ABSTRACT

Head and neck squamous cell carcinomas (HNSCCs) are characterized by a marked propensity for local invasion and dissemination to cervical lymph nodes, with distant metastases developing in 30–40% of cases. Overexpression of the epidermal growth factor receptor (EGFR/c-erbB-1) and/or its ligands and high levels of certain matrix metalloproteinases (MMPs) have been associated with poor prognosis. The aim of this study was to examine the effects of EGFR ligands on gelatinase expression and invasion in HNSCC cell lines. We tested epidermal growth factor (EGF), transforming growth factor α, betacellulin, heparin-binding EGF, and amphiregulin and measured expression of gelatinases MMP-9 and MMP-2 in an established squamous carcinoma cell line (Detroit-562) and in two cell lines newly derived from patients with head and neck cancers (SIHH-005A and SIHH-006). Incubation of the cell lines with EGF-like ligands up-regulated MMP-9 (but not MMP-2) expression as measured by semiquantitative reverse transcription-PCR in a dose-dependent manner, with the effects being most marked in cells with high EGFR levels and undetectable in cells with low levels. Maximum stimulation was obtained in a concentration range of 10–100 nM. In addition, we confirmed by zymography that gelatinolytic activity consistent with MMP-9 (Mr, 92,000) was up-regulated in parallel with increases in gene expression. Betacellulin (which binds both to EGFR and c-erbB-4 receptors) consistently increased MMP-9 expression and activation to a significantly greater degree than the other four ligands when tested at equimolar concentrations. In parallel with MMP-9 up-regulation, all EGF-like ligands increased tumor cell invasion through Matrigel in vitro Transwell assays. These activities were independent of ligand effects on cell proliferation. Antagonist (ICR926) or agonist (ICR9) anti-EGFR monoclonal antibodies, respectively, inhibited or potentiated MMP-9 activity and tumor cell invasion induced by all ligands. Furthermore, a monoclonal antibody that neutralizes MMP-9 activity (Ab1) also inhibited ligand-induced invasion of HNSCC. We confirmed that tumor cell lines used in these studies (and a larger series not reported here) generally expressed multiple c-erbB receptors and ligands. These results indicate that autocrine or paracrine signaling through EGFR potentiates the invasive potential of HNSCC via the selective up-regulation and activation of MMP-9. Furthermore, ligands such as betacellulin (which is commonly expressed in HNSCC), which can bind to and activate other c-erbB receptors, may be especially potent in this regard.

INTRODUCTION

HNSCC is the sixth most common malignancy and is a major cause of cancer morbidity and mortality worldwide. In the Western world, HNSCCs represent 5% of newly diagnosed cancers, but the incidence accounts for up to 40% of all malignancies in India and South East Asia. Worldwide, more than 500,000 new cases are projected annually. Whereas the management of HNSCC has improved, there is no evidence to suggest that therapeutic advances have resulted in increased survival rates. Indeed, the improvements in local control have led to an increase in presentation of distant metastases. The clinical observation that patients with HNSCC in comparable stages may run different clinical courses and may respond differently to similar treatments has yet to be adequately understood, but several potential prognostic markers have been proposed.

One such factor, the EGFR, is a Mr, 170,000 transmembrane phosphoglycoprotein whose overexpression has been shown to correlate with decreased disease-free survival and increased metastasis in tumors including HNSCC (3–5). EGFR has at least seven cognate ligands including EGF itself (6), TGF-α (7), BTC (8), HB-EGF (9), AR (10), and epieregulin (11). Expression of EGF and TGF-α in HNSCC has been documented by several groups (12, 13); however, expression of the other major ligands (BTC, HB-EGF, AR, and epieregulin) has not been explored.

Head and neck cancers are characterized by local invasiveness and a propensity for dissemination to cervical lymph nodes. Tumor invasion is a complex process that requires active interactions between the invading cell and the ECM and other stromal elements (14, 15). At least three coordinated processes are necessary for cell invasion: (a) changes in cell-cell and cell-matrix adhesion; (b) degradation of the ECM; and (c) cell migration. MMPs, a family of zinc-dependent endopeptidases, are key enzymes involved in these processes (16–18). Two members of the MMP family, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), have been shown to be highly expressed and strongly correlated with the malignant phenotype in HNSCC (19–21). MMP-2 and MMP-9 substrates include ECM components and collagen type IV, a key component of endothelial basal laminae. Their up-regulation and activation may therefore be directly linked to HNSCC angiogenesis, invasion, and metastasis.

Increasing evidence suggests a correlation between EGFR activation and some members of the MMP family in malignant keratinocytes (22, 23). However, the mechanisms regulating gelatinase expression in HNSCC are largely unknown. Previous studies demonstrated stimulation of MMP-9 expression by EGF/TGF-α in keratinocytes (24), colon cancer cells (25), and metastatic human breast cancer cells (26). The relative contributions of EGF-like ligands other than EGF and TGF-α to HNSCC gelatinase expression have not been clearly defined. We therefore compared the effects of five EGF-like ligands (EGF, TGF-α, BTC, HB-EGF, and AR) on gelatinase expression and invasive capacity in human HNSCC cell lines expressing different levels of EGFR.

MATERIALS AND METHODS

Cell Culture. Three cell lines were selected to represent a spectrum of EGFR expression and contrasting mitogenic responses to ligands. Human pharyngeal SCC line Detroit-562 was purchased from the American Type Culture Collection (Manassas, VA). Two new SCC lines, namely, SIHH-005A
and SHN-006, recently established in our laboratory from primary SCCs of tongue base and anterior tongue, respectively, were used between passages 5 and 10. Ethical permission was granted for the studies. All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. All HNSCC cell lines were maintained in DMEM containing 10% FCS, 2 mM l-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. All cells were routinely screened for and found to be free of Mycoplasma contamination using the Mycoplasma PCR Primer Set (Stratagene). Five EGF-like ligands were obtained from R&D Systems (Abingdon, United Kingdom). The two rat mAbs (ICR9 and ICR62) directed against different epitopes on the extracellular domain of the human EGF have been described previously (27, 28), as has the rat mAb against the extracellular domain of c-erbB-2 (ICR12; Ref. 29). Mouse anti-c-erbB-3 mAb (Ab-5, Clone H3.105.5) and anti-c-erbB-4 mAb (Ab-3, Clone H4.72.8) were obtained from NeoMarkers. The MMP-9 neutralizing mAb (Ab1) was obtained from CalBiochem (Nottingham, United Kingdom). Oligonucleotides were purchased from Genosys Biotechnologies (Cambridge, United Kingdom). Growth factor-reduced Matrigel, cell culture inserts (BD Biosciences, San Jose, CA), and all chemical reagents were obtained from Sigma (Dorset, United Kingdom) unless otherwise stated. Before the addition of growth factors, preconfluent cells were serum-starved in DMEM containing 0.1% BSA for 48 h. Cells were then incubated with human recombinant EGF, TGF-α, BTC, HB-EGF, or AR (0.1–100 nM) in DMEM/0.1% BSA for the time periods indicated. In the combined antibody experiment, mAbs (10–100 nM) were added at the same time as growth factors.

Flow Cytometric Analysis of EGF, c-erbB-2, c-erbB-3, and c-erbB-4 Expression. Near-confluent cells were trypsinized, washed with ice-cold PBS, resuspended in 1 ml of serum-free DMEM, and analyzed by using a FACScan (Becton Dickinson). First we determined the expression of four c-erbB receptors in head and neck cell lines used in this study by flow cytometric analysis. T47D cells, previously shown to express all four receptors, were used as a positive control. As shown in Fig. 1, the expression of c-erbB receptors was confirmed by flow cytometric analysis. T47D cells were used as a positive control. Each value is the mean of three independent experiments.

RESULTS

Expression of c-erbB Receptors and Five EGF-like Ligands in HNSCC Cells. First we determined the expression of four c-erbB receptors in head and neck cell lines used in this study by flow cytometric analysis. T47D cells, previously shown to express all four receptors, were used as a positive control. As shown in Fig. 1, the HNSCC cell lines expressed variable levels of all four c-erbB receptors.

Statistical Analysis. All proliferation experiments were performed in triplicate, and values are given as the means ± SE. For evaluation of statistical differences, Student’s unpaired t test was used. All experiments were performed at least twice, unless stated otherwise.

In Vitro Invasion. The in vitro invasion assay was used with the Transwell system, which is a widely used in vitro assay, to measure the invasion of cells through a porous membrane. This assay measures the ability of cells to invade through a matrigel matrix-coated inserts relative to the uncoated control inserts. Nonspecific rat or mouse IgG was used as a control in antibody studies. Cells under the same conditions were also set up in 24-well plates, stained with trypan blue, and counted to assess cell numbers and viability.

Expression of c-erbB Receptors and Five EGF-like Ligands in HNSCC Cells. First we determined the expression of four c-erbB receptors in head and neck cell lines used in this study by flow cytometric analysis. T47D cells, previously shown to express all four receptors, were used as a positive control. As shown in Fig. 1, the HNSCC cell lines expressed variable levels of all four c-erbB receptors.

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\text{Log value of fluorescent intensity} = \frac{B - A}{C - A} \times 100
\]

where A = A_{620\, nm} at start of incubation, B = A_{620\, nm} after treatment with ligands and/or mAbs, and C = A_{620\, nm} after incubation in medium alone.

Semiquantitative RT-PCR. The semiquantitative RT-PCR assay was carried out as described previously (30). Data regarding gene sequences were obtained from GenBank. Primers for PCR were designed based on strict criteria using the Primer Designer program version 2.0 (S&K Software, PA). Sequences of PCR primer sets for MMP-2, MMP-9, and β-actin were as described previously (30). Sequences of PCR primer sets of EGF, TGF-α, BTC, HB-EGF, and AR (in the 5'-3' direction) were as follows: (a) EGF, CACCTGGAAAAGACTCCTCAG (forward) and AGTGCAAGCATTGCAG (reverse); (b) TGF-α, CACATCTAGTCTGTTCTCCA (forward) and TAGGTGAACAGGGAGGCGGTCTG (reverse); (c) BTC, TTCATCTGGTGTGGCGAGAT (forward) and ACAGCATGTCGACACCCGA (reverse); (d) HB-EGF, ATGGAAGTGCTGCCGCGTGT (forward) and CAGTGCTGGTGGCTGAGG (reverse) and (e) AR, CTTCGAGACGGCGCCACACT (forward) and TATCAAGAGCGAGACACCA (reverse).

Conditioned Media Preparation. Cells were seeded in 96-well plates at 5 × 10^3 cells/well in DMEM with 10% FCS. After 24 h, the cells were washed twice with PBS, and medium was replaced with DMEM containing 0.1% BSA. Forty-eight h later, cells were washed with serum-free DMEM and incubated with 100 nM of DMEM/0.1% BSA with or without ligands (0.1–100 nM) for 48–72 h at 37°C. Conditioned media were collected, clarified by cold centrifugation at 2000 rpm for 5 min, and stored at −70°C until assays. Cells were trypsinized and counted in triplicate.

Quantitative Zymography. Conditioned media were analyzed under non-reducing conditions and separated in 11% SDS-polyacrylamide gels copolymerized with 0.1% (w/v) gelatin to demonstrate gelatinolytic activity (MMP-2 and MMP-9) as described previously (31). Duplicate gels were incubated as controls in buffer containing 10 mM EDTA to inhibit MMP activity. The gel was dried with a gel drier and scanned three times using an Arcus scanner, and the intensity of the bands (pixel unit) was measured by Quantiscan Image Analysis Software (Cambridge, United Kingdom). Pilot studies using serial dilution of standard MMP-2 and MMP-9 (Chemicon International) showed a good linear correlation between the amount of loading MMPs and the measured intensity in a range of 312.5 pg to 250 ng. Conditioned medium from the TPA-treated HT-1080 fibrosarcoma cell line served as a positive control and a standard for interexperimental variation.

Quantified in Vitro Invasion. The in vitro invasion assay used the Transwell system, which is a widely used in vitro assay, to measure the invasion of cells through a matrigel matrix-coated inserts relative to the uncoated control inserts. A nonspecific rat or mouse IgG was used as a control in antibody studies. Cells under the same conditions were also set up in 24-well plates, stained with trypan blue, and counted to assess cell numbers and viability.

Statistical Analysis. All proliferation experiments were performed in triplicate, and values are given as the means ± SE.
Using RT-PCR analysis, we then determined the mRNA expression of five ligands (EGF, TGF-\(\alpha\), BTC, HB-EGF, and AR) in the three HNSCC cell lines. We found that all cell lines consistently expressed TGF-\(\alpha\), BTC, HB-EGF, and AR, although to different extents (Fig. 2). Furthermore, we also observed frequent expression of these ligands in an additional 12 HNSCC cell lines and 5 tumor-derived fibroblast cell lines (data not shown).

**Effects of EGF-like Ligands and Anti-EGFR mAbs on HNSCC Proliferation.** We examined the biological activities of the five EGF-like ligands by comparing their effects on the growth of tumor cells. The proliferation of SIHN-006 (highest EGFR) was inhibited by all five ligands (Fig. 3A). At the highest concentration tested (100 nM), SIHN-006 cell proliferation was inhibited approximately 90% by BTC. Detroit-562 carcinoma cell proliferation was stimulated in the picomolar range (maximum at 100 pM) of all five ligands (Fig. 3B). This growth stimulatory effect was diminished upon treatment with higher concentrations of ligands. This biphasic growth response has been shown previously in other EGFR-overexpressing cell lines (32). It is generally accepted that the mitogenic response in such cell lines follows a “bell-shaped curve,” with stimulation of proliferation at low ligand concentrations, and inhibition at higher concentrations. The precise dose response for a particular cell line is likely to be a function of its receptor density and the level of autocrine growth factors produced because cells expressing high levels of receptor are exquisitely sensitive to very low ligand concentrations. The growth of SIHN-005A cells was stimulated by treatment with all five ligands in a dose-dependent manner up to 100 nM (Fig. 3C). This pattern of response has been found in other cell lines expressing low levels of EGFR (32).

The two rat anti-EGFR mAbs in this study were selected because mAb ICR9 binds to epitope A and increases the binding of EGF and TGF-\(\alpha\) to tumors expressing EGFR (27), whereas mAb ICR62, which binds to epitope C, has been shown under test to be the most effective mAb for inhibiting the binding of the five EGF-like ligands to tumor cells expressing EGFR (28). The effect of treatment with different ligands in the presence or absence of mAbs on the growth of Detroit-562 cells was examined. The results (Fig. 4) demonstrated that the mitogenic responses induced by 100 pm of each ligand (EGF, TGF-\(\alpha\), BTC, HB-EGF, or AR) were potentiated in the presence of mAb ICR9 and were reversed in the presence of mAb ICR62. The same agonist/antagonistic effects on growth were also found in SIHN-005A and SIHN-006 (data not shown). These results confirm that the effects of the five EGF-like ligands on cell proliferation are mediated directly via the EGFR.

**Effect of EGF-like Ligands on the Production of MMP-9 and MMP-2.** Under basal growth conditions, the levels of pro-MMP-9 secreted into the serum-free conditioned media paralleled the EGFR.
status of the cell lines (SIHN-006 > Detroit-562 > SIHN-005A; data not shown). However, the levels of MMP-2 secretion were approximately equal in all cell lines and were not linked to EGFR expression. Exposure of two cell lines with relatively high EGFR (Detroit-562 and SIHN-006) to all EGF-like ligands resulted in increased gelatinase activity in the culture medium as assayed by zymography. The stimulatory effect was much more evident in Detroit-562 than in SIHN-006, possibly due to the relatively low basal level of MMP-9 expression in Detroit-562 (only the results of Detroit-562 cells are shown in Fig. 5, A and B). The activity was observed consistently at $M_r$ 92,000, which corresponds to the latent form of MMP-9. The $M_r$ 72,000 gelatinase (MMP-2) was not induced by ligands over the 72-h time course examined. The level of MMP-9 secretion increased in a dose-dependent manner with increasing ligand concentrations. This effect was not seen in SIHN-005A cells expressing low EGFR (data not shown). When there was a marked up-regulation of MMP-9 (e.g., treatment with 10 nM BTC), a presumptive activated form of MMP-9 ($M_r$ 84,000) was also observed (Fig. 5A). Interestingly, differential up-regulation of MMP-9 was found with EGF-like ligands. For example, BTC was found to exert a much more potent effect on MMP-9 induction than other ligands (on a nanomolar basis). This ligand significantly up-regulated MMP-9 activity at 1 nm, with a peak activity at 10 nm; other ligands produced detectable increases at 10 nm, with a peak at 100 nm. All gelatinase activities were inhibited by EDTA or the zinc ion chelator 1,10-phenanthroline (data not shown), confirming that these were due to MMPs.

**Effect of EGF-like Ligands on the mRNA Expression of MMP-9 and MMP-2.** We then examined the effect of five EGF-like ligands on the mRNA expression of MMP-2 and MMP-9 in Detroit-562 cells. In a time course experiment, MMP-9 mRNA expression was shown to be gradually increased by all five ligands over a 24-h period (Fig. 6A). No detectable change in MMP-2 mRNA expression...
EGF-LIKE LIGANDS DIFFERENTIAL UP-REGULATE MMP-9

Fig. 6. Effect of EGF-like ligands on the mRNA expression of MMP-9 and MMP-2 in Detroit-562 carcinoma cells. A, graphic depiction of RT-PCR analysis of MMP-9 mRNA expression in ligand (EGF (●), TGF-α (▲), BTC (■), HB-EGF (□), or AR (●))-stimulated cells at different time points. All ligands did not increase the mRNA expression of MMP-2 above constitutive levels (data not shown). B, graphic depiction of MMP-2 (▲) and MMP-9 (■) expression in ligand-stimulated cells at 12 h compared to that in controls and normalized to β-actin. The results are representative of two or more independent experiments.

Effect of mAbs against EGFR on MMP-9 Activity. We first studied the effects of EGFR mAbs on MMP-9 under basal growth conditions (Fig. 7A). MAb ICR62 significantly reduced endogenous MMP-9 production in SIHN-006 cells, but the effects on Detroit-562 cells and SIHN-005A cells were hardly detectable due to the much lower levels of basal MMP-9 activity. In contrast, mAb ICR9 (which has been shown to increase the binding of EGF-like ligands to their receptor; Ref. 28) was found to up-regulate the MMP-9 activity of SIHN-006 and Detroit-562 cells. Furthermore, we found that secretion of MMP-9 into the culture media of Detroit-562 and SIHN-006 cells induced by treatment with all five EGF-like ligands (10 nM) was inhibited by the presence of ICR62 in a dose-dependent manner (illustrated by data from Detroit-562 cells treated with TGF-α, Fig. 7B). At a mAb concentration of 100 nM, the effect of ligand-induced MMP-9 secretion was completely abolished by ICR62. In addition, mAb ICR9 was found to increase the MMP-9 activity further. These results support the important role of the autocrine/paracrine EGFR signaling pathway in MMP-9 induction in HNSCC.

Effects of EGF-like Ligands on In Vitro Invasion. Invasion of cancer cells into artificial basement membranes is used as an effective in vitro model of invasiveness in vivo (33). Because up-regulation of MMP-9 expression might be expected to contribute to an invasive phenotype, we first compared the invasiveness of three cell lines under basal conditions. The invasiveness of HNSCC cells correlated with their EGFR status (data not shown). We then chose the intermediately invasive Detroit-562 cells to examine the effects of exogenous ligands. A 48-h incubation with five EGF-like ligands induced a dose-dependent stimulation of invasiveness over the range of 1–100 nM (Fig. 8). At a concentration of 1 nM, BTC showed a significantly higher induction of invasion than the other ligands (P < 0.001).

Effect of mAbs against EGFR or MMP-9 on In Vitro Invasion. Pilot studies showed that coinubation of TGF-α-treated Detroit-562 cells with mAb ICR62 significantly reduced invasion in a dose-dependent manner. With 100 nM ICR62, the stimulatory effect of TGF-α was completely abolished (data not shown). We then examined the inhibitory effects of ICR62 in combination with other EGF-like ligands. In accordance with the effect on MMP-9 activity, 100 nM ICR62 blocked the stimulatory effect of all five ligands (10 nM) on invasion, but the inhibitory effect was not significant under basal conditions, where invasion was low. (Fig. 9A). However, in a separate
of triplicate samples from two independent experiments. BSA in the absence or presence of increasing concentrations (0.1, 1, 10, and 100 nM) of reduced Matrigel matrix as a barrier. Tumor cells were incubated for 48 h in DMEM/0.1% a, TGF-
(EGF ( ), TGF-α ( ), BTC ( ), HB-EGF ( ), or AR ( ). Each value is the mean ± SE of triplicate samples from two independent experiments.

experiment with highly invasive SIHN-006 cells, treatment with ICR62 alone significantly reduced the percentage of invading cells from 71.18 ± 3.45% to 13.33 ± 0.88% (Fig. 9B). We also found this inhibitory effect in a larger panel of high EGFR-expressing HNSCC cells (30). This effect was not the result of growth inhibition because trypan blue exclusion showed that more than 90% of cells were still viable after a 48-h exposure to these concentrations of mAbs.

To determine the contribution of MMP-9 to tumor cell invasion, Detroit-562 cells were stimulated to invade by ligands in the absence or presence of a mAb that blocks the proteolytic activity of MMP-9 (Ab1; Ref. 34). Ligand-induced invasion was significantly inhibited by coincubation with the optimal concentration of mAb Ab1 (10 μg/ml; Fig. 9C). This inhibitory effect varied between 40.5–64.4%, depending on the ligand used. Additional studies with a higher concentration of this antibody (up to 1 mg/ml) did not exert a greater inhibitory effect, suggesting that other proteolytic enzymes may also contribute to invasion by HNSCC. We did not observe a significant inhibitory effect of mAb Ab1 on the weak invasive capacity of Detroit-562 cells under basal conditions (without exogenous ligand), although the inhibitory effect was found in a panel of highly invasive HNSCC cell lines including SIHN-006 cells (30).

**DISCUSSION**

The majority of HNSCCs show overexpression of EGF or TGF-α and the cognate receptor EGFR compared with normal squamous epithelia (12, 13). Previous studies have shown that high levels of EGFR and TGF-α expression correlate with aggressive behavior, increased metastasis, and decreased survival in human HNSCC (4, 5). Using RT-PCR analysis, we demonstrated here for the first time that mRNA of BTC, HB-EGF, and AR is also frequently expressed in HNSCC cell lines (and also in tumor-associated fibroblasts; data not shown). Thus, expression of all EGF-like ligands and their receptors may play a direct role in expression of the metastatic phenotype in HNSCC via autocrine or paracrine activation.

Recent attention has focused on a possible correlation between erbB signaling and the expression of MMPs (23, 35). MMPs assist tumor cell invasion and angiogenesis by degrading either cell surface-associated molecules or the subjacent matrix itself (17). In addition, MMPs may also release active growth factors such as TGF-α and basic fibroblast growth factor from cell-bound or ECM-sequestered precursors, further potentiating EGFR autocrine/paracrine signaling pathways and facilitating neoangiogenesis (36). Evidence supporting an important role for gelatinases (MMP-2 and MMP-9) in the invasive potential of malignant keratinocytes has been reported both in vitro and in vivo (19, 21, 24). EGFR and/or TGF-α have been shown to enhance the invasive and metastatic potential of various human cancer cells (37–39). Although the induction of gelatinases by EGF/TGF-α has been reported (23, 25, 26, 40), the role of other EGF-like ligands (BTC, HB-EGF, and AR) remains unclear. Our study therefore aimed to define the role of EGF-like ligands and their receptor in the regulation of these specific MMPs in HNSCC.

Under basal growth conditions, the level of MMP-9 expression and tumor cell invasion was found to parallel the EGFR status of the cell lines. Upon blockade of EGFR signaling by antagonistic EGFR mAb (ICR62), the basal level of MMP-9 expression of SIHN-006 cells was reduced, but it was difficult to detect an effect in Detroit-562 cells,

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**Fig. 8.** Effect of EGF-like ligands on Detroit-562 cells invasion. The invasive capacity of tumor cells was evaluated by the in vitro Transwell assay containing growth factor-reduced Matrigel matrix as a barrier. Tumor cells were incubated for 48 h in DMEM/0.1% BSA in the absence or presence of increasing concentrations (0.1, 1, 10, and 100 nM) of EGF ( ), TGF-α ( ), BTC ( ), HB-EGF ( ), or AR ( ). Each value is the mean ± SE of triplicate samples from two independent experiments.

**Fig. 9.** Effect of anti-EGFR mAb ICR62 or anti-MMP-9 mAb on in vitro invasion of Detroit-562 cells. A, Detroit-562 cells were incubated for 48 h in the absence or presence of 10 nM EGF, TGF-α, BTC, HB-EGF, or AR alone ( ) or with 100 nM mAb ICR62 ( ) or with 100 nM mAb ICR62 and 10 nM mAb Ab1 ( ). B, SIHN-006 cells were incubated for 48 h in the absence or presence of 30 nM EGFR mAb ICR62. C, Detroit-562 cells were incubated for 48 h in the presence of 10 nM ligand alone ( ) or with 10 μg/ml MMP-9 neutralizing mAb Ab1 ( ). A nonspecific rat IgG was used as a control. Each value is the mean ± SE of triplicate samples from two independent experiments.
where the level of MMP-9 was already low. This implies that the autocrine loop between EGFR and its endogenous ligands is responsible, at least in part, for the expression and production of MMP-9 in some highly invasive head and neck cells. We also observed this strong correlation between EGFR status, MMP-9 expression, and invasive capacity in a larger series of HNSCC cells (30).

Our unpublished results and previous studies (4, 5, 12, 13) showed that EGF-like ligands can be derived not only from tumor cells but also from connective tissue stromal cells. We found that concentrations of EGF-like ligands as low as 1 nm induced secretion of pro-MMP-9 with or without its active form in cell lines that have moderate to high levels of EGFR. This induction of MMP-9 expression was dose dependent (up to more than 40-fold at the optimal concentration of some ligands). The effect of EGF, TGF-α, BTC, HB-EGF, and AR on MMP-9 secretion was specific for this enzyme because MMP-2 expression was not affected with these ligands in any HNSCC cell line examined. This is consistent with the differential transcriptional regulation of MMP-9 and MMP-2 genes due to the presence of different promoter elements on the gelatinase genes (41). To the best of our knowledge, this is the first demonstration that all five EGF-like ligands up-regulate MMP-9 expression and that some ligands are more potent than others. SIHN-005A cells, which express low levels of EGFR, did not secrete MMP-9 in response to any ligands, suggesting that the MMP-9 induction effect by EGF-like ligands in HNSCC is critically dependent on receptor density.

The concentration of EGF-like ligands required to up-regulate MMP-9 expression and enhance invasion was at least a log higher (in the nanomolar range) than that required to influence cell proliferation (picomolar range) in EGFR-overexpressing HNSCC cell lines. In addition, the proliferation of tumor cells with low levels of EGFR can be stimulated by EGF-like ligands in the absence of effects on MMP-9 expression or invasion (e.g., SIHN-005A). What is more, although the ligands had different (and sometime inhibitory) effects on proliferation, they uniformly stimulated MMP-9 proteolysis and invasion of cell lines overexpressing EGFR independently of mitogenic effects. These data provide evidence that ligand-induced MMP-9 induction and tumor cell invasion are separable from the mitogenic response and perhaps activate different pathways downstream of EGFR or induce different durations of response.

The EGFR is the prototype of the type I receptor tyrosine kinases, which include three additional members: (a) c-erbB-2/neu/HER2; (b) c-erbB-3; and (c) c-erbB-4 (35). Abnormal expression of other erbB family members, apart from EGFR, has been shown in both HNSCC cell lines and clinical material (Refs. 42–44 and our results in the three cell lines used in this study). Some of the ligands that we examined bind to more than one erbB receptor and can activate receptors in trans via heterodimerization (45). Our finding that BTC consistently was the most active ligand in inducing MMP-9 may be explained by these differential patterns of receptor activation. Indeed, BTC has been reported to bind with a high affinity to erbB-4 and to stimulate the tyrosine phosphorylation of both EGFR and erbB-4 (46). Other possible explanations might be differences in the autocrine production of the ligands in each cell line and the differential binding efficiency of each ligand, leading to alternative endocytic routes of homo- and heterodimeric receptor complexes. We are currently exploring these possibilities.

In conclusion, our studies demonstrate for the first time that exposure of HNSCC cells overexpressing EGFR to five EGF-like ligands results in significant up-regulation of MMP-9 and that BTC produces the most potent effect. Furthermore, an inhibitory mAb directed against the external domain of human EGFR (ICR62) inhibited MMP-9 induction by all EGF-like ligands, whereas a mAb acting as an agonist (ICR9) further potentiated the ligand effects. Also, the autocrine production of MMP-9 was found to correlate with the EGFR status of HNSCC cells and was inhibited by ICR62 in at least one cell line. In addition, the observation that inhibition of MMP-9 activity impedes basal or ligand-mediated tumor cell invasion through a reconstituted basement membrane suggests that EGF-like ligands may play an important functional role in the process of HNSCC invasion by modulating expression of MMP-9. However, the incomplete inhibitory effect of anti-MMP-9 mAb suggests that other MMPs or other proteolytic enzymes (e.g., cathepsins, plasminogen activator) are also involved in the process of ligand-mediated invasion. Finally, these results add another potentially important therapeutic aspect to the use of mAbs against EGFR in terms of blocking the induction of specific members of the MMP family that play a major role in the process of invasion and metastasis.

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4 Unpublished observations.


Epidermal Growth Factor-like Ligands Differentially Up-Regulate Matrix Metalloproteinase 9 in Head and Neck Squamous Carcinoma Cells

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