HB-EGF and PDGF Mediate Reciprocal Interactions of Carcinoma Cells with Cancer-Associated Fibroblasts to Support Progression of Uterine Cervical Cancers

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Abstract

Tumor stroma drives the growth and progression of cancers. A heparin-binding epidermal growth factor–like growth factor, HB-EGF, is an EGF receptor ligand that stimulates cell growth in an autocrine or paracrine fashion. While elevated expression of HB-EGF in cancer cells and its contribution to tumor progression are well documented, the effects of HB-EGF expression in the tumor stroma have not been clarified. Here, we show that HB-EGF is expressed in stromal fibroblasts where it promotes cancer cell proliferation. In uterine cervical cancers, HB-EGF was detected immunohistochemically in the stroma proximal to the cancer epithelium. Proliferation of cervical cancer cells in vitro was enhanced by coculture with fibroblasts isolated from tumor tissues of patients with cervical cancer. Inhibition of HB-EGF function or treatment with platelet–derived growth factor (PDGF) inhibitors abrogated cancer cell growth enhanced by cervical cancer–associated fibroblast (CCF) coculture. Furthermore, tumor formation in a mouse xenograft model was enhanced by cotransplantation of CCF or mouse embryonic fibroblasts, but not with embryonic fibroblasts from HB-EGF–deficient mice. Conversely, conditioned medium from cancer cells induced HB-EGF expression in CCF. Mechanistic investigations established that PDGF was the primary factor responsible. Together, our findings indicate that HB-EGF and PDGF reciprocally mediate the interaction of cancer cells with cancer-associated fibroblasts, promoting cancer cell proliferation in a paracrine manner that has implications for novel combinatorial cancer therapies.

Cancer Res; 71(21); 6633–42. ©2011 AACR.

Introduction

Tumor-surrounding tissues or the tumor stroma influence the behavior of carcinomas (1). The tumor stroma consists of extracellular matrix (ECM) and various types of cells including fibroblasts, immune and inflammatory cells, and blood vessel cells. Invasive carcinoma is often associated with expansion of the tumor stroma and increased deposition of ECM. Cancer cells can alter their adjacent stroma to form a permissive and supportive environment for tumor progression by producing growth factors, proteases, protease inhibitors, and ECM molecules. Fibroblasts within the tumor stroma, referred to as "cancer-associated fibroblasts" (CAF), have a modified phenotype and a prominent role in the progression, growth, and spread of cancers (2–4). Crosstalk between cancer cells and the stromal fibroblasts is assumed. Cancer cells secrete various kinds of growth factors that stimulate their own proliferation in an autocrine manner. Some cancer cell–secreted growth factors, including TGFβ, platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF)-2, act as key mediators of fibroblast activation (2, 3, 5). The activated CAFs have a variety of effects on both cancer cells and the surrounding stroma. Their effects include altering the ECM environment, inducing an inflammatory response, and activating angiogenesis, as well as stimulating cancer cell proliferation and invasion by growth factor secretion. Thus, cancer–CAF interaction constitutes critical routes for cancer development and progression.

Cervical cancer is one of the most life-threatening diseases for women worldwide (6). Infection with human papilloma viruses (HPV) may be the first step of its carcinogenesis (7). Despite infection with HPV, most of the resultant precancerous lesions, termed squamous intraepithelial lesions (SIL), do not progress to an invasive carcinoma, suggesting that HPV does not act alone in the development of cervical cancer. Cervical cancer has a prominent stromal compartment. In the course of its progression, carcinoma cells invade the stroma, interact with fibroblasts, and change the stromal environment...
in a manner termed desmoplastic change or stromal reaction. Accordingly, the gene expression patterns of stromal cells are largely altered in cervical carcinogenesis (8). A study using a genetically-engineered mouse model suggested the presence of crosstalk between cancer cells and stromal fibroblasts, in which PDGF secreted from cancer cells stimulated PDGF receptor–expressing CAFs, leading to the induction of FGF-2 and FGF-7 production by CAFs which in turn stimulated angiogenesis and cancer cell proliferation (5).

Heparin-binding epidermal growth factor–like growth factor (HB-EGF) is an EGF receptor (EGFR) ligand, synthesized as a transmembrane precursor protein (pro-HB-EGF; ref. 9). Its extracellular domain is then cleaved by proteases, via the so-called ectodomain-shedding mechanism, which yields the soluble mature growth factor (sHB-EGF; ref. 10). sHB-EGF shows strong mitogenic and motility activity by acting through the EGFR (11–13). HB-EGF is involved in malignancy. Upregulation of HB-EGF has been reported in many types of malignant tumors (14–22). In the case of ovarian cancer, HB-EGF expression was increased in advanced cancers compared with that in normal ovaries and was associated with poor clinical outcome (14, 23). Although increasing evidence has accumulated on the role of HB-EGF in tumor cell growth and tumor progression (24), no study on the role of HB-EGF expressed in stromal fibroblasts has been reported.

Here, we show that HB-EGF is produced by stromal fibroblasts in uterine cervical cancer. Coculturing the human cervical cancer cell line ME180 with cervical cancer–associated fibroblasts (CCF) in vivo and in vitro indicated that stromal HB-EGF contributes to the enhanced proliferation of ME180 cells. On the other hand, PDGF produced in ME180 cells induced HB-EGF expression in fibroblasts, suggesting that these growth factors contribute to the reciprocal interaction of cancer cells and stromal fibroblasts.

Materials and Methods

Antibodies and reagents
Details about the antibodies and reagents are described in the Supplementary Materials and Methods.

Pathology scoring
HB-EGF immunostaining was simultaneously evaluated by 3 independent observers (including one gynecologic oncologist and one gynecologic pathologist), and a consensus was reached for each score. The positive reaction for HB-EGF was scored into 4 grades according to the following intensities of staining: 0, 1+, 2+, and 3+. The percentage of the positive staining area was also scored into the following 4 categories: 1 (0%–25%), 2 (26%–50%), 3 (51%–75%), and 4 (76%–100%). The sum of the intensity and percentage scores was used as the final staining score (25). The staining pattern of the frozen sections was defined as follows: 1, negative; 2–3, weak; 4–5, moderate; and 6–7, strong.

Cell culture
The cervical cancer cell line HeLa and squamous cell carcinoma (SCC)-derived ME180 cells were purchased from the American Type Culture Collection. The TCS and CaSki cell lines were obtained from the RIKEN BioResource Center. All the cell lines were passaged in our laboratory soon after receipt from cell banks, divided, and stocked in liquid nitrogen vessels. Each experiment was carried out using thawed cells without further authentication. Mouse embryonic fibroblast (MEF) cells from wild-type mice (MEF-HB+/-) or HB-EGF knockout (KO) mice (MEF-HB-/-) were obtained as described (26). All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin. CCF cells were obtained as described in the Supplementary Materials and Methods and cultured in DMEM supplemented with 20% FBS and antibiotics.

Coculture of ME180 cells and fibroblasts in 3-dimensional collagen gels

Coculture of ME180 cells and fibroblasts in 3-dimensional collagen gels. Cell culture inserts containing 3-dimensional collagen gels (13) using a Falcon cell culture insert (0.4-μm pore size membrane; Becton Dickinson Labware). HB-EGF–neutralizing IgG (10 μg/mL), control goat IgG (10 μg/mL), CRM197 (500 ng/mL), or other inhibitors were added to the collagen gel. The cell culture inserts containing 3-dimensional–cultured ME180 cells were set into 24-well dishes at day 0 and cocultured for 14 days. HB-EGF–neutralizing antibody and the viable cell numbers were counted (13). The effects of inhibitors on the growth of cervical cancer cells in coculture with CCF cells were expressed as a percentage of the control calculated by the following formula: total cell number of cervical cancer cells cocultured with CCF1 cells in the presence of a particular inhibitor divided by that of cervical cancer cells cocultured with CCF1 cells in the absence of a particular inhibitor × 100.

Mouse xenografting experiments
ME180 cells alone or mixtures of ME180 cells with either CCF cells or MEF cells were subcutaneously injected into the left flank of female athymic nude mouse at the cell numbers indicated in the figure legends. Unless otherwise stated, the tumor size was measured in 3 dimensions with calipers, and the tumor volume (mm3) was calculated using the formula: \( V = 0.52 \times \text{length} \times \text{width} \times \text{height} \).

Statistical analysis
Data are expressed as the mean ± SD. Statistical analysis of HB-EGF staining in various stages of cervical cancer progression was conducted via the \( \chi^2 \) test. The statistical significance of other experimental data was evaluated using one-way ANOVA. Values of \( P < 0.05 \) were considered significant.
Results

**HB-EGF expression in the stroma of uterine cervical cancers and its correlation with cancer progression**

Frozen sections of clinical samples were immunostained with an anti-human HB-EGF monoclonal antibody. In normal cervical tissues, the stroma was not immunostained, whereas the basal and parabasal epithelial layers were slightly reactive [Fig. 1A (i and ii)]. In cases with low-grade SILs (LSIL) and high-grade SILs (HSIL), faint immunostaining was observed in the stroma [Fig. 1A (iii–vi)]. However, in cervical cancer tissues, HB-EGF was strongly immunostained in the stromal region [Fig. 1A (vii–xii)]. An irrelevant antibody did not immunostain the stroma, whereas a similar immunostaining pattern was observed with other anti-HB-EGF monoclonal antibodies (ref. 27; data not shown).

To evaluate the relationships between stromal HB-EGF immunostaining and clinical features, the cases with positive staining in the stroma and epithelium were scored (Fig. 1B, Supplementary Tables S1 and S2). No HB-EGF immunostaining was recorded in normal cervical stroma, whereas moderate to strong stromal HB-EGF immunostaining was observed in 33% of LSIL cases, 66% of HSIL cases, and all cases of SCC. In contrast, all the tissues had negative to weak immunostaining in the epithelium. These results indicate that HB-EGF protein is increased in the stromal tissues during cervical carcinogenesis, suggesting that it is involved in its progression. In addition, stromal HB-EGF immunostaining was observed in adenocarcinoma tissue and small cell carcinoma tissue, as well as in SCC of the uterine cervix (Supplementary Table S1 and Fig. S1).

Strong HB-EGF immunostaining was observed in stromal fibroblasts localized at the invasion front of cancerous epithelium [Fig. 1A (x and xii)]. The antibody used recognizes both pro-HB-EGF and sHB-EGF. HB-EGF has strong affinities for heparin-like molecules and heparan sulfate proteoglycans.

**Figure 1.** Expression of HB-EGF in cervical cancer stromata. A, hematoxylin and eosin (H&E) staining (i, iii, v, vii, ix, and xi) and immunohistochemical staining (brown; ii, iv, vi, vii, x, and xii) of normal cervix (i and ii), LSIL (iii and iv), HSIL (v and vi), and invasive SCC (vii–xii). Frozen sections of clinical samples were immunostained with an anti-human HB-EGF monoclonal antibody. Stromal cells contacting the invasion front of cancerous epithelial cells were strongly stained (x, arrows). HB-EGF was expressed in cancer-associated fibroblasts (xii, arrows). Bars, 0.1 mm. B, scoring of HB-EGF staining. A significant difference in the stromal staining of HB-EGF was observed between normal cervix (NC), LSILs, HSILs, and SCCs (P = 0.011; χ² test). C, laser microdissection revealed that HB-EGF was mainly expressed in stromal cells. P1 to P5 were tissues from patients with SCCs, whereas P6 and P7 were from patients with adenocarcinoma [P = 0.009 for P1–P7 (n = 7); P = 0.015 for P1–P5 (n = 5); one-way ANOVA].
HB-EGF produced by fibroblasts contributes to ME180 cell proliferation in a coculture system

The effect of stromal HB-EGF on cervical cancer cell proliferation was examined by coculture of ME180 cells with CCF1 cells derived from cervical cancer–associated fibroblasts (Fig. 2A). Because growth promotion by HB-EGF is masked in common monolayer culture (13), ME180 cells were embedded in collagen gel using cell culture inserts. These inserts have 0.4-μm diameter pores in the membrane, so that soluble factors can pass between the 2 chambers. The proliferation of ME180 cells was largely enhanced by coculture with CCF1 cells compared with ME180 cells cultured alone. ME180 cells, cocultured with CCF1 cells, formed larger cell colonies than ME180 cells without CCF1 cells (Fig. 2B). The total number of ME180 cells produced when cocultured with CCF1 cells was about 5 times greater than that of ME180 cells cultured alone (Fig. 2C). The enhanced cell growth by coculture with CCF1 cells was almost abrogated by an anti-HB-EGF neutralizing antibody, whereas growth inhibition by an anti-HB-EGF antibody was not observed in ME180 cells cultured alone (Fig. 2C). CRM197, which inhibits the mitogenic activity of HB-EGF (28), inhibited the cell growth of ME180 cells in the coculture system, but did not significantly affect the growth of ME180 cells cultured alone (Fig. 2E). An EGFR inhibitor, erlotinib, also strongly inhibited the cell growth of ME180 cells in the coculture system but did not significantly affect the growth of ME180 cells cultured alone (Fig. 2F).

Enhanced proliferation by coculture with CCF1 cells was similarly observed in another cervical cancer cell line, CaSkii cells, and the enhancement was inhibited by CRM197 (Supplementary Fig. S2). Enhanced proliferation of ME180 cells was also observed in coculture with MEF cells. ME180 cells were cocultured with either MEF cells derived from a wild-type mouse (MEF-HB+/+) or MEF cells derived from an HB-EGF KO mouse (MEF-HB−/−). The total cell number of ME180 cells produced when cocultured with MEF-HB+/+ cells was about 8.5 times higher than that of ME180 cells cocultured with MEF-HB−/− cells (Fig. 2F). Thus, ME180 cell proliferation was enhanced by coculture with CCF or MEF cells, and HB-EGF produced by fibroblasts contributed to this enhancement.

The coculture experiments suggested that HB-EGF promotes ME180 cell proliferation by acting directly on ME180 cells through phosphorylation of their EGFR. However, it is also possible that HB-EGF acts on fibroblasts and indirectly enhances ME180 cell proliferation. To address this point, we carried out the following sets of experiments: First, addition of HB-EGF to the medium was found to enhance ME180 cell proliferation in the absence of CCF1 cells (Supplementary Fig. S3). Second, examination of EGFR expression and its phosphorylation revealed that ME180 cells expressed abundant EGFR and that its phosphorylation level was enhanced by coculture with CCF1 cells (Supplementary Fig. S4). Third, expression of dominant-negative (dn) EGFR in ME180 cells

Thus, sHB-EGF might be deposited at the ECM-enriched stromal region, even though HB-EGF is not synthesized in stromal cells. To clarify which cells produce HB-EGF, cervical cancer cells, or cancer-surrounding stroma cells, the transcription levels of HB-EGF in cancer epithelium and stromal regions were determined by quantitative reverse transcription PCR using microdissected frozen tissues as described in the Supplementary Materials and Methods. For these assays, tissues from SCCs and adenocarcinoma of the uterine cervix were examined. As shown in Fig. 1C, HB-EGF mRNA was higher in the stroma than in the epithelium in total cervical cancer tissues (n = 7, P = 0.009) and in SCCs (n = 5, P = 0.015), indicating that HB-EGF is mainly produced by stroma cells of cervical cancer lesions.
completely diminished the enhanced cell growth by coculture with CCF1 cells (Fig. 2G). These results indicate that HB-EGF directly enhances ME180 cell proliferation in coculture conditions through activation of EGFR on ME180 cells. HB-EGF binds to ErbB4, as well as to EGFR. However, ME180 cells had very low expression of ErbB4 (Supplementary Fig. S4). dnEGFR expression in ME180 cells completely inhibited the enhanced proliferation by coculture with CCF1 cells. Thus, ErbB4 is unlikely to contribute to this process.

**Cotransplantation of fibroblasts enhances tumor growth in ME180 cells**

To assess the contribution of fibroblasts and HB-EGF expressed in fibroblasts on tumor growth in vivo, we carried out cotransplantation experiments of ME180 cells with CCF or MEF cells into athymic nude mice. In the initial set of experiments, ME180 cells were used alone or mixed with various CCF cells (CCF1, CCF2, and CCF3) isolated from the cervical cancer stromata of different patients. Compared with ME180 cells alone, coinjection of ME180 cells with CCF1, CCF2, or CCF3 cells produced significantly larger tumors (Fig. 3A). The histology of the tumors showed a prominent stromal compartment, resembling SCC tissues of uterine cervical cancer (Fig. 3B). Immunostaining with an anti-human HB-EGF antibody showed the presence of human HB-EGF in fibroblasts surrounding the ME180 cells (Fig. 3B). Thus, the CCF cells enhanced the growth of ME180 cells in a mouse xenograft model to form tumors that were histologically similar to human uterine cervical cancer.

The enhanced tumor growth of ME180 cells by CCF cells was also examined by monitoring the tumor size using fluorescence imaging. Green fluorescent protein (GFP)-labeled ME180 (ME180/GFP) cells were subcutaneously transplanted with or without CCF1 cells into nude mice. Consistent with the results shown in Fig. 3A, fluorescence imaging confirmed that ME180 cell proliferation in the mouse xenograft model was enhanced by cotransplantation of CCF cells (Fig. 3C and D).

To examine whether stromal HB-EGF contributed to the enhanced tumor growth of the cervical cancer cells, ME180/GFP cells were mixed with either Ds-Red–labeled MEF-HB−/− (MEF-HB−/−/Red) or Ds-Red–labeled MEF-HB+/− (MEF-HB+/−/Red) cells and the mixtures were transplanted subcutaneously into nude mice. As shown in Fig. 3E and F, the tumors formed by ME180/GFP cells cotransplanted with MEF-HB−/−/Red cells were approximately twice as large as those formed by ME180/GFP cells cotransplanted with MEF-HB−/−/Red cells after 21 days. The latter tumors were similar in size to ME180 cells transplanted alone (see Fig. 3A and E), indicating that MEF-HB−/−/Red cells did not enhance ME180 cell tumor growth. These results suggest that HB-EGF expressed in fibroblasts played a major role in accelerating the growth of ME180 cells in this mouse xenograft model.

Both hematoxylin and eosin and immunofluorescence staining (Fig. 3F) indicated that Ds-Red–positive MEF cells (red) were incorporated into the ME180/GFP tumors (green) in both the MEF-HB−/−/Red and MEF-HB+/−/Red transplanta
tion experiments. Necrotic fields were more prevalent inside the tumor tissue when MEF-HB−/−/Red were cotransplanted, compared with MEF-HB+/−/Red cells, suggesting enhanced angiogenesis due to HB-EGF. Interestingly, the fluorescence intensity of ME180/GFP cells cotransplanted with MEF-HB+/−/Red cells tended to be higher than that of ME180/GFP cells cotransplanted with MEF-HB−/−/Red cells. GFP synthesis in the former combination might have resulted from a more abundant supply of nutrients and oxygen, given the enhanced angiogenesis in these tumors.

**PDGF secreted by cervical cancer cells induces HB-EGF expression in fibroblasts**

HB-EGF staining was preferentially observed at the stromal regions contacting the cancer invasion front. This suggested that cervical cancer cells might provide certain factors to the cancer-surrounding fibroblasts to induce the expression of HB-EGF and that fibroblasts could respond to these factors and upregulate HB-EGF mRNA synthesis. To test this hypothesis, conditioned media (CM) prepared from cervical cancer cell lines were added to the fibroblast primary cultures. When CM from CaSki or ME180 cells was added to CCF1 cells, the HB-EGF mRNA levels in CCF1 cells were markedly upregulated (Fig. 4A). Moderate upregulation was observed with CM from HeLa cells, whereas no upregulation was observed with CM from TCS or NIH3T3 cells (Fig. 4A). Enhanced HB-EGF mRNA expression by ME180 CM was observed in CCF2 and CCF3 cells as well as in CCF1 cells but not in ME180 cells themselves (Fig. 4B). Upregulation of HB-EGF mRNA expression was observed 3 hours after the addition of CM and peaked at 6 to 9 hours (Fig. 4C). Western blot analysis of CCF1 cell lysates confirmed the induction of HB-EGF protein synthesis by ME180 CM but not by control CM from CCF1 cells (Fig. 4D).

Examination of the expression levels of other EGFR ligands revealed that ME180 CM preferentially induced HB-EGF expression in CCF1 cells, although HeLa CM slightly induced epiregulin expression in these cells (Supplementary Fig. S5). These results indicate that CM from cervical cancer cell lines specifically induced HB-EGF among the EGFR family of growth factors in CCF cells.

To characterize the putative HB-EGF inducer in CM from ME180 cells, various inhibitors were tested to see whether they could prevent the induction of HB-EGF expression in CCF1 cells. FGF, insulin-like growth factor, PDGF, and TGFβ are known to be secreted by carcinoma cells to activate CAF cells. An FGE/VEGF receptor tyrosine kinase inhibitor, PD173074, did not inhibit HB-EGF expression in CCF1 cells, whereas the insulin-like growth factor receptor inhibitor AG1295 completely inhibited HB-EGF expression (Fig. 5A), suggesting that PDGF contributed to the HB-EGF induction in CCF cells. PDGF is composed of heterodimers or homodimers of 4 subtypes. To test whether PDGF could induce HB-EGF expression in CCF1 cells, various forms of PDGF were added to CCF1 cell cultures. PDGF-AB, PDGF-BB, and PDGF-DD induced HB-EGF expression in CCF1 cells, whereas PDGF-AA and PDGF-CC almost had no effect (Fig. 5B). Western blot analysis confirmed...
that ME180 cells produced PDGF-BB (Fig. 5C). Consistent with this result, CCF1 cells produced PDGFRβ (Supplementary Fig. S4). Moreover, tissue sections stained with anti-PDGF-B and anti-PDGFRβ antibodies indicated that these proteins were localized in cancer cells and the cancer-surrounding stromal region, respectively, in a mouse xenograft model coinjected with ME180 and CCF1 cells (Fig. 5D).

The PDGF receptor inhibitors AG1295 (Fig. 6A) and imatinib (Fig. 6B) prevented the enhanced proliferation of ME180 cells by coculture with CCF1 cells but did not inhibit ME180 cell proliferation when the cells were cultured alone (Fig. 6A and B). Coculture of CCF1 cells with ME180 cells resulted in PDGFRβ degradation in CCF1 cells (Supplementary Fig. S6), suggesting that the PDGFRβ activation by the ligand produced by ME180 cells was followed by ubiquitin-dependent degradation (29). Furthermore, the expression of dnPDGFRβ, but not that of dnEGFR, in CCF1 cells or MEF cells reduced the enhanced proliferation of ME180 cells by coculture with CCF1 or MEF cells (Fig. 6C). These results indicate that PDGF-BB does not contribute directly to ME180 cell growth but indirectly stimulates ME180 cell growth by inducing fibroblasts to produce HB-EGF. Fig. 6C also shows that dnEGFR expression in fibroblasts did not inhibit the enhanced proliferation of ME180 cells in coculture, confirming that EGFR ligands did not directly cause the activation of CCF or MEF cells.

In addition to the induced expression of HB-EGF by PDGF, HB-EGF also enhanced PDGF expression in ME180 cells. As shown by Western blot analysis (Fig. 5C), PDGF-BB synthesis...
in ME180 cells was enhanced when HB-EGF was added to cultured ME180 cells. These results indicate that PDGF secreted by ME180 cells induced HB-EGF expression in CCF1 cells, whereas HB-EGF produced by CCF1 cells enhanced ME180 cell proliferation and PDGF production. Thus, ME180 cells and CCF cells stimulate one another reciprocally through PDGF and HB-EGF expression (Fig. 7).

Discussion

Elevated levels of EGFR are associated with a poor prognosis for patients with cervical cancer, suggesting that the EGFR signaling pathway plays an important role in cancer progression (30–32). Here, we focused on HB-EGF expression and its growth-promoting activity in cervical cancer cells. Immunohistochemical and laser microdissection analyses revealed that HB-EGF was expressed in cancer-associated stroma but not in cancer epithelial cells. HB-EGF accumulated in CAF cells proximal to the cancer invasion front, although other types of cells might also express HB-EGF. Therefore, we investigated the role of stromal HB-EGF in cancer cell growth using in vitro coculture assays. ME180 cell and CaSki-cell...
proliferation was greatly enhanced in the presence of CCF cells. An anti-HB-EGF–neutralizing antibody or CRM197 inhibited these effects. CRM197 not only inhibits the mitogenic activity of HB-EGF but also prevents its protein synthesis in cells that express high levels of pro-HB-EGF (33). No effects were observed on protein synthesis in CCF1 cells and ME180 cells at the concentration of CRM197 used here. Growth promotion of ME180 cells was also observed by coculture with MEF-HB+ cells but not with MEF-HB−/− cells. Thus, HB-EGF expressed in CCF or MEF cells promoted ME180 cell proliferation in this coculture system. To the best of our knowledge, this is the first report that HB-EGF expressed in CAF promotes epithelial cancer cell growth.

Coinjection of CCF cells with ME180 cells enhanced tumor formation by ME180 cells in an athymic nude mouse model. Similarly, MEF-HB+ cells enhanced the proliferation of ME180 cells much more than MEF-HB−/− cells. These results indicate that HB-EGF expressed in stromal cells enhances cancer cell growth in vivo, consistent with the results of our coculture assays. However, as HB-EGF is known to be involved in tumor angiogenesis (34, 35), the growth-enhancing effect of CCF or MEF cells in vivo might have been partly caused by the indirect growth-promoting effect of HB-EGF via neovascularization.

Another point we stress is that cervical cancer cells can produce a factor that induces HB-EGF synthesis in fibroblasts. Because PDGF is expressed in cervical cancer cells, we suspect that it is a candidate, although other factors may also contribute to this process. PDGF did not induce cancer cell growth itself but induced HB-EGF synthesis in CCF cells. Consequently, AG1295 and imatinib inhibited tumor growth by ME180 cells cocultured with CCF cells. Interestingly, addition of HB-EGF to ME180 cells in the absence of fibroblasts...
resulted in increased PDGF production. These results indicate that cancer epithelial cells and stromal fibroblasts interact reciprocally through PDGF and HB-EGF to form a positive feedback loop. PDGF ligands produced by cancerous epithelial cells stimulate the PDGF-expressing stroma to upregulate the synthesis of FGFs, thereby promoting angiogenesis and epithelial-cell proliferation in a genetically-engineered mouse model (5). Therefore, not just a single growth factor system, but multiple systems could contribute to cancer–stroma interactions in cervical cancers.

Immunohistochemistry indicated that HB-EGF was localized at the stromal region proximal to the invasion front of cancerous epithelia. HB-EGF enhances cell motility as well as cell proliferation (11, 36), suggesting that HB-EGF contributes to the invasion and metastasis of cancerous epithelia. Membrane type 1 matrix metalloproteinase (MT1-MMP), which is involved in the degradation of ECM proteins, is believed to play an important role in cancer cell invasion and metastasis (37). MT1-MMP is expressed in cervical cancer cells, and its expression increases with cervical tumor progression (38). Invasion assays in collagen gel cultures showed that an EGF signal, in addition to MT1-MMP, is required for inducing keratinocyte invasiveness. We previously reported that MT1-MMP cleaves the N-terminal region of HB-EGF to convey enhanced mitogenic activity (39). Therefore, such processing of the N-terminal region of HB-EGF at the invasion front of cancer cells by MT1-MMP could be a cause of cervical cancer invasion.

There are several reports indicating a positive correlation between HB-EGF expression and cancer progression (24, 40, 41). In most of these cases, HB-EGF was upregulated in cancerous cells. Here, we showed that HB-EGF is produced in CAF and does not originate from cancer cells. The CAF-produced HB-EGF enhanced cancer cell proliferation. There is a report that HB-EGF plays a role in tumor progression in pericytes in a mouse model of pancreatic neuroendocrine tumorigenesis (35). Therefore, HB-EGF might contribute to cancer progression by various means. Although the development of therapeutic strategies targeting HB-EGF is only beginning, the combination of an HB-EGF inhibitor with other therapeutic agents, including PDGF inhibitors or MT1-MMP inhibitors, could lead to effective treatments for patients with refractory cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (16790901 to Y. Ueda, 17014057 to T. Enomoto, and 18591832 and 23430163 to E. Mekada). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Published OnlineFirst October 18, 2011; DOI: 10.1158/0008-5472.CAN-11-0034


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Cancer Res 2011;71:6633-6642. Published OnlineFirst October 18, 2011.

Updated version
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