Contact-dependent Growth Inhibition and Apoptosis of Epidermal Growth Factor (EGF) Receptor-expressing Cells by the Membrane-anchored Form of Heparin-binding EGF-like Growth Factor*

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Heparin-binding epidermal growth factor-like growth factor (HB-EGF) transduces mitogenic signals through the EGF receptor (EGFR). There are two forms of HB-EGF, the membrane-anchored form (pro-HB-EGF) and the soluble form (sHB-EGF). We studied the biological activity of pro-HB-EGF by using a model in which pro-HB-EGF-expressing effector cells were co-cultured with EGFR-expressing target cells. The DER cell, an EGFR-expressing derivative of the interleukin-3-dependent hematopoietic 32D cell line, grows well in the presence of EGF or sHB-EGF without IL-3. When DER cells were co-cultured on a monolayer of Vero-H cells overexpressing pro-HB-EGF, growth inhibition and subsequent apoptosis were induced in the DER cells even in the presence of excess amounts of EGF or sHB-EGF. Such growth inhibition of DER cells was abrogated when specific antagonists for pro-HB-EGF were added in the culture medium or when direct contact of DER cells with Vero-H cells was prevented, indicating that pro-HB-EGF is involved in this inhibitory effect. Pro-HB-EGF-induced apoptosis of DER cells was also observed even in the presence of IL-3. This rules out the possibility of simple competition between soluble EGFR ligands and pro-HB-EGF. Moreover, 32D cells expressing EGFR mutant composed of the extracellular and the transmembrane domain of EGFR and the cytoplasmic domain of erythropoietin receptor did not undergo apoptosis by co-culture with Vero-H cells, indicating that the inhibitory signal induced by pro-HB-EGF-expressing Vero-H cells is mediated to DER cells via EGFR and that the cytoplasmic domain of EGFR is essential for pro-HB-EGF-induced apoptosis. From these results, we concluded that pro-HB-EGF has unique biological activity through cell-cell contact that is distinct from the activity of sHB-EGF.

In multicellular organisms, cells interact with each other to form and maintain the cellular society. A number of molecules of different functional categories play a role in this interaction. Growth factors, cytokines, and their receptors are important constituents of intercellular signaling. Until recently, growth factors or cytokines had been recognized to be soluble proteins usually found in blood or other body fluid, and many studies have been done to understand the function of these soluble factors. However, recent studies revealed that several growth factors and cytokines are synthesized as membrane-anchored proteins and that such membrane-anchored forms may have functions distinct from the soluble forms. For example, the epidermal growth factor (EGF)1 family of growth factors, tumor necrosis factor-α, colony-stimulating factor-1, c-Kit ligands 1 and 2, ligands for Eph family (B type of ephrins), and Fas ligand are synthesized as membrane-anchored forms, and these transmembrane forms are biologically active. In the case of transforming growth factor-α, the membrane-anchored form is mitogenically active and transduces the mitogenic signal to neighboring cells by cell-cell contact, termed “juxtacrine stimulation” (1–4). In the case of e-Kit ligand (5–7), ephrins (8), and Fas ligand (9), the membrane-anchored form is fully biologically active, but the soluble form shows limited or no biological function. Thus, it is now clear that the membrane-anchored forms of growth factors and cytokines are not only precursor proteins of the soluble factors but also biologically active proteins having unique roles in cell-to-cell interaction. Despite the importance of studying membrane-anchored growth factors, not many studies on the biological functions of membrane-anchored growth factors and the molecular mechanisms through which they act have been done due to the lack of suitable methods.

Heparin-binding EGF-like growth factor (HB-EGF) was first identified as a 20–22-kDa glycoprotein in a conditioned medium of macrophage-like cells (10). It is structurally a member of the EGF family, which encompasses a number of structurally homologous mitogens including EGF, transforming growth factor-α, vaccinia virus growth factor, amphiregulin, β-cellulin, epi-reulin, and neuregulin-1 and -2 (for a review, see Ref. 11). Similar to other EGF-family growth factors, HB-EGF binds to the EGFR, thereby inducing phosphorylation. More recently, HB-EGF has been shown to bind and stimulate HER4 as well as EGFR (12). HB-EGF can bind to heparin and cell surface heparan sulfate proteoglycans (11). Like other members of the EGF family, HB-EGF is synthesized as a transmembrane pro-

1 The abbreviations used are: EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; pro-HB-EGF, membrane-anchored form of HB-EGF; sHB-EGF, secreted form of HB-EGF; IL-3, interleukin-3; DT, diphertheria toxin; EGFR, EGF receptor; EpoR, erythropoietin receptor; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; FCS, fetal calf serum.
tein (pro-HB-EGF) and can be cleaved on the plasma membrane to yield soluble HB-EGF (sHB-EGF) (13, 14). sHB-EGF is a potent mitogen for a number of cells including NIH3T3 cells, smooth muscle cells, epithelial cells, keratinocytes, and kidney tubule cells (for a review, see Ref. 11).

Not only is pro-HB-EGF the precursor protein of sHB-EGF, but pro-HB-EGF itself is a biologically active protein. Pro-HB-EGF is known to be the specific receptor for diphtheria toxin (DT) and mediates endocytosis of the receptor-bound DT, resulting in entry of its A fragment into the cytoplasm (15, 16). As a growth factor, pro-HB-EGF has mitogenic activity to neighboring cells in a juxtacrine mode when pro-HB-EGF-expressing cells are fixed with formalin (17). A feature of pro-HB-EGF that is distinct from other membrane-anchored growth factors is that it forms a complex with other membrane proteins. CD9, a tetramembrane-spanning protein family, forms complexes with pro-HB-EGF (15, 18), thereby up-regulating the biological activity of pro-HB-EGF by protein-protein interaction (15, 17). In addition to CD9, pro-HB-EGF forms a complex with integrin αβ1 (19). Heparan sulfate proteoglycan(s) also binds to the heparin-binding domain of pro-HB-EGF (20). The fact that the complex composed of pro-HB-EGF, CD9, and integrin αβ1 co-localizes at cell-cell contact sites supports the notion that pro-HB-EGF plays a role in intercellular communication in a juxtacrine manner (19).

We previously showed that pro-HB-EGF-expressing cells pretreated with formalin, stimulate cell growth of neighboring cells in a juxtacrine manner (17). Although these results clearly indicate that pro-HB-EGF itself is a biologically active protein and affects the cell growth of neighboring cells, formalin treatment may modulate the biological functions of pro-HB-EGF. In order to examine the biological activities of pro-HB-EGF under more physiological conditions, we used a co-culture system in which intact cells expressing pro-HB-EGF were incubated with EGFR-expressing cells. The juxtacrine activities of pro-HB-EGF were evaluated by measuring the cell growth of the EGFR-expressing recipient cells. Using this system, we provide evidence of different biological activity of pro-HB-EGF from that of the soluble form.

**EXPERIMENTAL PROCEDURES**

**Materials**

Mouse anti-human EGFR mAb (LA-1) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Recombinant human EGFR was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Recombinant human HB-EGF, murine IL-3, and anti-human HB-EGF neutralizing antibody were purchased from R & D Systems (Minneapolis, MN). CRM197 was prepared as described previously (21). DNA ladder marker was purchased from New England Biolabs, Inc. (Beverly, MA). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody was purchased from Cappel (Durham, NC).

**Binding of 125I-Labeled DT**

Purified DT was labeled with Na125I (Amersham Pharmacia Biotech) as reported previously (22), using Sepharose beads conjugated with lactoperoxidase and glucose oxidase (Sigma) (23). Binding of 125I-labeled DT to Vero cells and Vero-H cells was carried out as described previously (24).

**Cell Culture and Transfection**

Vero cells, a cell line derived from the African green monkey kidney, and Vero-H cells, Vero cells stably expressing human pro-HB-EGF (13), were grown in Eagle’s minimum essential medium supplemented with nonessential amino acids MEM-NEAA supplemented with 10% FCS, penicillin G (100 units/ml), and streptomycin (100 μg/ml). Murine 32D cells and their derived cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS, penicillin G (100 units/ml), and streptomycin (100 μg/ml), and 5% WEHI-3 cell-conditioned medium as a source of IL-3 (25). All cell lines used in this study were determined to be mycoplasma-free. Contamination of mycoplasma would greatly affect the experimental results in the present study.

To obtain stable transformants expressing EGFR and EGFR/EpoR chimera, 32D cells were transfected with plasmids containing human EGFR cDNA (pTJNeo-EGFR) (26) and cDNA of EGFR-EpoR chimera (designated Δ108, obtained from Dr. A. Yoshimura, Kurume University (27), respectively, by electroporation using the Cell-Portor Electroporation System I (Life Technologies, Inc.). The transfected cells were cultured in the presence of 600 μg/ml G418, and clones of G418-resistant cells were isolated. The level of expression of EGFR in the stable transformants was determined by flow cytometry analysis as described below.

**Flow Cytometry**

Cells were allowed to react with anti-human EGFR mAb (LA-1) followed by incubation with FITC-conjugated goat anti-mouse IgG. The cells were then analyzed using an EPICS XL Flow Cytometer (Coulter Co., Miami, FL). The scatter window was set to eliminate dead cells and cell debris. For each measurement, 1 × 10^6 cells were analyzed.

**Co-culture Assay**

Effector cells (Vero cells or Vero-H cells) were placed in tissue culture dishes at a density of 1–2.5 × 10^5 cells/cm^2 and cultured in MEM-NEAA containing 10% FCS for 18 h. The cells were washed twice with RPMI 1640 containing 10% FCS. Then 1–10 × 10^6 cells/ml of target cells (DER cells or 32D-derived cell lines) were added to the monolayer, followed by incubation in RPMI 1640 containing 10% FCS. Recombinant EGF (0.3 nM), recombinant HB-EGF (1 nM), or recombinant IL-3 (1.5 μg/ml) was added to the culture medium. In some cases, Transwell (0.4 μm pore size, Millipore Corp., Bedford, MA) was introduced into the well to separate the recipient cells from the effector cells (Fig. 1). After co-culture for 24–48 h, the rate of DNA synthesis or degree of apoptosis of the target cells was determined.

**Measurement of DNA Synthesis**

The degree of DNA synthesis was determined by measuring the incorporation of [3H]thymidine into the DNA of the target cells by incubation with [3H]thymidine (74 kBq/ml) for 4 h. Then the target cells were harvested by gentle pipetting, and the radioactivity of the trichloroacetic acid-insoluble fraction was measured with a scintillation counter.

**Determination of Apoptosis**

The degree of apoptosis was measured by three methods.

**Detection of DNA Ladder**—Target cells were harvested by gentle pipetting, washed once with phosphate-buffered saline, and lysed in 300 μl of TE buffer (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100, pH 8.0). After centrifugation at 15,000 rpm for 20 min, the supernatant was subject to digestion with ribonuclease A (0.4 mg/ml) for 1 h at 37 °C, followed by incubation with proteinase K (0.4 mg/ml) for 1 h at 37 °C. The sample was then extracted with isopropl alcohol overnight at −20 °C. The precipitated DNA was resuspended in 20 μl of TE buffer (10 mM Tris, 10 mM EDTA, pH 8.0) and analyzed by electrophoresis on a 0.7 or 1.3% agarose gel in the presence of 0.5 μg/ml ethidium bromide.

**Detection of Cytoplasmic Fragmented DNA-Histone Complex** (28)—Target cells were harvested and analyzed by Cell Death Detection ELISAPLUS (Roche Molecular Biochemicals), according to the manufacturer's instructions.

**Detection of Phosphatidylinerse Expression on Cytoplasmic Membranes by Flow Cytometry** (29)—Target cells were harvested and analyzed for the Annexin V-EDC (Phosphatidylinerse) Detection Kit (Coulter Diagnostics, Inc., Cambridge, MA), according to the manufacturer's instructions. The percentage of apoptosis-positive cells was determined by the ratio of the cell number in the second peak of the stronger relative fluorescence intensity to the total cell number (1 × 10^5), which was set to 100%.

**RESULTS**

**Pro-HB-EGF Antagonizes the Mitogenic Activity of EGF and sHB-EGF to DER Cells**—In order to study the biological activity of pro-HB-EGF, we employed a co-culture assay in which target cell lines expressing EGFR were incubated with a monolayer of an effector cell line expressing pro-HB-EGF (Fig. 1). Vero-H cells (13), stable transformants of Vero cells that express pro-HB-EGF at a level 20 times higher than the parental Vero cells, were used as the effector cell line (Fig. 2a). As target cells, we used the DER cell line, which is a stable transformant of IL-3-dependent 32D cells expressing human EGFR (Fig. 2b).
Similar to other 32D-derived cell lines expressing EGFR (30), DER cells can grow in a medium containing EGFR ligands without IL-3. Fig. 2c shows that DNA synthesis in DER cells was maximally promoted by EGF at concentrations over 30 pM or by sHB-EGF at concentrations over 300 pM.

DER cells were co-cultured on a monolayer of Vero-H cells or Vero cells in a medium containing 10% FCS. After incubation for 48 h, [3H]thymidine was added to the medium, followed by incubation for 4 h. The DER cells were then separated from the Vero-H cells or Vero cells by gentle pipetting, and the amount of DNA synthesis was determined. Vero-H cells and Vero cells do not detach by this pipetting procedure. Furthermore, under these culture conditions, incorporation of [3H]thymidine into Vero-H cells or Vero cells was negligible; thus, co-culture does not affect the incorporation of [3H]thymidine into DER cells. As shown in Fig. 3a, only a little DNA synthesis was observed in DER cells under these co-culture conditions with the monolayer of Vero cells or Vero-H cells, compared with that of DER cells cultured alone in the presence of 0.3 nM EGF. However, growth of DER cells, monitored by DNA synthesis, was enhanced to a great degree when DER cells were separated from the Vero cells or Vero-H cells by Transwell. Although Vero cells and Vero-H cells express pro-HB-EGF molecules on their cell surface, they also constitutively secrete significant amounts of sHB-EGF into the medium (13). Therefore, DER cells would be affected by both pro-HB-EGF and sHB-EGF under these co-culture conditions, with detachment of Vero cells or Vero-H cells, compared with that of DER cells cultured alone in the presence of 0.3 nM EGF. However, growth of DER cells, monitored by DNA synthesis, was enhanced to a great degree when DER cells were separated from the Vero cells or Vero-H cells by Transwell. Although Vero cells and Vero-H cells express pro-HB-EGF molecules on their cell surface, they also constitutively secrete significant amounts of sHB-EGF into the medium (13). Therefore, DER cells would be affected by both pro-HB-EGF and sHB-EGF under these co-culture conditions, but affected by only sHB-EGF when separated by Transwell. The present results, therefore, imply the possibility that the secreted sHB-EGF in the medium is mitogenic, but the membrane-anchored form (pro-HB-EGF) does not efficiently support DER cell growth in a juxtacrine manner or rather antagonizes the mitogenic activity of sHB-EGF.

In order to examine whether pro-HB-EGF exhibits the antagonizing property to the mitogenic activity of soluble EGF receptor ligand, a co-culture assay was carried out in the presence of 0.3 nM of EGF in the culture medium. This concentration of EGF is enough to support DER cell growth (Fig. 2c). As shown in Fig. 3b, DER cell growth was markedly reduced in the presence of EGF when they were co-cultured with Vero-H cells, as compared with DER cells cultured alone in the same amount of EGF. Co-culture with Vero cells resulted in a slight reduction of DER cell growth. Moreover, the inhibitory effect of Vero-H cells and Vero cells on the growth of DER cells was abrogated when the DER cells were separated from the effector cells by Transwell, indicating that direct contact of DER cells with Vero-H cells is required for inhibitory activity.

The higher inhibitory activity seen by co-culture with Vero-H
cells than by co-culture with Vero cells, suggests that pro-HB-EGF is involved in this inhibitory activity. To further show that pro-HB-EGF is involved in the inhibitory activity of Vero-H cells, pro-HB-EGF activity was neutralized by CRM197. CRM197, a nontoxic mutant protein of DT (21), specifically binds to the EGF-like domain of HB-EGF; thus, CRM197 neutralizes the activity of human HB-EGF but not other EGF ligands (31). When CRM197 was added to the medium, it reduced the growth-inhibitory activity of Vero-H cells (Fig. 3c). CRM197 per se did not affect the rate of DER cell growth (data not shown). These results indicate that pro-HB-EGF is implicated in the inhibitory activity of Vero-H cells.

The amount of pro-HB-EGF expressed on the cell surface of Vero-H cells was about 1 x 10^6 molecules/cell, determined by DT binding. Therefore, the concentration of pro-HB-EGF in the present co-culture conditions is estimated at about 0.03 nM (0.03 pmol/2 x 10^4 cells/ml). Since the concentration of EGF added to this co-culture assay was 0.3 nM, this suggests that the inhibitory activity of pro-HB-EGF is unlikely to be due to simple competition with EGF.

**Pro-HB-EGF Induces Apoptosis of DER Cells**—The growth of DER cells was inhibited by co-culture on the monolayer of Vero-H cells. Since cell death was observed under a microscope in the co-culture of DER cells with Vero-H cells (data not shown), we examined whether apoptosis of DER cells is induced when their growth is inhibited by co-culture on Vero-H cells. Apoptosis was examined by detection of DNA ladder formation (Fig. 4a). Consistent with cell growth, a DNA ladder was observed in DER cells cultured in the absence of EGF, while a DNA ladder was not observed in DER cells cultured in the presence of EGF. When DER cells were co-cultured for 24 h on Vero-H cells, a DNA ladder was observed in DER cells even in the presence of EGF. CRM197 abrogated such DNA ladder formation of DER cells. A DNA ladder was also observed when DER cells were co-cultured on Vero-H cells in the presence of recombinant HB-EGF instead of EGF. A DNA ladder was not observed when DER cells and Vero-H cells were separated by Transwell.

The level of apoptosis was examined more quantitatively by measuring the amount of fragmented nucleosomal complex by enzyme-linked immunosorbent assay (28). As shown in Fig. 4b, the level of apoptosis of DER cells co-cultured on Vero-H cells was reduced by about 60% when DER cells and Vero-H cells were separated by Transwell and by 90% by the addition of CRM197.

To examine the percentage of the population of DER cells that undergo apoptosis when co-cultured with Vero-H cells, we also measured the level of apoptosis of DER cells by flow cytometry using the FITC-labeled Annexin V method (29). Under this assay condition, apoptosis was detected in 50% of DER cells cultured alone for 36 h without any EGFR ligands (data not shown). When DER cells were cultured with 0.3 nM EGF, only 2.5% of the cells were counted as apoptosis-positive (Fig. 4c). When DER cells were co-cultured with Vero-H cells, more than 15% of DER cells were apoptosis-positive even in the presence of EGF. When the contact of DER cells with Vero-H cells was abrogated by Transwell, the percentage of apoptotic DER cells was reduced to about 6%. When the activity of pro-HB-EGF was inhibited by CRM197 or anti-HB-EGF neutralizing antibody, the percentage of apoptotic DER cells was reduced to less than 6%. Neither CRM197 nor anti-HB-EGF neutralizing antibody per se affected the rate of apoptosis of DER cells (data not shown). These results indicate that pro-HB-EGF, or pro-HB-EGF-expressing Vero cells, have the capability of inducing not only growth inhibition but also apoptosis of DER cells, even in the presence of the soluble form of EGF or sHB-EGF. Pro-HB-EGF-mediated apoptosis was also observed when EGFR-expressing Ba/F3 cells were used as the target cells (data not shown), indicating that the apoptosis is not a

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**FIG. 3. Growth inhibition of DER cells by co-culture with Vero-H cells.** a, Vero cells and Vero-H cells do not support DER cell growth. DER cells (1 x 10^7) were cultured alone in the presence (bar 1) or absence (bar 2) of 0.3 nM EGF or co-cultured with 2 x 10^7 Vero cells (bar 3) or 2 x 10^7 Vero-H cells (bar 4). DER cells were allowed direct contact with the effector cells (−), or direct contact was hampered by Transwell (TW). After culture for 48 h, DER cells were collected by gentle pipetting, and the incorporation of [3H]thymidine into the DNA of DER cells was measured. The rate of DNA synthesis is indicated as a percentage of the rate of DNA synthesis in DER cells cultured alone in the presence of EGF. Data represent the mean ± S.D. obtained in three independent experiments. b, Vero-H cells antagonize growth of DER cells even in the presence of EGF. DER cells (1 x 10^7) were cultured alone in the absence (bar 1) or presence (bar 2) of 0.3 nM EGF or co-cultured with 2 x 10^7 Vero cells (bar 3) or 2 x 10^7 Vero-H cells (bar 4) in the presence of 0.3 nM EGF. DER cells were allowed direct contact with the effector cells (−), or direct contact was hampered by Transwell (TW). After culture for 48 h, the rate of DNA synthesis was determined. Data represent the mean ± S.D. obtained in three independent experiments. c, neutralization of growth-inhibitory activity of Vero-H cells by CRM197. DER cells (1 x 10^4 cells) were cultured alone (bar 1), or co-cultured with Vero-H cells (2 x 10^7) (bars 2 and 3) in the presence of 0.3 nM EGF (bars 1–3). CRM197, a nontoxic mutant protein of DT that specifically inhibits mitogenic activity of HB-EGF, was added at a concentration of 1 μg/ml (bar 3). After culture for 48 h, the rate of DNA synthesis was determined. Data represent the mean ± S.D. of the results obtained in three independent experiments.
After culture for 24 h, fragmented DNA was prepared from DER cell case of lane 7 obtained in five independent experiments. apoptosis determined by means of fragmented DNA-histone complex. Similar results were obtained from histograms obtained by flow cytometric analysis as described under “Experimental Procedures.” Similar results were obtained from four independent experiments are shown. M, DNA ladder marker.

FIG. 4. Apoptosis of DER cells co-cultured on Vero-H cells. a, detection of apoptosis by DNA ladder. DER cells (1 × 10⁶) were cultured alone (lanes 1 and 2) or co-cultured with 2 × 10⁶ Vero-H cells (lanes 3–7) with the following 0.3 nM EGF (lanes 2, 4, and 5), 1 nM sHB-EGF (lanes 6 and 7), 1 μg/ml CRM197 (lane 5), or no addition (lanes 1 and 3). In the case of lane 7, Vero-H cells and DER cells were separated by Transwell. After culture for 24 h, fragmented DNA was prepared from DER cell lystate and analyzed by electrophoresis on a 1.3% (left panel) or 0.7% (right panel) agarose gel. The results shown are representative data obtained in five independent experiments. M, DNA ladder marker. b, apoptosis determined by means of fragmented DNA-histone complex. DER cells (1 × 10⁶) were co-cultured with 2 × 10⁶ Vero-H cells in the presence of 0.3 nM EGF under the following conditions. DER cells were separated from Vero-H cells by Transwell (TW) or with the addition of 1 μg/ml CRM197 (CRM). After culture for 48 h, the fragmented chromatin fraction was prepared from DER cell lystate and analyzed using the Cell Death Detection Kit. The rate of apoptosis was determined by absorbance at 405 nm. Data are shown as specific absorbance values, which, were obtained by subtracting the background value determined by DER cells growing randomly in the presence of EGF. Similar results were obtained in two independent experiments. c, detection of apoptosis by the Annexin-V-FITC method. DER cells (1 × 10⁶) were cultured alone (bar 1) or co-cultured with 2.5 × 10⁶ Vero-H cells (bars 2–5) with 0.3 nM EGF and under the following conditions. Bar 4, in the presence of 1 μg/ml CRM197; bar 5, in the presence of 1 μg/ml anti-HB-EGF neutralizing Ab; bar 3, DER cells were separated from Vero-H cells by Transwell. After culture for 36 h, DER cells were harvested and analyzed using the Annexin V-FITC apoptosis detection kit. The data were scored from histograms obtained by flow cytometric analysis as described under “Experimental Procedures.” Similar results were obtained in two independent experiments.

specific phenomenon of the 32D cell lines.

Pro-HB-EGF Induces Apoptosis of DER Cells in the Presence of IL-3—We have shown that pro-HB-EGF induces apoptosis of DER cells even in the presence of an abundant amount of EGF or sHB-EGF. This inhibitory effect could be explained by the following possibility. Pro-HB-EGF might not have any biological activity, but it might inhibit the mitogenic activity of EGF and sHB-EGF by competitive binding to EGFR. To examine this possibility, we analyzed whether pro-HB-EGF induces apoptosis of DER cells that are cultured in the presence of IL-3. DER cells, derived from IL-3-dependent 32D cells, grow well in the presence of IL-3, and DER cell growth would not be affected by co-culture on Vero-H cells if the inhibitory effect of pro-HB-EGF is due to competitive binding activity. However, this was not the case. Apoptosis of DER cells was induced even in the presence of IL-3 by co-culture with Vero-H cells, although the degree of apoptosis was lower than that in the presence of EGF (Fig. 5). CRM197 and anti-EGFR mAb each inhibited apoptosis of DER cells co-cultured on Vero-H cells in the presence of IL-3, indicating that the apoptosis-inducing activity of pro-HB-EGF is not due to simple competition with soluble EGFR ligands to bind to EGFR. Anti-EGFR mAb did not affect the rate of apoptosis of DER cells when used alone (data not shown).

The Cytoplasmic Domain of EGFR Is Essential for Pro-HB-EGF-induced Apoptosis of 32D Cell Lines—Vero-H cells that overexpress pro-HB-EGF inhibit the growth of EGFR-expressing DER cells in a cell contact-dependent manner, and they consequently induce apoptosis of DER cells even in the presence of soluble mitogens. This inhibitory effect was neutralized by anti-HB-EGF antibody and CRM197, indicating that pro-HB-EGF is involved in this inhibitory activity. To examine whether this inhibitory activity of pro-HB-EGF is mediated to DER cells through EGFR, 32D cells expressing EGFR-EpoR chimeric receptor (32D/EGFR-EpoR) were used as the effector cells instead of DER cells. This chimeric receptor is composed of the extracellular and transmembrane domains of EGFR and the cytoplasmic domain of the erythropoietin receptor. 32D/EGFR-EpoR cells, similar to DER cells, grow well in the presence of EGF or sHB-EGF without IL-3 (data not shown). When 32D/EGFR-EpoR cells were co-cultured with Vero-H cells, their cell growth was not inhibited; thus, apoptosis was not induced (Fig. 6a). The level of expression of the chimeric receptor on the cell surface was low compared with that of EGFR on DER cells. However, the insusceptibility of 32D/EGFR-EpoR cells is not.
due to lower expression of the chimeric receptor, since apoptosis was also observed in another 32D cell line expressing EGFR (DER2), which had a much lower number of EGFR than the number of chimeric receptors on 32D/EGFR-EpoR cells (Fig. 6b). Furthermore, when 32D/EGFR-EpoR cells were cultured alone in the absence of EGF or IL-3, apoptosis was observed similar to DER cells and DER2 cells (data not shown). Thus, the difference of the responsiveness of EGFR-expressing 32D cells and the chimeric receptor-expressing 32D cells to apoptotic induction by Vero-H cells would not be due to the difference in level of expression of receptor molecules. A similar result was also observed when EGFR-EpoR-expressing Ba/F3 cells were used as the target cells (data not shown). These results indicate that the inhibitory activity toward cell growth and induction of apoptosis by pro-HB-EGF are transmitted to target cells through their EGFR and that the cytoplasmic domain of EGFR is required for transducing the apoptotic signal.

**DISCUSSION**

**Pro-HB-EGF-induced Growth Inhibition and Apoptosis**—In this study, we have shown that co-culture of EGFR-expressing target cells with pro-HB-EGF-expressing effector cells resulted in growth inhibition and consequent apoptosis in the target cells. Neutralization experiments on the inhibitory activity of Vero-H cells with CRM197 or anti-HB-EGF antibody indicated that pro-HB-EGF is involved in this inhibitory activity. This is also supported by the weak inhibitory activity of Vero cells expressing a lower amount of pro-HB-EGF than Vero-H cells. Moreover, experiments using Transwell indicated that direct contact of effector cells with target cells is required for growth inhibition and apoptosis. From these results, we concluded that pro-HB-EGF is involved in inhibiting the growth of target cells and induces apoptosis in a contact-dependent manner. The results also show that pro-HB-EGF has biological activity that is distinct from that of sHB-EGF.

Pro-HB-EGF did not induce apoptosis in 32D cells expressing EGFR-EpoR chimeric receptors. This indicates that the apoptotic activity of pro-HB-EGF-expressing cells is transmitted to the target cells via EGFR and that the cytoplasmic domain of EGFR is required for induction of apoptosis. Although recent reports have shown that HB-EGF binds to HER4 as well as EGFR (HER1) (12), at least HER1 is implicated in this inhibitory response. Both direct contact of the target cells with pro-HB-EGF-expressing cells and expression of EGFR with intact cytoplasmic domain on the surface of the target cells were required for induction of apoptosis of the target cells. This suggests that direct interaction between pro-HB-EGF and EGFR in a juxtacrine manner is necessary for the inhibitory activity of pro-HB-EGF.

Pro-HB-EGF-expressing effector cells inhibit the cell growth of target cells. However, it has not been clear whether this growth inhibition and apoptosis are due to innate activity of pro-HB-EGF itself or a secondary effect induced by pro-HB-EGF. In either case, however, it should be emphasized that interaction of pro-HB-EGF with EGFR is the initial trigger for growth inhibition and apoptosis. In addition, the present study does not discriminate between growth inhibition and apoptosis.

The cell lines used in our study are of 32D or Ba/F3 origin, which are both IL-3-dependent. Pro-apoptosis was concomitant with growth inhibition in these cells when cultured without IL-3 or EGF/F ligands. Thus, it is difficult to determine whether pro-HB-EGF induces growth inhibition, which consequently causes apoptosis, or if pro-HB-EGF generates a direct apoptotic signal.

**Pro-HB-EGF Generates a Different Signal from That of sHB-EGF**—As shown in this study, EGF and sHB-EGF stimulate the growth of DER cells, while pro-HB-EGF inhibits their growth even in the presence of EGF or sHB-EGF. Why do two forms of HB-EGF, the soluble form and membrane-anchored form, exhibit opposite biological effects?

One possible explanation might be that pro-HB-EGF is a mitogenically inactive molecule but that it inhibits the mitogenic activity of EGF and sHB-EGF by competitive binding to EGFR. However, this possibility is denied by the following results: 1) growth inhibition and apoptosis of target cells were observed in the presence of a sufficient amount of EGF (300 pm), while the concentration of pro-HB-EGF molecule in the co-culture conditions with Vero-H cells is estimated to be about 30 pm; 2) apoptosis of target cells was also observed in the presence of IL-3, which transduces mitogen signal to the target cells in an EGFR-independent manner; and 3) apoptosis induced by the co-culture with Vero-H cells was strongly reduced in target cells expressing EGFR mutant (EGFR-EpoR), although the EGFR mutant binds to HB-EGF.

The addition of EGF to the culture medium of A431 cells, a cell line that naturally overexpresses EGFR, causes growth inhibition (32). It is plausible that a too strong signal generated by EGFR causes growth inhibition of cells. In fact, constitutive and strong induction of Ras, Raf or mitogen-activated protein kinase can lead to apoptosis (33–35). The target cells used in this study also express a large amount of EGFR. Therefore, we can assume that the binding of pro-HB-EGF to EGFR causes...
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constitutive activation and/or hyper-activation of EGFR and its downstream signaling molecules. However, neither constitutive phosphorylation nor hyperphosphorylation of EGFR was observed in DER cells co-cultured with Vero-H cells (data not shown). In addition to EGFR phosphorylation, activation of mitogen-activated protein kinase was also examined by using the specific antibody to phosphorylated mitogen-activated protein kinase. However, significant activation of mitogen-activated protein kinase was not observed (data not shown). Thus, pro-HB-EGF-induced growth inhibition and apoptosis cannot be explained by hyperactivation of EGFR.

A more plausible explanation for pro-HB-EGF-induced growth inhibition and apoptosis is that interaction of EGFR with pro-HB-EGF may generate a downstream signal linked to growth inhibition and apoptosis. As shown here, pro-HB-EGF-induced growth inhibition and apoptosis were not seen in 32D cells expressing EGFR/EpoR chimeric receptor, despite the fact that these cells can grow well in the presence of EGF. These suggest a requirement of the cytoplasmic domain of EGF for the inhibitory activity of pro-HB-EGF and support the notion that a qualitatively different signal is generated from EGF by interaction with pro-HB-EGF.

What differences in the molecular nature of pro-HB-EGF and sHB-EGF are responsible for inducing an opposite biological response in EGF-expressing cells? The importance of the clustering of membrane-anchored ligands has generally been suggested. In the case of the B type of ephrin, the secreted form has no function, but clustering of the soluble form by antibody suggests. In the case of the B type of ephrin, the secreted form by antibody is important for its inhibitory activity to EGFR-expressing target cells, as shown in this paper. We have found that the saturation density of Vero cells is lower than that of the parental Vero cells, consistent with the notion that pro-HB-EGF is involved in arresting the growth of confluent cells. It has also been reported that hepatoma cells transfected with the construct expressing pro-HB-EGF, proliferate slower than the same cells transfected with the construct expressing only sHB-EGF (37). Thus, the growth-inhibitory activity of pro-HB-EGF may partly fill the role of the growth arrest mechanism seen in confluent cells or in so-called "contact inhibition" state.

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