesthetized, then the medial and left hepatic

lobes were removed (70% partial hepatectomy) according to the method of Higgins and Anderson (21). Sham-operated rat served as the control. On the 3rd day after the operation, hepatocytes and non-parenchymal cells were isolated from hepatectomized and sham-operated rats livers by *in situ* perfusion method (18). Total RNA was extracted from each fraction and then analyzed by Northern blot analysis.

Results

Hepatotrophic Activity of HB-EGF. The DNA synthesis of rat hepatocytes in primary culture was stimulated by HB-EGF in a dose-dependent manner up to 30 ng/ml, but decreased at the concentration of 100 ng/ml (Fig. 1). No reduction of DNA synthesis at high doses of the growth factor was observed when hepatocytes were treated with HGF. The maximal stimulation of the DNA synthesis given by 30 ng/ml of HB-EGF represented more than 80% of the stimulation induced by HGF which is the most potent mitogen for hepatocytes. The half-maximal response doses of HB-EGF and HGF were about 3 and 5 ng/ml, respectively. On a molar basis, HB-EGF was one-fourth to -fifth as potent as HGF in hepatotrophic activity.

Additive Effect of HB-EGF to HGF on Hepatocyte Growth. In order to investigate the *in vitro* interaction between HB-EGF and HGF on DNA synthesis, primary hepatocyte cultures were treated with HB-EGF in the

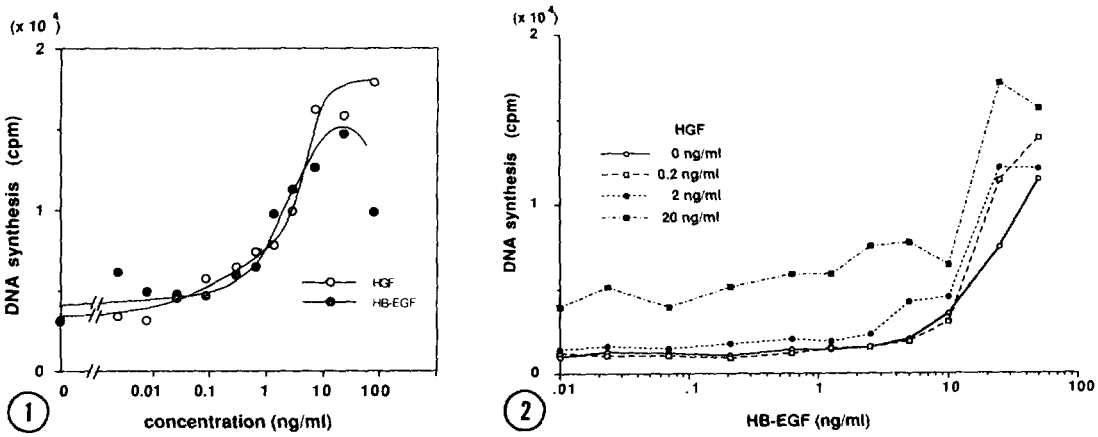

Fig. 1. Effects of HB-EGF and HGF on [^{3}H]thymidine incorporation of rat hepatocytes in primary culture. Hepatocytes obtained by the *in situ* perfusion method were seeded at the cell density of 1 x 10^5/ml in collagen-coated 96-well plates and incubated for 24 hr in WE medium supplemented with 10% FCS. Cells were incubated for 24 hr in serum-free WE medium containing serially diluted HGF or HB-EGF. Five hours after the addition of [^{3}H]thymidine, the radioactivity incorporated into the DNA was counted with a Betaplate system. All values were expressed as means of quadruplicates.

Fig. 2. Additive effect of HB-EGF to HGF on hepatocyte growth. Effect of HB-EGF on DNA synthesis of hepatocytes in the presence of 0, 0.2, 2.0, or 20 ng/ml of HGF was investigated.

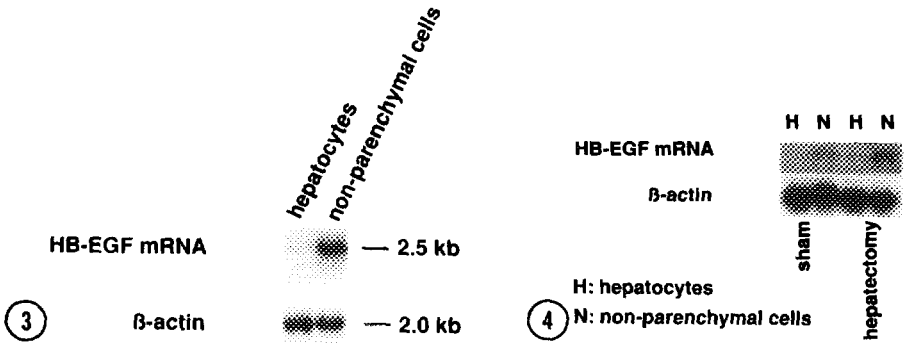

Fig. 3. HB-EGF gene expression in the hepatocyte and non-parenchymal cell fractions. Total RNA was isolated from hepatocytes and non-parenchymal cell fractions and then subjected to Northern blot analysis. Hybridization was carried out using a [^{32}P] labeled rat HB-EGF cDNA fragment.

Fig. 4. HB-EGF gene expression in the hepatocyte and non-parenchymal cell fractions on the 3rd day after hepatectomy. Hepatocytes and non-parenchymal cells were separated from the remnant liver of a hepatectomized rat or the liver of a sham-operated rat on the 3rd day after operation. HB-EGF gene expression in each fraction was assessed by Northern blot analysis.

presence of 0, 0.2, 2.0 or 20 ng/ml of HGF. As shown in Fig. 2, the curves displaying DNA synthesis became translocated in parallel to the upper side of the figure in association with the increase in the dose of coexisting HGF. This result indicates that HB-EGF stimulates the DNA synthesis of hepatocytes additively to the hepatotrophic effect of HGF.

HB-EGF Gene Expression in Hepatocytes and Non-parenchymal Cells. Hepatocytes and non-parenchymal cells were separated from a normal adult rat liver by *in situ* perfusion method (18) and subjected to the analysis for the expression of HB-EGF gene by Northern blot analysis (Fig. 3). An approximately 2.5 kb transcript of HB-EGF gene was detected in the fraction of non-parenchymal cells, but a very low level in that of hepatocytes.

HB-EGF Gene Expression During Liver Regeneration. On the3rd day after the operation, the level of HB-EGF mRNA in non-parenchymal cells from hapatectomized liver was about fivefold higher than that from sham-operated rat liver (Fig. 4) by a densitometric analysis. However, hepatocytes from both hepatectomized and sham-operated rat livers expressed very low levels of HB-EGF mRNA and showed no alteration in the levels.

Discussion

We demonstrated here that HB-EGF is a potent hepatotrophic factor and may act in the regenerating liver through a paracrine mechanism. Among various hepatotrophic factors, HGF is the most potent mitogen for

hepatocytes in primary culture, being 5 to 10-fold more potent than EGF (12). In our experiments, HB-EGF stimulated DNA synthesis of rat hepatocytes *in vitro* , of which the mitogenic activity was one-fourth of that of HGF on a molar basis (Fig. 1). The effect on DNA synthesis of HB-EGF was dose-dependent up to 30 ng/ml, but showed a reduction inversely at 100 ng/ml of HB-EGF. Because the signal of HB-EGF may be transduced *via* EGF receptors (2), the reduction of DNA synthesis at a high dose of HB-EGF could be explained by the down-regulation of EGF receptors.

Sequence analysis indicates that mature HB-EGF polypeptide, in addition to containing an EGF-like domain at the C-terminal site, has an N-terminus rich in lysine and arginine residues (2). This basic amino acid sequence confers heparin-binding activity which facilitates the binding of HB-EGF to EGF receptors and subsequently induces its mitogenic activity. Some hepatotrophic factors, for example, HGF, bFGF and a FGF are known to have heparin-binding property. The local concentration of heparin-binding growth factors are likely to be regulated, in part, by binding to heparan sulfate proteoglycan. Furthermore, recent studies have demonstrated that interaction of FGF and heparin or heparan sulfate proteoglycan are essential for the presentation and subsequent binding of the ligand to signal transducing receptors (22, 23). Thus, heparan sulfate proteoglycan appears to participate in regulating the functions of growth factors possessing heparin-binding property (24, 25). On this basis, although HB-EGF and HGF share different receptors, they might influence to their respective heparin-binding abilities, resulting in alternation of their mitogenic activity. However, with hepatotrophic activity, HB-EGF acted additively to the effect of HGF (Fig. 2), as was reported in the case of EGF (12).

All *in vitro* hepatotrophic factors are candidates for promoters of liver regeneration *in vivo* (26). Several lines of evidence indicate that HGF may act in the early phase of liver regeneration as a trigger for initiating DNA synthesis of hepatocytes after hepatectomy. However, expression of HGF mRNA is more markedly induced in the lung rather than in the remnant liver after hepatectomy (27, 28), suggesting that HGF may act mainly through an endocrine rather than a paracrine mechanism in liver regeneration.

HB-EGF gene transcript in normal adult rat has been reported to be detected in lung, spleen, skeletal muscle, heart and brain, and at very low levels in the liver (20). In this study, we clarified that the HB-EGF transcript was detected mainly in the non-parenchymal cells rather than hepatocytes, by separating the normal liver into non-parenchymal cell and hepatocyte fractions (Fig. 3). Physiologically important growth factors for liver regeneration can be expected to be produced in the remnant liver and modulated during liver regeneration. Therefore, to better assess the role of HB-EGF in liver regeneration, we investigated the modulation of HB-EGF

gene expression during liver regeneration. On the 3rd day after hepatectomy, the level of HB-EGF mRNA increased in the non-parenchymal cells from the remnant liver to fivefold of that from sham-operated rat liver (Fig. 4), suggesting a physiologically important role of HB-EGF in liver regeneration.

The levels of TGF-α gene transcripts have also been reported to rise after hepatectomy (7). A recent study indincates that an increase of TGF-α mRNA level occurred, starting at 6 hr after hepatectomy and peaking at twice the initial TGF-α mRNA level after 12-24 hr, suggesting that TGF-α may act an important role in liver regeneration *via* an autocrine mechanism (29). Because TGF-α would share EGF receptors to bind with HB-EGF, some interaction may occure between these growth factors during liver regeneration. Further studies are needed to clarify the precise mechanisms of these growth factors during liver regeneration.

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