Membrane-anchored Heparin-binding Epidermal Growth Factor-like Growth Factor Acts as a Tumor Survival Factor in a Hepatoma Cell Line*

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Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), which belongs to the EGF family, is produced as a membrane-anchored form (pro-HB-EGF) and later processed to a soluble form (sHB-EGF). It is known that high expression of pro-HB-EGF occurs in hepatoma tissues, although its biological meaning remains unknown. We established two types of hepatoma cell lines (AH68tc), which stably produce pro-HB-EGF and sHB-EGF, respectively. While sHB-EGF-producing cells (sHB-AH) showed rapid growth, pro-HB-EGF-producing cells (pHB-AH) showed markedly suppressed cell growth as compared with the parental cells. Transforming growth factor β or serum-starved conditions induced apoptosis of mock and sHB-AH as well as the parental cells, but not of pHB-AH. The resistance to apoptosis upon serum-starved treatment was correlated with an increase in the rate of the G1 phase in the cell cycle due to up-regulation of the cyclin-dependent kinase inhibitor p21. The mechanism underlying this resistance of pHB-AH to apoptosis was thought to be related to the prolonged half-life of the EGF receptor followed by continuous phosphorylation of the tyrosine residues. These observations demonstrate a unique function of pro-HB-EGF that is not observed for the mature form and show that pro-HB-EGF may act as a tumor survival factor in hepatoma cells.

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plastic progression, tumor growth, and resistance to cytotoxic anti-cancer agents (19). Some oncogenes, and tumor suppressor genes, such as bcl2 and p53, are involved in the prevention of apoptosis. While HB-EGF has been thought to be linked to hepatocarcinogenesis, there is no direct evidence of this. In this study, we transfected two forms of the HB-EGF gene; to produce pro- and soluble forms, into a rat hepatoma cell line and investigated their biological functions with emphasis on the relationships to apoptosis. We found that sHB-EGF stimulated the growth of hepatomas and pro-HB-EGF suppressed it through up-regulation of EGF-R and that hepatomas expressing high levels of pro-HB-EGF were resistant to apoptosis induced under several conditions. Possible mechanisms for these functions of pro-HB-EGF are proposed.

MATERIALS AND METHODS

Cell Culture—The rat hepatoma cell line, AH66tc (20), was donated by the Japanese Cancer Research Resources Bank and cultured in RPMI 1640 medium (Nikken BioMedical Laboratory, Kyoto, Japan) containing 100 μg/ml of kanamycin (Sigma), 50 units/ml of penicillin (Banyu Corp., Tokyo, Japan) and 10% fetal calf serum (FCS). Cell numbers were determined on a hemocytometer under a microscope (Banyu Corp., Tokyo, Japan) and 10% fetal calf serum (FCS). Cell containing 2.2 M formaldehyde and then transferred onto a Zeta-probe (Bio-Rad) and the hybridization probe was kindly provided by Dr. M. Nakanishi (Department of Geriatric Research, National Institute for Longevity Sciences). Northern blot analysis was performed according to the method reported by Yan and Goldberg with a small modification. Each band was measured by a densitometer.

Establishment of Soluble HB-EGF or Pro-HB-EGF Gene-transfected AH66tc Cells—An expression vector of sHB-EGF was constructed as follows. A point mutation to yield a stop codon (PstI-stop) was introduced into the transmembrane domain of mouse HB-EGF cDNA (21) by Sacchi (23). 20 and vector alone-transfected AH66tc cells (mock) were obtained, and transfection and selection was performed as to G418 (Sigma). The inserted sHB-EGF gene was directly sequenced, and then the site of mutation was confirmed. An expression vector of pro-HB-EGF was constructed by inserting the full sequence of mouse HB-EGF cDNA into the expression vector PE170.7 cells were cultured in 6-cm dishes and then incubated for 12 h. Cell surface proteins on the cells were biotinylated with 0.1 mg/ml of sulfo-NHS-biotin (Pierce) for 20 min on ice as described previously (27) and then cultured in 6-cm dishes with the medium in which each transfected was cultured. The cells were lysed with Nonidet P-40 buffer after 0, 1, 2, and 4 h. The cell lysates obtained with the Nonidet P-40 buffer described above were subjected to immunoprecipitation using anti-EGF-R. After SDS gel electrophoresis following by blotting onto a nitrocellulose membrane, the filter was incubated with avidin-peroxidase conjugates, an ABC kit (VECTOR Laboratories Inc.), and developed with an ECL kit (Amersham Corp.).

Effects of Immobilized HB-EGF on AH66tc Cells—250 ng of human recombinant HB-EGF (28) dissolved in 250 μl of 0.2% borate buffer, pH 9.5, was added to each well (16-mm diameter) of a 24-well plate (125 μl/cm² of 0.01% HB-EGF, 0.2% borate buffer, pH 9.5). The plate was incubated for 24 h at 4 °C. The HB-EGF solution was subjected to measurement of the immobilization efficiency of HB-EGF. The immobilization was calculated as an average of quadruplicate measurements of soluble HB-EGF activity in each of four independent wells. As judged on this measurement, 50 ng of soluble HB-EGF was immobilized. The wells were then washed five times with PBS, 5 × 10⁴ parental AH66tc cells were plated with 500 μl of DMEM, 2% FCS in each well. For estimation of the soluble HB-EGF activity of AH66tc cells, cells were plated with 500 μl of DMEM, 2% FCS containing an appropriate amount of HB-EGF in wells pretreated with 0.2 μl borate buffer, pH 9.6, alone. The cells were incubated for 3 days at 37 °C, trypsinized, and then counted with a Coulter counter (Coulter Corp., Hialeah, FL). The level of tyrosine phosphorylation on EGF-R was determined as follows: 6-cm dishes were pretreated with 125 μg/cm² of 0.2% borate buffer, pH 9.5, with or without 0.01% HB-EGF for 24 h at 4 °C. 1 × 10⁵ AH66tc cells harvested with PBS, 0.5 ml EDTA were plated with 1 ml of DMEM, 0.1% bovine serum albumin or 1 ml of DMEM, 0.1% bovine serum albumin, 5 ng of HB-EGF on an HB-EGF-immobilized or nonimmobilized dish, respectively. After 12.5 min of incubation at 37 °C, the cells were immediately harvested by pipetting and chilled in ice water. The chilled cells were immunoprecipitated with anti-EGF-R IgG, followed by Western blotting for detection of phosphorylated tyrosine residues on EGF-R as described above.

Analysis of Cell Cycle Distribution—AH66tc transfected in a subconfluent stage and a confluent stage were harvested with PBS containing 0.2% EDTA and fixed with 70% ethanol. The cells were treated with 1 mg/ml of RNase (Sigma) for 20 min and then stained with 100 μg/ml of propidium iodide (Sigma). After filtration of the cells through 50–70 μm nylon meshes, cell cycle distribution was measured with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Apoptosis on Treatment with TGF-β and Serum-starved Conditions—TGF-β treatment was as follows. 1 × 10⁵ AH66tc cells (mock) were plated in six-well plates with RPMI medium containing 10% FCS. After a 12-h incubation, the medium was changed to fresh medium, and the cells were incubated with 5 ng/ml TGF-β for the indicated times. sHB-AH and pHB-AH were treated with 5 ng/ml of TGF-β for 24 h. The
Establishment and Characterization of Two Types of HB-EGF Gene-transfected AH66tc Cells—Expression of HB-EGF mRNA in AH66tc transfectants. Total RNA extracted from parental AH66tc, mock, sHB-AH, and pHB-AH cells was analyzed by Northern blotting. The probe used was 32P-labeled mouse HB-EGF cDNA. β-Actin was used as a control. The numbers at the left indicate ribosomal RNAs.

DNA of each AH66tc transfectant was purified by proteinase K treatment, followed with phenol/chloroform. The DNAs were electrophoresed on a 0.9% agarose gel containing ethidium bromide. Apoptosis in situ was detected by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method. Briefly, sHB-AH and pHB-AH were cultured in an eight-well chamber slide (Lab-Tek, Nunc). After 5 ng/ml TGF-β treatment for 24 h, the cells were fixed with 3% paraformaldehyde for 20 min. The slide was rinsed three times with distilled water, submerged in terminal deoxynucleotidyl transferase buffer (Takara Shuzo, Shiga, Japan), and incubated in biotinated dUTP and terminal deoxynucleotidyl transferase at 37 °C for 90 min. Condensed DNA was visualized with a fluorescein isothiocyanate-labeled secondary antibody and was photographed through a fluorescence microscope. Detailed procedures were given in the manufacturer’s protocol of the in situ apoptosis detection kit (Takara Shuzo). Serum starvation was as follows. AH66tc transfectants were grown in medium containing 10% FCS until a confluent state was reached, and then the medium was changed to serum-free medium. Photographs were taken at 1, 7, and 14 days after the change to serum-free medium.

RESULTS

Cell Growth of HB-EGF Gene-transfected AH66tc—sHB-AH showed dramatically a rapid growth compared with the other two groups, pHb-AH and mock cells (Fig. 3), suggesting that HB-EGF stimulated the growth of AH66tc cells. Surprisingly, each type of pHb-AH showed much slower growth than mock cells. After 72 h of observation, the difference was prominent. Since pHb-AH also exhibit a juxtacrine activity, the mechanism of growth suppression of pHb-AH must be studied further.

Expression of EGF-R and Its Phosphorylation—HB-EGF is known to bind with EGF-R, followed by signal transduction.

The suppression of cell growth by pro-HB-EGF described above suggested that some modification occurred in EGF-R. To investigate this, we examined the expression of EGF-R by Western blotting (Fig. 4A). Protein expression of EGF-R was about 2–3 times increased in pHb-AH in comparison with the expression level in sHB-AH and mock. The data were almost the same under 70% confluent and 100% confluent conditions (data not shown). After EGF-R binds its ligands, signal transduction occurs with clustering of the receptor, followed by phosphorylation of the tyrosine residues, and then the receptor is internalized into the cytoplasm (10). The levels of phosphotyrosine residues on EGF-R were equally high in both sHB-AH and pHb-AH but low in mock (Fig. 4B). Since the protein level of total EGF-R was higher in pHb-AH than in sHB-AH, the net phosphorylation on EGF-R in sHB-AH and pHb-AH was almost the same. When exogenous HB-EGF was added to mock, a 6-fold increase in phosphorylation on EGF-R as compared with the untreated control was observed. In the case of sHB-AH and pHb-AH, this increase was 0.97 and 1.60-fold, respec-
tively. The effects of exogenous HB-EGF were not prominent in these cells, because EGF-R was already phosphorylated through the autocrine mechanisms of HB-EGF or the juxta- 
crine mechanisms of pro-HB-EGF produced by themselves. 
When phosphorylation on EGF-R was reexamined at 30 min 
after changing to fresh medium, the level of phosphorylation 
was markedly decreased in both sHB-AH and pHB-AH but 
not so changed in mock. The levels of decreases were significantly 
higher in sHB-AH than pHB-AH (80–90% versus 60–70%). 
These results suggested that EGF-R on sHB-AH was phospho-
rylated by HB-EGF in an autocrine manner and that phospho-
rylation of EGF-R on pHb-AH was decreased by unidentified 
mechanisms. This decrease may be caused by a cleavage of 
pro-HB-EGF due to a stress of changing the medium to a fresh 
one, which is similar to our previous observations (27). 
Although the signal via EGF-R was transmitted in pHb-AH, cell 
growth was suppressed. This inhibitory effect of pro-HB-EGF 
is controversial and may be involved with the increased EGF-R. 
To determine the details of the mechanism for up-regulation 
of EGF-R, the stability of EGF-R was investigated. Since EGF-R 
was internalized after ligand binding within a minute or so, 
levels of biotinylated EGF-R in sHB-AH were gradually con-
sumed in an autocrine manner and undetectable at 4 h (Fig. 
4C). In contrast, the level of biotinylated EGF-R in pHb-AH did 
not change for more than 4 h, suggesting that internalization of 
EGF-R followed by binding with pro-HB-EGF was extremely 
delayed. 

Growth Suppression in the Pro-HB-EGF Model—Although 
transfection of the pro-HB-EGF gene suppressed the growth of 
AH66tc cells with up-regulation of EGF-R, secondary effects 
including up- or down-regulation of other molecules such as 
adhesion molecules must be ruled out. To show the direct effect 
of pro-HB-EGF, we immobilized HB-EGF on a plate and then 
observed the growth of the parental AH66tc cells (Fig. 5). 
Although the addition of exogenous HB-EGF stimulated the 
growth of AH66tc cells, immobilized HB-EGF slightly sup-
pressed it (p < 0.03) compared with control. Tyrosine phospho-
rylation of EGF-R was observed upon both the addition of 
soluble HB-EGF and the addition of immobilized HB-EGF, but 
it was not observed in a control. These observations suggest 
that growth suppression of pHb-AH is a direct effect of pro-
HB-EGF itself. The same phenomenon was observed for human 
hepatoma cell lines HepG2 and Hep3B (data not shown).

Apoptosis Induced by Several Conditions—While HB-EGF 
has been thought to promote cell growth of hepatomas, specific 
roles of pro-HB-EGF in vivo have been suggested. Each AH66tc 
transfectant was treated with TGF-β or under serum-starved 
conditions. Treatment with TGF-β for 24–48 h induced apop-
tosis with a DNA ladder formation of AH66tc cells (Fig. 6A).

This was observed for sHB-AH but was completely inhibited in 
pHB-AH (Fig. 6B). While the DNA ladder formation seemed 
unclear in sHB-AH treated with TGF-β, some apoptotic cells 
were observed in sHB-AH by a TUNEL method, but not in 
pHB-AH (Fig. 6C). When these cells were treated with an 
anti-cancer drug, adriamycin or cisplatin, pHB-AH were also 
more resistant than the other two types (data not shown).

Furthermore, when three types of AH66tc cells were cultured 
under serum-starved conditions for a long time, many mocks 
and sHB-AH were dead after 1 week, but approximately 80% of 
the pHb-AH were still alive after 2 weeks (Fig. 7). When 
pHB-AH were cultured in a serum-starved medium containing 
5 mg/ml of exogenous HB-EGF, the number of surviving cells 
was decreased after 1 week, and the expression of EGF-R was 
slightly decreased (data not shown). As mentioned above, over-
expression of pro-HB-EGF enhanced the resistance against any
stress that we tested here.

Cell Cycle and Expression of p21 mRNA—While EGF-R has a stimulatory effect on the proliferation of a wide variety of normal and cancer cells (10), the addition of EGF to tumor cells with high levels of EGF-R causes growth inhibition through prolonged induction of the cyclin-dependent kinase inhibitor, p21 (WAF1) (29). Overexpression of pro-HB-EGF induced both increases of EGF-R and suppression of cell growth. To determine whether or not the mechanism of growth suppression is linked to p21 induction, the cell cycle distribution and mRNA expression of cyclin-dependent kinase inhibitors p21, p27, and p15 was investigated among three types of AH66tc cells. When they were in a sparse state, there were no differences in the cell cycle distribution among the three groups of cells. In a confluent state, however, the G1:G0 ratio in pHB-AH was extremely increased compared with in mock and sHB-AH (Fig. 8A). At this time, expression of p21 mRNA was little increased under confluent conditions in both mock and sHB-AH and dramatically increased in pHB-AH (Fig. 8, B and C), but expression of p27 and p15 mRNAs was not changed in the confluency of each transfectant despite different expression levels of p15 mRNA among each clone.

DISCUSSION

The present study demonstrates that ectopically expressed pro-HB-EGF has a specific role in the EGF-R-mediated signal transduction pathway for negative cell proliferation, which is completely the reverse effect of sHB-EGF. Pro-HB-EGF is expressed in various tissues that are not thought to be involved in cell proliferation, such as heart and vascular endothelial cells, suggesting that its original function in vivo is not always promotion of cell growth. Since pro-HB-EGF binds to EGF-R as well as sHB-EGF, the difference in their biological effects described here is thought to be due to how their signals after binding to the receptor are sent to an intracellular system. While both forms of HB-EGF induced the phosphorylation of EGF-R (Fig. 4B), its phosphorylation state may be prolonged in the case of pHB-AH, since the half-life of EGF-R in pHB-AH was longer than that of sHB-AH.

pHB-AH showed a marked increase of EGF-R protein compared with sHB-AH and mock cells (Fig. 4A), which depended on prolongation of the half-life of EGF-R (Fig. 4D). When the EGF-R ligands bind to EGF-R, internalization of the receptor followed by signal transduction occurs (10). In the case of pro-HB-EGF, it was thought that this internalization was delayed or inhibited and that the phosphorylation of EGF-R was continuous. Prolonged stimulation to EGF-R by pro-HB-EGF...
up-regulated the expression of p21 mRNA but not of other cyclin-dependent kinase inhibitors, p15 and p27 mRNAs (Fig. 8). Recently, cell growth arrest and induction of p21 in tumor cells expressing high amounts of EGF-R have been reported to be mediated by signal transducers and activators of transcription 1 (STAT1) (30). Whether STAT1 is induced by pro-HB-EGF has not yet been investigated.

Membrane-anchored forms of growth factors, which mostly belong to the EGF family, show the same growth factor activity as their soluble forms (31). The activity is not as strong as that of their soluble forms, and cell-cell contact is necessary for their signal transduction (27). The specific activity of membrane-anchored molecule is found in the c-Kit ligand, which is essential for hematopoietic cell proliferation and differentiation (32). Recently, Grell et al. (33) reported that membrane-anchored tumor necrosis factor was the prime activating ligand of the 80-kDa tumor necrosis factor receptor, which is the minor form of the receptor. These reports suggested the essential roles of membrane-anchored growth factors. The reasons for the different signal transduction between two forms of the c-Kit ligand are the prolonged activation and longer life span of the c-Kit protein (34). The mechanism of growth suppression by pro-HB-EGF is similar to the phenomenon in the case of the c-Kit ligand. An immobilized anti-Kit monoclonal antibody behaves like a membrane-anchored form of the c-Kit ligand rather than its soluble form (35). With a similar system, immobilized HB-EGF also inhibited the growth of AH66tc cells (Fig. 5).

What is the biological significance of overexpression of pro-HB-EGF in hepatomas? Hepatomas at the earliest stage do not always need rapid growth, but it is required for escape from various immune systems. Resistance against several factors may play a role in the early progression of hepatomas. A hepatoma overexpressing pro-HB-EGF showed strong resistance to several factors. Although the mechanism underlying resistance to TGF-β-induced apoptosis remains unknown, the resistance to serum-starved treatment is thought to be due to G1 arrest induced by up-regulation of p21 (Fig. 8). The same phenomenon of resistance to apoptosis was observed in myoblast differentiation with p21 induction (36). In some cases, cells under G1 arrest undergo apoptosis (15). However, pHBAH

**Fig. 7.** Morphological changes in AH66tc transfectants cultured under serum-starved conditions. After the three types of AH66tc cells had been grown to a confluent state, the medium was changed to new medium containing no FCS. Photographs were taken at 1, 7, and 14 days after the medium change (original magnification, × 100). We describe the results for mock 1, sHB-AH3, and pHBAH3 cells. The results for other clones were almost identical to those described here.

**Fig. 8.** Cell cycle distribution and expression of p21, p27, and p15 mRNAs. A, the cell cycle distribution of AH66tc transfectants in a subconfluent state or a confluent state was analyzed with a FACSscan flow cytometer (Becton Dickinson). B, total RNA extracted from AH66tc transfectants under 50% confluent conditions (lanes 1, 4, and 7), confluent conditions (lanes 2, 5, and 8), and 48 h after the attainment of confluent conditions (lanes 3, 6, and 9) were analyzed by Northern blot hybridization. Details were given under "Materials and Methods." A gel stained with ethidium bromide is shown for comparable amounts of RNAs. C, the density of each p21 mRNA band was measured with a densitometer. We present the results for mock 1, sHB-AH3, and pHBAH3 cells. The results for other clones were almost identical to those described here.
showed slow growth with a high percentage of the G₁ phase in the cell cycle. Greenberg et al. (37) reported opposing effects of extracellular signal-regulated kinase and c-Jun N-terminal kinase mitogen-activated protein kinase on apoptosis, using rat pheochromocytoma cells, PC-12. Whereas signaling via EGF-R is related to activation of extracellular signal-regulated kinase or c-Jun N-terminal kinase, there were no differences of extracellular signal-regulated kinase and c-Jun N-terminal kinase activities between sHB-AH and pHB-AH (data not shown). When pro-HB-EGF is cleaved by some unidentified proteases, sHB-EGF stimulates the growth of hepatoma cells. A large hepatoma that shows rapid growth cannot be caught only by the immune system. It is very interesting to speculate on whether the unidentified protease is activated in the large hepatoma. High expression of EGF-R was found in a subline of a human hepatoma cell line, and the mechanism underlying up-regulation of EGF-R is dependent on the stability of the receptor on the surface membrane (38). These authors stated that overexpression of EGF-R might contribute to the greater neoplastic potential of the hepatoma. The present study demonstrates that pro-HB-EGF is one of the factors that induces high expression of EGF-R. This is a unique function of pro-HB-EGF that the soluble form does not have. It may be related to very early development of hepatomas as a cell survival factor.

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