Soluble MMP-14 produced by bone marrow-derived stromal cells sheds epithelial endoglin modulating the migratory properties of human breast cancer cells

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It has been proposed that epithelial cells can acquire invasive properties through exposure to paracrine signals originated from mesenchymal cells within the tumor microenvironment. Transforming growth factor-β (TGF-β) has been revealed as an active factor that mediates the epithelial-stroma cross-talk that facilitates cell invasion and metastasis. TGF-β signaling is modulated by the coreceptor Endoglin (Eng), which shows a tumor suppressor activity in epithelial cells and regulates the ALK1-Smad1,5,8 as well as the ALK5-Smad2,3 signaling pathways. In the current work, we present evidence showing that cell surface Eng abundance in epithelial MCF-7 breast cancer cells is inversely related with cell motility. Shedding of Eng in MCF-7 cell surface by soluble matrix metalloproteinase-14 (MMP-14) derived from the HS-5 bone-marrow-derived cell line induces a motile epithelial phenotype. On the other hand, restoration of full-length Eng expression blocks the stromal stimulus on migration. Processing of surface Eng by stromal factors was demonstrated by biotin-neutravidin labeling of cell surface proteins and this processing generated a soluble form that competes with membrane-bound betaglycan for TGF-β binding, and in this manner, inhibits the Smad2,3 pathway. Stromal MMP-14 abundance was stimulated by TGF-β secreted by MCF-7 cells acting in a paracrine manner. In turn, the stromal proteolytic activity of soluble MMP-14, by inducing Eng shedding, promoted malignant progression. From these data, and due to the capacity of TGF-β to regulate malignancy in epithelial cancer, we propose that stromal-dependent epithelial Eng shedding constitutes a putative mechanism that exerts an environmental control of cell malignancy.

Introduction

There is strong evidence supporting the participation of stromal cells in carcinoma progression (1). Particularly, it has been demonstrated that cellular stromal components accomplish a crucial role in breast cancer growth and development (2). It is well known that the stroma and epithelium maintain an active dialog that produces functional reciprocal consequences. Thus, stromal soluble factors can modify the invasive potential of carcinoma cells and epithelial factors are responsible for the stromal desmoplastic response that characterizes some type of tumors (3). Carcinoma-associated fibroblasts have been identified as an established source of tumor and angiogenesis-promoting factors with a yet unraveled role in tumorigenesis (4). The origin of these carcinoma-associated fibroblasts is still a matter of debate, but there is no doubt that they exert an important influence in the invasive properties of carcinoma cells in either the primary or the metastatic niches (5). Homing and further proliferation of tumoral cells in the metastatic site is also strongly affected by the secondary stroma and its ability to provide signals that favor metastatic colonization as it occurs in breast metastasis to bone (6).

Endoglin (Eng; CD105) is an integral membrane-bound glycoprotein, which acts as a transforming growth factor-β (TGF-β) auxiliary receptor (7). Eng is highly expressed in endothelial cells and it has been proposed to play a key role during tumor neoangiogenesis (8). Besides the well-recognized proangiogenic role of endothelial Eng, it has also been demonstrated that epithelial Eng is a crucial player in the carcinogenic phenomenon (9). Current evidence suggests that the main role of Eng in epithelial carcinogenesis is acting as a tumor suppressor molecule. Thus, a suppressor role for Eng has been postulated in esophageal, prostate and breast carcinomas (10–12), although in the latter type of cancer some controversy still remains, since a report shows that overexpression of Eng in MDA-MB-231 breast cell line enhances TGF-β-induced chemotaxis/invasion through matrigel, suggesting a proinvasive role for Eng in breast cancer (13). Conversely, we have shown previously that knockdown of Eng in murine-transformed keratinocytes stimulates basal and TGF-β-mediated ALK5/Smad2/3 signaling activity, a route that has been proposed to play a central role in epithelial–mesenchymal transition and the stimulation of invasiveness (14).

To date, data linking the development of metastatic disease with the expression of Eng are mostly limited to the role of Eng in tumor angiogenesis (15). However, an interesting and complementary research field has emerged with the identification in breast and colorectal cancer of elevated levels of a soluble form of Eng associated to a poor prognosis (16,17). In search of the mechanisms by which Eng modulates cell motility, it has been demonstrated that overexpression of Eng in mouse fibroblasts lead to decreased migration in chemotactic and wound-healing assays, as well as changes in the cellular morphology (18). Using a genetic model of prostate cancer that combined Eng haploinsufficiency with the TRAMP (transgenic adenocarcinoma mouse prostate) mouse, Romero et al. (19) have suggested that Eng is required for multiple aspects of carcinoma-associated fibroblast function including viability, endothelial cell recruitment and tumour-induced migration.

The existence of a soluble form of Eng in plasma of breast cancer patients has been associated with the development of a metastatic disease (17). Also, in another study that evaluated serum Eng in patients with a variety of tumors, increased serum levels of Eng were associated with enhanced neovascularization of metastatic tumors (20). In these cases, it is possible to assume that the higher concentration of serum Eng was the consequence of a proteolytic processing of membrane-associated Eng, which seems to suggest that membrane shedding is a plausible possibility to control Eng protein levels in tumor cells. In fact, using a colorectal carcinoma model, it has been demonstrated that membrane-bound matrix metalloproteinase-14 (MMP-14; MT1-MMP) present in the surface of colorectal carcinoma cells cleaves Eng in close proximity to the transmembrane domain (21).

Membrane-bound receptor shedding has been proposed as a functional mechanism to regulate growth factor signaling and usually it involves the proteolytic cleavage of the receptors’ extracellular domain (22). A similar phenomenon occurs with betaglycan, also a coreceptor for TGF-β, which after the proteolytic processing by MMP-14, generates a soluble form that competes with membrane-bound betaglycan for TGF-β binding, and in this manner, inhibits the cellular effect of the factor (23).

The aim of this study was to analyze mechanisms that mediate the stromal-induced shedding of Eng in a human breast cancer cell line.
We describe for the first time that a stromal proteolytic activity, identified as a soluble form of MMP-14 present in the conditioned medium (CM) derived from a bone marrow-derived cell line (HS-5) (24) cleaves membrane-bound Eng present in the weakly invasive MCF-7 cell line. We found that this paracrine Eng shedding process gives rise to an increase in the migratory potential of tumor cells that is reversed by the overexpression of membrane-bound Eng. In addition, we also studied the influence of epithelial-derived TGF-β1 on stromal MMP-14 expression. Our data suggest that stromal-derived MMP-14 is responsible for epithelial Eng shedding that, in turn, favors the expression of a more motile epithelial phenotype.

Materials and methods

Cell culture, cells and chemicals

The human cell lines mammary breast MCF-7 and bone marrow-derived HS-5, obtained from the ATCC (Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12 and DMEM (Invitrogen Carlsbad, CA), respectively, supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and maintained in a humidified atmosphere of 37°C, 5% CO₂. Human recombinant TGF-[beta]1 and BMP-2 were purchased from R&D Systems (Minneapolis, MN). SB431542 inhibitor was from TOCRIS Bioscience (Business Park Ellisville, MO). GM6001 (Ilomastat) was purchased from Chemicon (Temecula, CA). Protein A Sepharose CL-4B was from Sigma (St Louis, MO). Sulfo-NHS- LC-Biotin and Neutra/Avidin Sepharose were from Thermo (Waltham, MA). CM were obtained by culturing confluent HS-5 and MCF-7 cells for 48 h, in serum-free media (1 ml per 10 cm² of culture area). CM was clarified by centrifugation and used freshly.

Western blot and antibodies

After treatments, cells were resuspended in lysis buffer (50 mM N-[2-hydroxyethyl]piperazine-N’-2-ethanesulfonic acid, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 2 mM ethyleneglycol-bis(aminooxyethy)-tetracetic acid, 1% Triton X-100 and 10% glycerol) supplemented with Complete protease inhibitors (Roche, Mannheim, Germany). MCF-7 cells were stimulated with 10 μg/ml of TGF-[beta]1 and/or 20 ng/ml of TGF-[beta]2 was determined by a Bradford method (Fermentas, Maryland, MD). Protein extracts were heat denatured in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis loading buffer 4X (240 mM Tris–HCl, pH 6.8, 8% SDS, 40% glycerol and 2% mercaptoethanol). Equal amounts of protein from different treatments were resolved by SDS–polyacrylamide gel electrophoresis in 8% acrylamide gels and electrotransferred to polyvinylidene difluoride membranes using a buffer containing 24 mM Tris, 194 mM glycin and 2% methanol. Proteins were further analyzed using the ECL chemiluminescence detection kit (Amersham, Arlington Heights, IL). The immunoreactions were achieved by incubation of the membranes, previously blocked with a solution containing 5% bovine serum albumin in Tris-buffered saline and 0.05% Tween 20 (Sigma), with the following antibodies: rabbit anti human Eng (ab9228), rabbit anti human phospho Smad3 (ab29033) (Abcam, Cambridge, MA), mouse anti human Eng (M3257; DAKO, Glostrup, Denmark), rabbit anti phospho Smad2/3 (sc-11769), goat anti Smad2,3 (sc-6032), mouse anti Smad1 (sc-7965) and rabbit anti c-Myc (sc-789) were from Santa Cruz (Santa Cruz, CA), rabbit anti p-Smad 1,5,8 (#9511; Cell Signaling, Danvers, MA), mouse anti EGFR-R (25), mouse anti MMP-14 (MAB918; R&D Systems), mouse anti MMP-2 (Ab-3; Calbiochem, Cambridge MA), rabbit anti human Smad1 (PA5-19748) and mouse anti Smad3 (MA5-15663) were from Thermo scientific (Rockford, IL) and mouse anti beta actin (A5441; Sigma). Identifications of proteins in soluble fractions were performed as above in concentrated (10x) CM using Vivaspin concentrators tubes (GE Healthcare, Hatfield, UK). Densitometric analysis of western blot bands was performed using Molecular Imaging Software, version 4.0 of Kodak (Rochester, NY).

Cell migration assay

MCF-7 cell migration was studied using a 6.5 mm Transwell chamber with a pore size of 8 μm (Corning, Corning, NY). The transwell membranes were coated with 10% fetal bovine serum in culture media for 2 h at 37°C only on the lower chamber. Media conditioned by HS-5 cells (CM-HS-5) was prepared by incubating 5 x 10⁶ cells in a total volume of 8 ml of serum-free culture media for 24 h. When required, MCF-7 cells were pretreated with a 50% (vol/vol in DMEM) solution of CM-HS-5 for 24 h. Control and M-CM-HS-5–pretreated MCF-7 cells (6 x 10⁶) were resuspended in serum-free medium and seeded on the upper compartment of the chamber. In migration experiments in which MCF-7 Eng overexpressing cells were used, control and CM-HS-5–pretreated cells were transiently transfected with a pSuper-C vector containing a wild-type version of the human Eng gene tagged with myc (26) using the TransIT 2020 reagent (Mirus, Madison, WI) according to the manufacturer’s instructions. A group of cells were pretreated with 10 μM GM6001 (Iomastat), a broad-range inhibitor of metalloproteinases (MMPs), prior to the migration assay, as indicated. In experiments in which the TGF-[beta]-dependent migratory capacity was evaluated, MCF-7 cells were transfected with expression vectors encoding HA-tagged wild-type, constitutively active ALK5 (T204D) and kinase-deficient ALK5 (K225R) kindly provided by Dr I.Attisano (University of Texas Southwestern Medical Center, Dallas, TX) and used as a control. After TransIT 2020 transfection, MCF-7 cells growing on coverslips were incubated for 24 h, using the TransIT-siQUEST transfection reagent (Mirus) according to the manufacturer’s instructions. Migration values were determined by counting five fields (20x) per chamber after fixing and staining the migratory cells on the lower side of the membrane with 0.2% crystal violet in 10% methanol for 5 min (28).

Immunofluorescence microscopy

MCF-7 cells growing on coverslips were incubated with 50% CM HS-5 for 48 h. Then, cells were treated with 4% parafomaldehyde for 30 min at room temperature. Fixed cells were blocked with gelatin 0.2% in PBS for 1 h at room temperature and then incubated with mouse anti-human Eng antibody (M3257; DAKO) at 1:50 dilution for 16 h at 4°C. Once washed, cells were incubated with Alexa Fluor 594 goat anti-mouse (A-11055; Life Technologies, Carlsbad, CA) at 1:2000 dilution as a secondary antibody, washed and mounted with fluorescence mounting medium (DAKO). Confocal images of cells were acquired using the Confocal Laser Scanning Microscope-510 META (Zeiss, Oberkochen, Germany) and processed using LSM Image Browser software (Zeiss) and Adobe Photoshop 7.0. All images were taken using objective Plan-Apochromat 63x/1.4 Oil at 1024 pixel resolution.

Reporter assays

Reporter assays using TGF-[beta]-responsive promoters were performed as described previously (29), using p(CAGA)₅-luc (30) and p(hLUX-31) constructs, in the presence or the absence of TGF-[beta], as indicated. All transient transfection experiments were carried out using TransIT 2020 reagent (Mirus, Madison, WI) and used as internal control to correct for transfection efficiency and for normalization.

Biotinylation of cell surface Eng

MCF-7 cells (10⁶ per point) were incubated with increasing concentrations of MC-HS-5 for 48 h. After treatment, cells were detached with ethylenediamine- tetraacetic acid (0.53 mM) and the cell suspension was washed two times with ice-cold PBS pH 7.4 and each time centrifuged at 2000 rpm at 4°C. Sulfo-NHS-LC-Biotin (Thermo) was dissolved in PBS pH 8.0 (final concentration 2 mg/ml) and incubated with cell suspension for 5 h at 4°C with rotation. Unreacted biotinylating reagent was blocked two times for 15 min with an equal volume of 10 mM of glycine in PBS. Cells pellet was lysed in a buffer containing 50 mM N-[2-hydroxyethyl]piperazine-N’-2-ethanesulfonic acid pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 2 mM ethyleneglycol-bis(aminooxyethy)-tetraacetic acid, 1% Triton X-100 and 10% glycerol, supplemented with complete protease inhibitors (Roche), at 4°C. Next, the solution was sonicated twice and in a total volume of 10 mmole of glycine in PBS. Cells pellet was lysed in a buffer containing 50 mM N-[2-hydroxyethyl]piperazine-N’-2-ethanesulfonic acid pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 2 mM ethyleneglycol-bis(aminooxyethy)-tetraacetic acid, 1% Triton X-100 and 10% glycerol, supplemented with complete protease inhibitors (Roche), at 4°C. Next, the solution was sonicated twice and centrifuged at 13 000 for 10 min. Cleared supernatants were incubated overnight with 250 μl of Neutra/Avidin Sepharose (Thermo) at 4°C. NeutraAvidin-bound proteins were separated by centrifugation at 2500 r.p.m. for 5 min and pellets were washed four times with PBS. Then, pellets were resuspended with 100 μl of sample buffer 4x (240 mM Tris–HCl, pH 6.8, 8% SDS, 40% glycerol and 20% mercaptoethanol) boiled for 10 min and the supernatant was analyzed by SDS–polyacrylamide gel electrophoresis.

MMP-14 immunoprecipitation

To assess the role of stromal MMP-14 in the proteolytic capacity of CM-HS-5, MMP-14 was immunoprecipitated out of the HS-5 CM. Briefly, 8 μg of anti-human MMP-14 (MAB 918; R&D Systems) were incubated overnight with 250 μl of previously complexed Protein A Sepharose CL-4B (Sigma). Then, 1.0 ml of a solution of 50% CM (in DMEM) was subjected to two rounds of immunoprecipitation with the matrix-bound antibody. The resulting supernatant was used to stimulate MCF-7 migration as described above.
Quantitative PCR

Total RNA was isolated with Trizol (GIBCO) from CM MCF-7 and TGF-β1-treated HS-5 cells according to the manufacturer’s instructions. Reverse transcription to complementary DNA was performed using 1 μg of RNA from each sample using M-MLV reverse transcriptase and oligo d(Ts) (Promega) as primer, according to the manufacturer’s protocol. MMP-14 messenger RNA (mRNA) expression was assessed by real-time PCR using a LightCycler instrument (Roche, Mannheim, Germany). The reaction was performed using 100 ng of complementary DNA and LightCycler® FastStart DNA Master SYBR Green 1 kit (Roche) in a final volume of 20 μl. All the reactions were performed in duplicate and negative controls were included. The primers used were: MMP14F (forward), 5′ CCTG CTCCCAT AACACT 3′; MMP14R (reverse), 5′ GCCCTGAATGACCCTCCT 3′; GPDHF (forward), 5′ CAAAATCA AGTGGGGCGATGC TG 3′; GPDHR (reverse), 5′ TGTTGGTCA TGATGCTTCCAGCAT 3′. The level of MMP-14 expression was normalized using glyceraldehyde 3-phosphate dehydrogenase as loading control.

Statistical Analysis

Experimental data were presented as mean ± SD of experiments performed in triplicate. Data were subjected to a variance analysis, and comparison among groups was performed by Kruskal–Wallis/Dunn’s multiple comparison test and Mann–Whitney unpaired t-test. In both cases the GraphPad Prism 5 software (GraphPad Software, San Diego, CA) was used. A value of P < 0.05 was considered as a statistically significant difference.

Results

Media conditioned by HS-5 cell line stimulates the migratory capacity of MCF-7 cells by diminishing the abundance of Eng

Bone marrow constitutes one of the main metastatic sites for breast cancer cells (32). Therefore, we hypothesized that soluble factors produced by a bone marrow-derived cell line could modulate Eng expression and by this means, the migratory capacity of a breast cancer cell line. To evaluate this hypothesis, breast MCF-7 cells were pretreated with media conditioned by the bone marrow-derived HS-5 stromal cell line (CM-HS-5) and their migratory capacity was measured using a transwell assay. In these experiments, CM-HS-5 was not present during the migration assays. We also measured the expression of Eng in control and pretreated MCF-7 cells by western blot. Figure 1A shows that MCF-7 cells previously exposed to CM-HS-5 exhibit an enhanced migratory behavior. These cells also expressed a diminished amount of Eng. To verify that indeed the increase in migration was a consequence of a decrease in Eng expression, we subjected MCF-7 cells overexpressing a Myc-tagged Eng to the stimulus of CM-HS-5. Figure 1B shows that the forced expression of Eng causes the reversion of the CM-HS-5-dependent enhancement of migration, highlighting the role of Eng in epithelial migration. Although migratory values of Eng-overexpressing cells are apparently stimulated by CM, we found no statistically significant differences between basal and CM-stimulated migration in the presence of Eng overexpression (Figure 1B).

To confirm that CM HS-5 treatment diminished the abundance of Eng in MCF-7 cells, the surface-associated Eng was analyzed in control and CM HS-5-treated cells by confocal microscopy. Figure 1C shows that treated cells display a significantly reduced amount of immunoreactive Eng compared with control cells. To reinforce the hypothesis that decrease of Eng constitutes a more general mechanism to control cell motility, we performed the same migration experiment using a weakly invasive MCF-10 cell line and an invasion experiment using the strongly invasive MDA MB-231 cell line. In this case, cells migrate through a filter coated with 10 μg/ml of matrigel. In both cases, irrespective of the basal Eng expression, CM HS-5 increased migratory (or invasive) behavior and decreased the abundance of Eng (Figure 1D and E). With the purpose to evaluate whether CM HS-5 does not affect Eng transcriptional expression, we subjected MCF-7 to the stimulus of increasing concentrations of CM and evaluated mRNA expression by quantitative PCR. We found that the stromal stimulus showed a discrete effect on Eng expression at the transcriptional level that does not follow a dose–response pattern. These results rule out the possibility of a transcriptional regulation of Eng by the CM HS-S5 (Supplementary Figure S1, available at Carcinogenesis Online).

Pretreatment of MCF-7 cells with CM HS-5 enhanced Smad2/3 and inhibited Smad1,5,8 signaling

To investigate whether the decrease of Eng by CM HS-5 pretreatment has a consequence on TGF-β1-dependent Smad signaling, we analyzed the phosphorylation pattern of Smad2,3 and Smad 1,5,8 in intact and pretreated cells. Figure 2A shows that CM HS-5-pretreated MCF-7 cells displayed a diminished activation pattern of Smad1,5,8 that is more evident during the first 20 min of activation. In contrast, the same cells showed increased phosphorylation levels of Smad2,3, even at time zero and maintained this activation for 30 min after stimulation. These results suggest that the decreased amount of Eng in CM-HS-5-pretreated cells provoke a shift in TGF-β1 signaling enhancing the activation of the canonical Smad2,3 pathway. To confirm the inhibition of ALK1/Smad1,5,8 pathway by CM-HS-5 pretreatment, we performed the same experiment using BMP2, the primary ligand of this route (33). As Figure 2B shows, pretreatment of MCF-7 cells with CM-HS-5 caused an evident inhibition of BMP2-induced phosphorylation of pSmad1,5,8.

To assess whether this shift was consistent with the TGF-β1-dependent transcriptional activity, intact and CM-HS-5-pretreated cells were used to activate pCAGA (30) and pId-lux (31) reporter constructs which are specific for Smad3 and Smad1,5,8, respectively. We found that both basal and TGF-β1-stimulated pCAGA reporter activation was significantly stimulated in CM-HS-5-pretreated cells (Figure 2C). On the other hand, pId-lux reporter responded poorly to TGF-β1 stimulus in both experimental conditions (Figure 2D). However, when cells were stimulated with BMP-2, we observed a robust reporter activity which was significantly decreased in pretreated cells, supporting the view that the Smad1,5,8 pathway is inhibited by CM-HS-5. To test the differential involvement of both signaling routes in the acquisition of migratory capacity, we performed TGF-β1-stimulated migration experiments using MCF-7 cells that overexpress either a wild-type version of ALK5 or a dominant-negative mutant of the receptor. Also, to assess the influence of Smad1,5,8 signaling pathway, we expressed a constitutively active mutant of ALK1. As shown in Figure 2E, overexpression of ALK5 results in an enhanced migratory activity that is strongly counteracted by the expression of the dominant-negative version of the same receptor. In contrast, overexpression of constitutively active ALK1 (and its consequent Smad1,5,8 activation, see Figure 2E inset) reduced the TGF-β1-stimulated migration. Additionally, we also assayed MCF-7 migration upon Smad3 and Smad1 knockdown using a small interfering RNA strategy. As expected, knocking down of Smad3 provoked an inhibition of cell motility. However, knocking down Smad1 by a similar procedure does not provoke changes in cell migration reconfirming the notion that cell motility is under the control of ALK5-Smad2/3 pathway (Figure 2F and G).

To investigate whether the decreased expression of Eng is indeed responsible for the increased migratory capacity and the changes observed in TGF-β1 signaling, we performed migration and Smads activation experiments. We used MCF-7 cells whose basal level of Eng was downmodulated with a short hairpin RNA approach. As expected, shEng MCF-7 cells displayed an enhanced capacity to migrate and showed both a decreased activity of Smad1,5,8 pathway and an stimulation of the Smad2,3 route (Supplementary Figure S2, available at Carcinogenesis Online). When we compared the effect of either CM-treated or shEng MCF-7 cells on Smad2/3 activation, we found a similar result. Supplementary Figure S3, available at Carcinogenesis Online shows a quantification of the TGF-β1-dependent activation of Smad2/3 pathway in both experimental conditions that display a similar activation pattern. To assess whether the differential activation of both signaling pathways by their ligands also produces a differential migratory response, we evaluated MCF-7 migratory potential using TGF-β1 and BMP-2, which activate ALKS/Smad2,3 and ALK1/Smad1,5,8, respectively. As expected, TGF-β1
Soluble MMP-14 sheds epithelial endoglin behaved as a potent stimulator of MCF-7 migration, while BMP-2 inhibited cell movement. Migration rate reached after the stimulus of both agents suggest that, under these experimental conditions, the inhibitory stimulus of BMP-2 was not able to counteract the TGF-β1 stimulus (Supplementary Figure S4, available at Carcinogenesis Online).

Decreased MCF-7 Eng level after CM-HS-5 pretreatment is partially due to a metalloproteinase-dependent shedding process

It has been demonstrated that a shedding process may occur during Eng posttranslational regulation (21). To test if a proteolytic processing mechanism is involved in the CM-HS-5-dependent reduction of Eng and the associated migratory enhancement, MCF-7 cells were pretreated with CM-HS-5 in the presence or absence of 10 μM GM6001 (Iломastat), a broad-range inhibitor of metalloproteinase (MMP) activity. Figure 3A shows that inhibition of MMP activity reduced the CM-HS-5-induced migratory enhancement of MCF-7 cells. These results are in agreement with the expression of Eng after Iломastat treatment showing that Eng recovery after metalloproteinase inhibition was associated with a lower migration (Figure 3A, inset).

To confirm this hypothesis, MCF-7 cells were labeled at their surface with biotin (Biotin-NeutrAvidin procedure; see Materials and methods) and the avidin-bound Eng was identified by western blot analysis. Figure 3B shows that the abundance of cell surface Eng decreases, whereas soluble levels of Eng increase, in a dose-dependent manner after treatment with media conditioned by HS-5 cells. These results suggest that the decrease of membrane Eng is due to a proteolytic processing that leads to an increase in the soluble levels of Eng (sol-Eng). To verify that the soluble form of Eng found after CM-HS-5 treatment is of epithelial origin, we also analyzed the soluble Eng content of CM-HS-5. We found that HS-5 cells also produced immunoreactive soluble Eng, but at much lower...
Fig. 2. Pretreatment of MCF-7 cells with CM HS-5 enhances Smad 2,3 and inhibits Smad 1,5,8 signaling. (A) Western blot analysis of Smad1,5,8 and Smad2,3 phosphorylation relative to the total protein expression levels in MCF-7 cells preincubated or not (control) with CM-HS-5 and stimulated with TGF-β1 (5 ng/ml) at the indicated times. Values at the bottom of each lane are indicated relative to unstimulated control cells, in which an arbitrary value of 1 was given. (B) Western blot analysis of Smad1,5,8 phosphorylation relative to the total protein expression levels in MCF-7 cells preincubated or not (control) with CM-HS-5 and stimulated with 25 ng/ml of BMP2 at the indicated times. (C and D) TGF-β1 and BMP2-induced transcriptional activation of pCAGA (C) and pId1-Lux (D) luciferase reporter genes. Control and CM-HS-5-treated MCF-7 cells were transiently transfected with the reporter constructs and treated or not with TGF-β1 or BMP2, as indicated. Figures are representatives of three separate experiments. (E) ALK5 activation stimulates MCF-7 cell migration, whereas activation of ALK1 inhibits it. Cells were transiently transfected with the wild-type form of ALK5 (ALK5wt), a dominant-negative version of ALK5 (dnALK5) and an activated...
levels than the epithelial soluble (Figure 3B). Therefore, HS-5 soluble Eng levels were negligible compared with those of epithelial origin.

**TGF-β1 secreted by MCF-7 cells stimulates the production of MMP-14 by HS-5 cells**

The main hypothesis underlying our work is that stromal and epithelial compartments interchange soluble signals that modulate the course of tumoral progression. A previous work has shown that Eng shedding by a MMP-14 activity was a phenomenon that resulted of the close proximity of both molecules (Eng and membrane-bound MMP-14) in endothelial cell membrane (21). To assess if a similar process occurs in our system, the production of soluble and membrane-bound MMP-14 and MMP-2 (the most abundant stromal metalloproteinase) was measured in the epithelial and stromal cells used in our system. Figure 4A shows that HS-5 cells were the unique source of MMPs in their soluble and insoluble form, thus ruling out the possibility that Eng on MCF-7 cells was processed by endogenous MMPs of these cancer cells. Moreover, both soluble and insoluble forms of stromal MMP-14 show slightly increased levels when HS-5 cells were preincubated (16 h) with medium conditioned by MCF-7 cells. No change was observed in the production of stromal MMP-2 upon treatment with MCF-7-derived soluble factors (Figure 4A, left panel). In a previous work we have demonstrated that MCF-7 cells produce and secrete TGF-β1 (34). To test whether this soluble factor is involved in the production of stromal MMP-14, HS-5 cells were treated for 48 h with medium conditioned by MCF-7 cells pretreated or not with CM-HS-5 in the presence or absence of GM6001 (Ilomastat), as indicated. Inset shows the abundance of Eng protein in the different conditions, as determined by western blotting. (**P < 0.05) and (***) indicates statistically significant differences with P < 0.01.

To assess if epithelial TGF-β1 exerts its activity on stromal MMP-14 production through a transcriptional mechanism, we replicated the experiments of Figure 4B and 4C and measured the MMP-14 mRNA expression by quantitative PCR. Figure 4D shows that the expression of MMP-14 mRNA in HS-5 cells treated with CM-MCF-7 or TGF-β1 followed a similar response as the MMP-14 protein, suggesting a transcriptional regulation of MMP-14 under these conditions.

**Depletion of MMP-14 from the medium conditioned by HS-5 cells reverts its promigratory effect on MCF-7 cells**

To address whether MMP-14 present in CM-HS-5 was directly involved in the acquisition of motile properties by MCF-7 cells, we performed a subset of migration experiments in which MMP-14 present in CM was previously depleted by a serial immunoprecipitation procedure. Figure 5A shows that pretreatment of MCF-7 cells with increasing concentrations of CM-HS-5 gave rise to a dose-dependent enhancement of their migratory capacity. When the most concentrated solution (50% CM HS-5) was subjected to immunoprecipitation using an anti MMP-14 antibody, the stromal-induced migratory capacity decreased in direct proportion with the decline of residual MMP abundance in CM-HS-5, as demonstrated by western blot analysis (Figure 5B). The diminution of MMP-14 content in CM HS-5 has also a consequence in the abundance of TGF-β1 Eng. As Figure 5C shows, pretreatment of MCF-7 cells with increasing concentrations of CM HS-5 stimulate their migratory capacity in a linear relation with declination of cellular Eng abundance. Serial immunoprecipitation of MMP-14 allows the recovery of Eng abundance and the inhibition of the migratory potential. These results allow us to propose that MMP-14 present in the medium conditioned by HS-5 cells is responsible for shedding of epithelial membrane Eng and, in consequence, of the increased MCF-7 cells migration observed after preincubation of MCF-7 cells with CM-HS-5.

version of ALK1 [ALK1(+)] and then tested for cell migration as in Figure 1. Inset shows that ALK1(+) expressing cells display an activated Smad1 pathway. MCF-7 cells transfected with either a small interfering RNA for Smad3 (F) or Smad1 (G) and non-related small interfering RNA controls, were assayed for their migratory capacities in the presence or absence of 5ng/ml of TGF-β1 using the transwell method. Cells were allowed to migrate for 24h and migration rates were evaluated as described in Materials and methods. The expressions of total and phosphorylated Smad3 (F) and Smad1 (G) proteins treated or not with TGF-β1 for 30 min were determined by western blotting. (*), (**) and (***) indicates statistically significant differences with P < 0.05, P < 0.01 and P < 0.001, respectively.
Fig. 4. TGF-β1 secreted by MCF-7 cells stimulates the production of MMP-14 by HS-5 cells. (A) Stromal HS-5 and epithelial MCF-7 cells were preincubated or not with 50% CM-MCF-7 or CM-HS-5, respectively, for 16 h. Afterward, cells were washed and incubated for 24 h in serum-free medium. Then, media were collected and cells lysed. The expression of MMP-14 and MMP-2 was analyzed by western blotting in the collected media and lysates. (B) HS-5 cells were incubated with medium conditioned by MCF-7 cells (50%) for 48 h in the presence or absence of increasing concentrations of SB431542, and MMP-14 protein levels were analyzed both in the collected media and in the cell lysate by western blotting. (C) HS-5 cells were incubated in the presence of increasing
Soluble MMP-14 sheds epithelial endoglin

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Discussion

Interactions with stromal fibroblasts at the primary site of tumors create a favorable microenvironment supporting cancer growth, survival, evasion of immune surveillance and resistance to therapy (2). Results in the present work support the hypothesis that a stromal soluble form of the MMP-14 enzyme, acting in a paracrine manner, can modulate the migratory behavior of MCF-7 breast cancer cells by proteolytically processing the membrane-bound Eng present in these epithelial cells. This stromal stimulus on mammary carcinoma cell migration/invasion seems to be a more general response, because very similar responses to that of MCF-7 were obtained using a less migratory breast cell line, such as MCF-10, or a highly invasive MDA MB-231 cell line.

The production of proinvasive proteases (as MMPs) is a common feature in numerous carcinomas, including breast, stomach, lung, colon and head-and-neck tumors (35). MMP-14 represents a type I transmembrane MMP that is expressed by both stromal fibroblasts and carcinoma cells (36,37). Membrane-bound MMP-14 undergoes a complex process of shedding producing a series of soluble active and inactive fragments. Among them, the 50–53 kDa fragments correspond to the entire ectodomain of the enzyme and are active species. Moreover, the 44 kDa fragment is derived from a self-regulatory mechanism that reduces pericellular proteolytic activity and corresponds to an inactive form (38).

Increased levels of serum MMP-14 have been identified in breast cancer patients without clinically apparent metastases, suggesting that this MMP is produced through a shedding process attributable to an enhancement of proteolytic environment that characterizes tumor progression (39). In line with these data, our results show that a soluble form of MMP-14, generated by HS-5 bone marrow-derived cells, sheds the membrane-bound Eng on MCF-7 cell surface by a mechanism that involves a MCF7-derived stimulus mediated by TGF-β1. The inductive role of bone marrow stromal cells on the acquisition of the malignant properties of breast cancer cells has been evaluated in a variety of experimental models (40). Thus, the paracrine modulation of Eng expression in tumor cells by CM-HS-5, demonstrated here, may contribute to the growth and malignant transformation of tumors. In this regard, using different cellular models, silencing of epithelial Eng has been proposed as a relevant mechanism to control tumor cell growth, as well as cell migration and invasion in vitro (10,14). In a human mammary cells model, it has been hypothesized that Eng expression attenuates promigratory and proinvasive effects of TGF-β1 in cells containing active oncogenes; in this case, the expression level of Eng does not affect TGF-β1 signaling (11). Experiments performed in a mouse model of skin carcinogenesis have also demonstrated that shedding of surface-bound Eng, which in turn disrupts its TGF-β-signaling regulatory function is an event associated with malignant progression in vivo (14). Other authors provided evidences that Eng shedding constitutes a plausible regulatory mechanism of angiogenesis (21). These authors showed evidence that a surface-located MMP-14 was able to cleave Eng in endothelial cells, a phenomenon that may regulate the angiogenic potential in a tumor microenvironment.

Our experiments were performed using mainly a weakly invasive tumor carcinoma cell (MCF-7) that express Eng at low levels and does not express detectable amounts of MMPs either at the pericellular and soluble levels. Conversely, the stromal cellular component, represented here by the HS-5 cell line, is an active producer of MMPs, a common fact in many human peritumoral fibroblasts (37). O’Connor et al. (12) also demonstrated that this stromal cell line expresses Eng and that its level is under the control of prostatic soluble factors.

From our data we propose that stromal HS-5 cells produce and secrete a soluble form of MMP-14 that sheds epithelial Eng through a proteolytic process triggered by TGF-β1 secreted by the same breast epithelial cells (Figure 6).

Thereby, overexpression of epithelial TGF-β1, acting in a paracrine manner, could serve as a signal to produce a stromal proteolytic activity (MMP-14) that, in turn, stimulates the shedding of epithelial Eng, reinforcing the establishment of a malignant state. This potential mechanism is obviously restricted to weakly invasive carcinoma cells, which express detectable levels of Eng, and might represent an early step in the onset of tumor progression. In fact, in a study that evaluated by in situ reverse transcription–polymerase chain reaction the expression of MMPs in breast tumor samples, MMP-14 appears to have the highest levels of gene expression in the preinvasive tumors examined, suggesting a role for MMP-14 in the tumor invasion process (41). Similarly, in pancreatic ductal adenocarcinoma, a deadly concentrations of TGF-β1 for 16 h, and MMP-14 protein levels were analyzed by western blotting. (D) HS-5 cells were subjected to the same experimental conditions as in B and C. At the end of the incubation period, cells were lysed with Trizol and mRNA levels of MMP-14 were measured by reverse transcription–quantitative PCR.
human malignancy characterized by its desmoplastic nature, an increased expression of MMP-14 is correlated with an upregulation of TGF-β1, a factor that promotes MMP-14 expression (42).

In mechanistic terms, we propose that changes in migratory behavior resulting from Eng shedding may be associated with changes in TGF-β signaling. Previous work has proposed that, as a result of the extracellular and cytoplasmic interactions of Eng with ALK5 and ALK1, the latter route is potentiated (9). Our data shows that, after pre-incubation of MCF-7 with CM-HS-5, the expression of surface-bound Eng declines and consequently, the Smad1,5,8 pathway is inhibited, whereas Smad2/3 phosphorylation is stimulated. In many cellular systems it has been demonstrated that TGF-β1-induced activation of Smad3 constitutes a critical step for cellular events that play a key role in the induction of cell migration (43,44). Our data, showing that cell migration is blocked by both overexpression of a dominant-negative version of ALK5 or knocking down Smad3, reinforce the importance of ALK5/Smad2/3 activation in cell motility (Figure 2E and Fig.S2A in Supplementary Figure S2A, available at Carcinogenesis Online).

Prior studies performed in prostatic cell lines have shown that the loss of Eng protein expression is associated with decreased cell adhesion and concomitant increased cell migration (45). Moreover, in an in vivo model of prostate cancer, Lakshman et al. (46) reinforces the suppressor role for Eng providing evidence that epithelial Eng loss leads to progressive increases in the number of circulating metastatic tumor cells as well as the formation of distant soft tissue metastasis.

Taken together, this study supports the idea that the fate of a malignant epithelial cell depends largely on its interaction with an active stroma (2). In this case, we propose (Figure 6) that the endogenous autocrine production of TGF-β, a characteristic trait of epithelial malignancy, may act in a paracrine manner, stimulating the production of a soluble form of stromal MMP-14 that, in turn, cleaves the membrane-bound epithelial Eng. It has been clearly demonstrated that disruption of membrane Eng is an important event in tumor progression. Therefore, it is highly possible that Eng-processed cells acquire a whole set of malignant traits (autocrine TGF-β production, among others) that perpetuates the expression of a more motile malignant phenotype.

**Supplementary material**

Supplementary Figures S1–S4 can be found at http://carcin.oxfordjournals.org/

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