ERM/ETV5 Up-regulation Plays a Role during Myometrial Infiltration through Matrix Metalloproteinase-2 Activation in Endometrial Cancer

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Abstract
We have described recently the Ets family transcription factor, ERM/ETV5, specifically up-regulated in endometrioid endometrial carcinoma (EEC) and associated with myometrial infiltration. Ets family members have been correlated to tumor progression by up-regulating the expression of matrix-degrading proteases. In the present study, we investigated the possibility that in EEC, ERM/ETV5 may act by inducing the expression of genes involved in extracellular matrix remodeling. Unraveling the molecular events associated with the initiation of tumor invasion would represent an obvious improvement for EEC patients. The overexpression of ERM/ETV5 induced scattering in the endometrial cancer cell line Hec-1A, correlating to increased matrix metalloproteinase-2 (MMP-2) gelatinase activity. Both chromatin immunoprecipitation and reversion experiments with RNA interference showed a functional link between ERM/ETV5 overexpression and MMP-2 activation. The increased MMP-2 activity associated with overexpressed ERM/ETV5 in a mouse model conferred invasive capacity to endometrial tumors. Ortophically implanted overexpressing ERM/ETV5 tumors presented a more aggressive and infiltrative pattern of myometrial invasion. Finally, the specific localization of ERM/ETV5 and MMP-2 at the invasive front of myometrial infiltrating human endometrial carcinomas further reinforced the hypothesis of a role for ERM/ETV5 in the early steps of endometrial dissemination. Taken together, these results lead us to propose that in EEC, ERM/ETV5 acts through MMP-2 gelatinolytic activity to confer invasive capabilities, associated with an initial switch to myometrial infiltration. They also postulate ERM/ETV5 as a valuable marker for patient stratification and a transcription pathway that should be evaluated for therapies specifically targeting the initial steps of EEC dissemination. [Cancer Res 2007;67(14):6753–9]

Introduction
The tumorigenesis of sporadic endometrial cancer, the most common gynecologic malignancy encountered in western countries, is commonly explained based on a dualistic model that discriminates between type I endometrioid [endometrioid endometrial carcinoma (EEC)] and type II nonendometrioid endometrial tumors from both biological and clinical variables (1). EECs represent the majority of cases of sporadic endometrial cancer (70–80%), with unopposed estrogen stimulation as the etiologic factor associated with the development of the carcinoma (2). EECs usually develop in premenopausal and perimenopausal women, express estrogen and progesterone receptors (3), and are associated with elevated levels of serum estradiol (4). Histologically, most tumors are of low grade, are frequently preceded by endometrial hyperplasia and, overall, are characterized by a favorable prognosis. Nonetheless, myometrial affectation, as the initial event in tumor invasion and distant dissemination, determines an increase in the rate of recurrence after a first surgical treatment and a decrease in survival at the 5-year follow-up.

From a molecular point of view, PTEN gene silencing, microsatellite instability associated with defects in DNA mismatch repair genes (i.e., MLH1), and mutations in the K-Ras gene have been described as the major alterations defining the activation of EEC tumorigenesis, from normal endometrium to hyperplasia and then on to carcinoma. Nevertheless, the molecular determinants for steps toward advanced tumorigenesis, such as the transitions from a localized to an infiltrating tumor and to a metastatic stage, remain more elusive (5). We have characterized recently the up-regulation of the ERM/ETV5 gene in EEC with a specific and significant increase restricted to those tumor stages associated with myometrial invasion (6, 7). ERM/ETV5 belongs to the PEA3 subfamily, included in the Ets family of transcription factors. This family is characterized by a sequence of ~85 amino acids in an evolutionarily conserved DNA-binding domain that regulates the expression of a variety of genes by binding to a central A/GGAA/T core motif, in cooperation with other transcriptional factors and cofactors (8, 9).

More to the point, tumor progression and the increased expression of matrix metalloproteinases (MMPs), a family of neutral proteases that catalyze the destruction of the extracellular matrix, were found to be closely related with the tumor-invasive capacities promoted by Ets transcription factors (10). Active gelatinases have been described in endometrial carcinoma, resulting in alterations to the microenvironment that promotes tumor invasion and metastasis (11, 12). In the present study, we
investigated the possibility that ERM/ETV5 may play a part in the initial switch to myometrial infiltration by inducing the expression of the genes involved in extracellular matrix remodeling.

Materials and Methods

Cell culture, constructs, and stable cell line generation. Hec-1A cells (provided by C. Simon from the Instituto Valenciano de Infertilitat, Valencia, Spain) were cultured in McCoy's 5A medium with GlutaMAX supplemented with 10% fetal bovine serum (Life Technologies).

Plasmid construction consisted of the full-length human ERM/ETV5 being removed with EcoRI from the pSV-HERM/ETV5 construct (a generous gift from Dr. Chotteau-Lelièvre, Institut de Biologie de Lille, Lille, France). It was then inserted at the EcoRI position at the multiple cloning site of the pEGFP-C2 vector (BD Biosciences). The correct ERM/ETV5 orientation and reading frame were confirmed by sequencing.

Hec-1A cells were transfected with Fugene 6 (Roche Diagnostics) and either the pEGFP-C2 vector alone or the hERM/ETV5 containing pEGFP-C2 vector. Hec-1A cells stably expressing the green fluorescent protein (GFP) or GFP-ERM/ETV5 were established by incubation with 500 µg/ml genetin G418 (Life Technologies).

Western blot. Western blotting was done as described previously (13). The primary antibodies used were 1:100 monoclonal antibody (aAb) α-actin (NeoMarkers, LabVision Corp.) and 1:100 rabbit polyclonal antibody (rAb) ERM/ETV5, and membranes were revealed with horseradish peroxidase–conjugated goat anti-rabbit immunoglobulins (DakoCytomation) and the SuperSignal West Dura substrate (Pierce Biotechnology, Inc.).

Gelatin zymography. Active gelatinases A (MMP-2) and B (MMP-9) were analyzed in cell lysates by gelatin zymography as described previously (14). The negative bands correlating to gelatinase activity within the zymograms were quantified by densitometric analysis. At least five independent experiments were done.

Chromatin immunoprecipitation. GFP-ERM/ETV5 chromatin immunoprecipitation (ChIP) on Hec-1A GFP and Hec-1A GFP-ERM/ETV5 cells were done as per manufacturer's recommendations for mAb GFP (Roche Diagnostics) and rAb ERM/ETV5. Rabbit acetyl-histone H4 (Upstate) and normal rabbit IgGs (Upstate) were used as positive and negative controls, respectively. For PCR analysis, 1 µl of input DNA extraction and 10 µl of immunoprecipitated DNA were used for 35 cycles of amplification (annealing temperature, 60°C). The primers for MMP-2 were obtained from the Thermo Electron Corp. The sequences were as follows: TAAAGGA-AGCACCCACCAGT (reverse primer) and ACCCCAGACTCCACCTTT (forward primer). The HES gene [CGTGTACTGCTGATGTTTGC (reverse) and GATGGGGCGCGGGAAAT (forward)] was used as a control.

Small interfering RNA sequences and transfection. Transfected cells were incubated with 100 nM–lentil enhanced GFP (EGFP) and negative control small interfering RNA (siRNA; Ambion) using standard electrotansfection (10⁶ cells/125 µl in a Bio-Rad gene pulser cuvette 0.2 cm, 250 V, 250 µF, infinite resistance). Following electroporation, samples were immediately combined with fresh culture medium. Cells were plated and incubated at 37°C for 48 h followed by RNA and protein extraction and video-microscopy assays.

Total RNA isolation and assay by quantitative real-time PCR. Total RNA was extracted and purified using RNeasy Mini kit (Qiagen). Quantitative real-time PCR (RT-Q-PCR) was done as described previously (7). Human actin (Applied Biosystems) was used to normalize.

Video microscopy. Cells treated with the negative control siRNA or GFP siRNA, or incubated in the presence or not of a specific MMP-2 inhibitor III (12.5 nmol/L; Calbiochem), were kept in a computer-controlled mini-incubator, which provided stabilized temperature of 37 ± 0.5°C, with 95% humidity and 5% CO₂ and optical transparency for microscopic observations. The incubator was fastened to an inverted microscope (Live Cell Imaging CellR, Olympus). Images were taken with the 10x objective every 30 min at least for 24 h.

At least 100 cells per video were manually tracked using the WCIF Imagej software. Comparisons between cell trajectories from the different cell types and conditions were determined by the maximum relative distance to origin (MRDO) variable (15). All experiments were done in triplicate, at least.

Animal model. Five-week-old female athymic Swiss Webster mice (Charles River Laboratories, Inc.) were used for this study. The animals were housed in individually ventilated cage units and maintained under pathogen-free conditions. Food and water were provided ad libitum. Animal care and experiments were carried out in accordance with the guidelines of the Spanish Council on Animal Care.

S.c. xenografts were established by the implantation of 2 × 10⁶ Hec-1A, Hec-1A GFP, and Hec-1A GFP-ERM/ETV5 tumor cells in 200 µl PBS into the subcutaneous region of two nude mice per cell line. The animals were sacrificed at 6 weeks, and the tumors were dissected for orthotopic implantation.

For orthotopic implantation, four mice per cell line were operated on by medial laparotomy. The retroperitoneum was opened, and a tissue block of ~1 mm³ of the generated s.c. tumors was implanted onto the posterior face of the uterus, making a pocket to isolate the tumor tissue from the rest of the organs in the abdominal cavity. The organs were reintroduced into the abdominal cavity, and the retroperitoneum and skin were then closed with surgical suture. Two weeks later, the animals were sacrificed by cervical dislocation, the peritoneal cavity was opened, and the tumor was formalin fixed and processed for histologic examination by H&E staining and immunohistochemistry.

Immunohistochemistry and in situ zymography. The EEC invasive front tissue microarray (TMA), constructed with tumors from the Vall d’Hebron Hospital (Barcelona, Spain), included 100 endometrioid, 12 serous papillary, 7 clear cell, 1 mucinous, and 6 adenosquamous carcinomas. The TMA was constructed with cores corresponding to the inner part of the tumor and to the invasive border. The protocol was approved by the Institutional Review Board, and informed consent was obtained from all of the patients.

Immunohistochemistry was done as described (7). The primary antibodies used were ERM/ETV5 (1:50; Santa Cruz Biotechnologies) and MMP-2 (prediluted ab15478; Abcam).

Alternatively, DQ gelatin processing for gelatinase activity on 7-µm-thick unfixed frozen sections of stage IC EECs was done as per manufacturer's instructions (EnzCheck, Molecular Probes). Sections either incubated without DQ gelatin or incubated with 50 µmol/L 1,10-phenanthroline to block MMP activation were used as controls.

Statistical analysis. The Statistical Package for Social Science (version 12.0) was used. The nonparametric Kruskal-Wallis test was applied to gel and in situ zymography, Kruskal-Wallis test was followed by Dunn’s multiple comparison test to cell migration data, whereas comparisons between different groups of samples in the TMA were analyzed using Spearman ρ nonparametric statistics.

Results

ERM/ETV5 overexpression in the Hec-1A cell line resulted in enhanced MMP-2 activity. The human endometrial cancer cells, Hec-1A, were transfected with pEGFP-ERM/ETV5 or pEGFP without insert as a control (see Materials and Methods), and stable transfectants were selected with geneticin following transfection. Western blot analysis showed similar levels of endogenous ERM/ETV5 expression for all three cell lines, whereas only Hec-1A GFP-EGFP-ETV5 expressed the fusion protein GFP-ERM/ETV5 (Fig. 1A). As expected, both the endogenous and the fusion protein were localized in the nucleus (see Supplementary Fig. S1). The homogeneity of the selected cell lines was assessed by flow cytometry (data not shown).

Within the established cell lines, we determined whether the overexpression of ERM/ETV5 could influence protease activity. For this purpose, we examined the activity of the two main MMPs described in endometrial cancer, gelatinases A and B (MMP-2 and MMP-9, respectively), by enzymatic zymography (16).
A representative example of a gelatin-embedded gel is shown in Fig. 1, where the gelatinase activity of the total protein extracts from Hec-1A, Hec-1A GFP, and Hec-1A GFP-ERM/ETV5 was directly proportional to the band intensities. Increased intensity, corresponding to the active form of MMP-2, was found in the Hec-1A GFP-ERM/ETV5 cell line when compared with the Hec-1A cells and those transfected with GFP. The densitometry quantification of at least five gelatinase activity assays resulted in a statistically significant increase in MMP-2 activity in the Hec-1A GFP-ERM/ETV5 cell line (P < 0.005; Fig. 1C). No significant differences were found between the two control cell lines (Fig. 1C), and no gelatinase MMP-9 activity was found among the three cell lines (Fig. 1B). These data suggested a correlation between ERM/ETV5 overexpression and increased MMP-2 activity in the Hec-1A endometrial cancer cell line.

ChIP showed a direct interaction between ERM/ETV5 and the MMP-2 promoter. Next, by ChIP, we assessed whether ERM/ETV5 in vivo bound to the MMP-2 promoter in the established cell lines. Briefly, cells were fixed with formaldehyde, and soluble chromatin was fragmented by the sonication of cross-linked nuclei. Chromatin fragments in the range of 500 to 1,000 bp were immunoprecipitated by specific antibodies (GFP and ERM/ETV5). Purified genomic DNA from the immunoprecipitated chromatin was subjected to semiquantitative PCR using primers covering the 411-bp proximal region of the human MMP-2 promoter (Fig. 2A). Control reactions using IgG isotype did not exhibit specific immunoprecipitation, whereas reactions containing antibodies to ERM/ETV5 or GFP to a lesser extent showed specific binding to this region of the MMP-2 promoter in the Hec-1A GFP-ERM/ETV5 cell line (Fig. 2B). Further controls showed no specific GFP antibody-mediated immunoprecipitation with fragmented chromatin from the Hec-1A GFP cell line, neither when PCR amplification was done covering a promoter without putative Ets binding sites as for the gene 18S (Fig. 2B).

ERM/ETV5 overexpression promoted MMP-2–dependent cell migration and scattering in the Hec-1A endometrial cancer cell line. Hec-1A cells overexpressing GFP-ERM/ETV5 showed enhanced mobility compared with the control non-transfected or GFP-transfected cell lines, as evidenced by video microscopy and cell migration tracking (Fig. 3). Representative examples of the trajectories followed by the Hec-1A and Hec-1A GFP-ERM/ETV5 cells are represented (Fig. 3A–C). Enhanced MRDO values, as the maximal relative distance covered by the cells, corresponding to Hec-1A GFP-ERM/ETV5 cells showed statistical significance when compared with Hec-1A or Hec-1A GFP cells (P < 0.001; Fig. 3F). A gradual increase in MRDO levels was found when Hec-1A cells expressing low levels of the fusion

Figure 1. Enhanced MMP-2 activity on expression of the fusion protein GFP-ERM/ETV5. A, Western blot detection of endogenous human ERM/ETV5 protein and transfected GFP-ERM/ETV5 protein in the cell lines developed. Actin was used as a loading control. B, expression of GFP-ERM/ETV5 enhances gelatinase MMP-2 activity. Top, representative zymogram done with cell lysates from the Hec-1A (line 1), Hec-1A GFP (line 2), and Hec-1A GFP-ERM/ETV5 (line 3) cells. MMP-2 activity is shown as directly proportional to the band intensities. No MMP-9 activity was detected in these cell lines. C, quantification of at least five gelatinase activity assays among the three established cell lines.

Figure 2. Direct interaction of ERM/ETV5 to the promoter region of MMP-2. A, the analysis of the promoter region of MMP-2 shows one Ets and two PEA3 putative binding sites. Underlined, forward and reverse primers for MMP-2 promoter region PCR. B, PCR analysis of the chromatin immunoprecipitated with antibodies directed against the GFP and ERM/ETV5 shows specific binding to the promoter region of MMP-2. ChIP done with antibodies against the GFP in the Hec-1A GFP control cell line showed no specific binding of the GFP to the promoter region of MMP-2. Irrelevant IgGs and antibodies directed against the acetylated histone are shown as negative and positive controls, respectively. The 18S gene was used as a negative control for a promoter not regulated by Ets factors.
protein were compared with high GFP-ERM/ETV5–expressing cells (see Supplementary Fig. S2).

RNA interference (RNAi) directed against the GFP to knock down the overexpressed fusion protein GFP-ERM/ETV5, without interfering with the endogenous ERM/ETV5, reversed the activation of MMP2 to the levels found in the control cell lines, as shown by gel zymography (Fig. 3E). Likewise, GFP siRNA resulted in the reversion of the increased Hec-1A GFP-ERM/ETV5 migration (Fig. 3F). The resulting MRDO levels for the three cell lines treated with the negative control siRNA or with the GFP siRNA and in the presence or not of the specific MMP-2 inhibitor III. The MRDO levels from the siRNA-negative control and those obtained in the absence of the MMP-2 inhibitor were pooled together (control). Statistical significance (**, P < 0.001) was obtained when the MRDO levels from the Hec-1A GFP-ERM/ETV5 cells were compared with those from the Hec-1A and Hec-1A GFP control cell lines, as well as when treated with the GFP siRNA or the MMP-2 inhibitor III.

Altogether, the results obtained with the developed Hec-1A cell lines indicated that ERM/ETV5 promotes the migratory capacity of endometrial tumor cells by activating MMP-2 gelatinase through direct interaction with its promoter region.

Increased gelatinase activity in ERM/ETV5–overexpressing tumors was related to myometrial infiltrating phenotypes in a mouse model. To analyze the implications of overexpressed ERM/ETV5 for tumor invasion in a three-dimensional system within an accurate tissue environment, we generated in vivo orthotopic models for endometrial cancer. Briefly, we generated tumors in nude mice by s.c. injection of the Hec-1A GFP-ERM/ETV5, Hec-1A GFP, and untransfected Hec-1A cell lines. Then, tumor pieces of 1 mm³ originating from each cell line were implanted onto the posterior face of the uterus, and 2 weeks postimplantation, the tumors were analyzed for MMP-2 expression and activity and for myometrial infiltration.

Immunohistochemistry showed the basal levels of MMP-2 expression associated with the endogenous ERM/ETV5 in the tumors originating from the control Hec-1A (data not shown) Hec-1A. Again, this effect could be reversed by incubation with an inhibitor of MMPs (50 μmol/L 1,10-phenanthroline), reinforcing a functional link between ERM/ETV5 and MMP-2.

Figure 3. GFP-ERM/ETV5 expression results in enhanced cell migration mediated by increased MMP-2 activity. A, sequential phase-contrast images showing representative examples of the trajectories followed by Hec-1A cells stably expressing or not the GFP-ERM/ETV5. Hec-1A cells transfected with the GFP behaved as the nontransfected Hec-1A cells (data not shown). Elapsed time is indicated in hours. Bar, 10 μm. B and C, phase-contrast images of Hec-1A and Hec-1A GFP-ERM/ETV5 cells, at the end of video recording, showing representative examples of the increased migration that resulted in larger trajectories and in enhanced scattering in those cells overexpressing ERM/ETV5. Bar, 0.1 mm. D, histogram representing the percentage of ERM/ETV5 expression determined by RT-Q-PCR, after treatment of the cell lines with the negative control siRNA and the GFP siRNA. E, Western blot showing the specific silencing of the fusion protein GFP-ERM/ETV5 and not the endogenous ERM/ETV5 in the Hec-1A GFP-ERM/ETV5 cells treated as in (C), with the resulting MMP-2 activity reduction shown by gel zymography. F, MRDO levels for the three cell lines treated with the negative control siRNA or with the GFP siRNA and in the presence or not of the specific MMP-2 inhibitor III.
Hec-1A GFP (Fig. 4A) cell lines. In contrast, the tumors derived from the Hec-1A GFP-ERM/ETV5 cell line markedly stained for MMP-2 (Fig. 4B). Extracts from these tumors were incubated in the presence of a fluorescent conjugate of gelatin, DQ gelatin, to assay their gelatinolytic activity (see Materials and Methods). In those tumors derived from the Hec-1A GFP-ERM/ETV5, the increase ($P = 0.008$) in fluorescence was indicative of further processing of the gelatin conjugate (Fig. 4C).

Interestingly, when we analyzed the interface between the orthotopic tumors and the myometrium of the mice, we observed differences at the initial steps of myometrial invasion. Tumors originating from Hec-1A GFP-ERM/ETV5 presented an infiltrative pattern at 2 weeks postimplantation (Fig. 4E) compared with a more expansive pattern of invasion in the Hec-1A (data not shown) or Hec-1A GFP (Fig. 4D) tumors. Three of four animals presented tumoral lesions that internalized into the myometrium associated with the overexpression of ERM/ETV5 compared with only one of four animals in the Hec-1A GFP-derived tumors. The median number of invasive lesions for the Hec-1A GFP-ERM/ETV5 tumors was 16 ± 5.9, whereas 12 lesions/2 mm of myometrium interface in the unique GFP control animal that presented myometrial infiltration.

These results permitted us to translate the association between ERM/ETV5 and MMP-2 to the three-dimensional structure of an in vitro model. Furthermore, they revealed the contribution of ERM/ETV5 overexpression to a more aggressive and infiltrative pattern at the initial steps of myometrial invasion in endometrial cancer.

**Gelatinolytic activity in human endometrial carcinoma.** Using in situ zymography with the fluorescent conjugate gelatin, DQ gelatin, we next examined the gelatinase activity in tumoral tissue sections from human samples with elevated levels of ERM/ETV5 expression as assessed by RT-Q-PCR (7). A representative tissue section corresponding to a stage IC EEC showed, in an area with profuse tumoral glands [see 4',6-diamidino-2-phenylindole (DAPI) staining in Fig. 5B], a specific fluorescent labeling indicative of gelatinase activity, mainly within the epithelial glands and with low activity in the stromal compartment (Fig. 5A). The specificity of the assay was further shown on a consecutive section from the same stage IC EEC sample, incubated with DQ gelatin and the gelatinase inhibitor 1,10-phenanthroline (Fig. 5C), which showed almost completely reversed gelatinase activity in the same epithelial glands (Fig. 5D). A consecutive section incubated without DQ gelatin as an autofluorescence control rendered negative results (data not shown).

These data broaden the correlation found in the in vitro and in vivo models between ERM/ETV5 expression and MMP-2 gelatinase activity to human samples.

**MMP-2 protein expression correlated to ERM/ETV5 up-regulation in human endometrial carcinomas with a marked increase at the invasive front.** We finally examined the association between ERM/ETV5 and MMP-2 in human EEC tumor samples. First, we did immunohistochemistry on a TMA constructed with representative areas from 74 EECs (6). The statistical treatment of the TMA assay indicated a strong correlation between MMP-2 and ERM/ETV5 protein expression ($P < 0.001$; see Supplementary Fig. S3). Second, we analyzed in more detail the eventual role of these proteins in myometrial infiltration using an invasive front TMA constructed with a series of 126 infiltrative human endometrial cancer samples (see Materials and Methods). ERM/ETV5 was found positive in 107 (84.9%) cases and MMP-2 in 114 (90.5%), confirming that the high levels of these proteins were associated with myometrial infiltration. More interestingly, when we examined the interface between the tumor and the myometrium, we could find a marked increase in ERM/ETV5 and MMP-2 expression at the invasive cores in 77.2% of the MMP-2 positive cases and 61.7% of the ERM/ETV5–positive cases. In those areas where the tumor glands infiltrated the myometrium at the invasive front, the intensity of both ERM/ETV5 (Fig. 6B) and MMP-2 (Fig. 6D) staining increased compared with the inner areas of the tumor (Fig. 6A and C). These results showed the concomitant up-regulation of ERM/ETV5 and MMP-2 at the invasive front of myometrial infiltrating human carcinomas.

**Figure 4.** MMP-2 increased activity in the Hec-1A GFP-ERM/ETV5 cell line confers invasive properties to tumors in an orthotopic endometrial cancer model. Immunohistochemistry of primary tumors originating from Hec-1A GFP (A) or Hec-1A GFP-ERM/ETV5 cells (B) shows increased MMP-2 levels associated with the overexpression of ERM/ETV5. C, the quantification of gelatinase activity in these tumors presents enhanced gelatin processing in the Hec-1A GFP-ERM/ETV5 cell line. D and E, H&E staining of orthotopically implanted tumors, showing the interface between the generated tumor and the myometrium of the mice, shows a more aggressive pattern of invasion with an infiltrative profile associated with the overexpression of ERM/ETV5 (E) compared with GFP as a control (D). Dashed red line, the interface between the myometrium and the orthotopically implanted tumors.
Discussion

In the present study, we have characterized the gelatinase activity associated with ERM/ETV5 up-regulation during the early stages of endometrial tumorogenesis, associated with an initial switch to myometrial infiltration. First, we showed that the overexpression of the transcription factor ERM/ETV5 in the endometrial cancer cell line, Hec-1A, resulted in enhanced MMP-2 gelatinase activity. Gelatinase A (MMP-2, 72 kDa) is primarily responsible for the degradation of the helical domains of type IV collagen, the principal collagen of basement membranes. Conserved PEA3 elements, which bind members of the Ets transcription factors, have been found in all inducible MMP promoters (17). Correspondingly, we could show the specific binding of ERM/ETV5 to the promoter region of the MMP-2 in the established Hec-1A cell lines. Second, ERM/ETV5 up-regulation and MMP-2 gelatinase activity were further confirmed in orthotopic mouse models originating from the different cell lines. The overexpression of ERM/ETV5 resulted in both increased MMP-2 levels and proteolytic cleavage of a fluorescent derivative of gelatin. Finally, TMA immunohistochemistry showed a significant correlation between ERM/ETV5 and MMP-2 protein expression in the epithelial glands of more than 70 human carcinoma samples, representative of the tumorogenesis process in EEC. In accordance with these results, MMP-2 expression has been proposed recently to have a predictive value for stage I endometrial cancers (16). Likewise, the in situ zymography showed that increased MMP-2 expression corresponded to enhanced gelatinolytic activity, localized to the epithelial glands in endometrial carcinoma sections from stages IC, where ERM/ETV5 had been described to be specifically up-regulated (7). The other gelatinase, MMP-9, has been mainly associated with inflammatory infiltration (18, 19). The fact that no inflammatory infiltration was present in the EEC sections analyzed in this study suggests that the gelatinolytic activity we observed in our in situ zymography series was mainly the result of MMP-2 activity.

These results extend to endometrial cancer the results of those studies that describe a correlation between the expression of members of the Ets family and genes involved in extracellular matrix remodeling. Ets-1 controls the expression of two metalloproteinase gene promoters, stromelysin-1 (MMP-3; ref. 20) and collagenase-1 (MMP-13; ref. 21), and the urokinase-type plasminogen activator promoter (22). Induced by the hepatocyte growth factor, it stimulates the overexpression of MMP-7 in human hepatocellular carcinoma (23). ETV4, another Ets family transcription factor, has been implicated in tumor progression through the induction of MMP expression in human colorectal and gastric cancer (24). However, no correlation in ERM/ETV5 expression and MMP activity has been found in gastric and colorectal cancer (24).

We went further and analyzed what this correlation could mean to endometrial carcinoma. We were able to find a functional link between the overexpression of ERM/ETV5, resulting in enhanced motility and scattering of the Hec-1A cells, and the increased gelatinase activity that modifies the cell-substrate interface. Both RNAi and specific inhibition of the gelatinase activity were able to reverse the scatter effect of ERM/ETV5 overexpression. Accordingly, Ets-1 overexpression showed improved migratory properties in tumoral epithelial HeLa cells (25). Moreover, it has been found to be involved in cell migration and tumor invasion, correlating to the increase in the proteolytic activity of the invading cancer cells (26). Similarly, ETV4 has been involved in the invasive potential of cancer cell lines, such as neuroblastoma (27), oral squamous cell carcinoma (28), and ovarian cancer (29).

Furthermore, we showed within the animal model that the migratory capabilities conferred by ERM/ETV5 were translated into a more aggressive pattern of myometrial invasion, with an infiltrative profile at the invasion front of the tumor. These results also reinforced the accuracy of orthotopic models, compared with s.c. tumor implantations, for the study of the mechanisms involved in the metastasis process, comprising the initial steps of adjacent tissue invasion (i.e., myometrial infiltration in EEC). Tumor implantation at the original tissue site is essential because the outcome of metastasis depends on the interaction of the metastatic cells with the different organ environments (30). Finally, the invasive properties of ERM/ETV5-overexpressing tumors were corroborated at the human carcinoma level. In addition to the

Figure 5. In situ zymography of endometrial carcinoma tissues. Serial frozen sections incubated with 40 μg/mL fluorescein-conjugated DQ gelatin A, DAPI localized to the profuse epithelial glands of IC endometrial cancer tissue (B) and with 50 μmol/L 1,10-phenanthroline, as a control (C and D). Note that in the same glands from a consecutive section, specific fluorescence corresponding to gelatinase activity in the tumoral epithelial glands was abolished when the gelatin conjugate was incubated in the presence of the MMP inhibitor (see corresponding DAPI in D). Magnification, ×20.

Figure 6. Marked increase in ERM/ETV5 and MMP-2 proteins at the invasive front in human endometrial carcinomas. ERM/ETV5 (A and B) and MMP-2 (C and D) staining at the invasive front of human endometrial carcinomas (B and D) present enhanced intensity levels compared with the inner parts of the tumors (A and C). Magnification, ×40.
characterization of the specific ERM/ETV5 up-regulation in IC stage EECs, associated with >50% of myometrial infiltration (7), we were able to show a marked increase in ERM in ETV5 and MMP-2 labeling at the invasive front of the tumor, suggesting an active and coordinated role for these proteins at the time of myometrial infiltration. In accordance with all these data, high MMP-2 and low tissue inhibitor of metalloproteinase-2 expression were described as the most potant markers of endometrial tumors with a high risk for local and distant spread (31). In addition, MMP-2 has been involved recently in the sequential promotion of pulmonary metastasis when expressed in human breast cancer cells (32).

All of this prompts us to suggest that in EEC, ERM/ETV5 could be acting through MMP-2 to confer the invasive capabilities associated with an initial switch to myometrial infiltration. Unraveling the molecular events in EEC associated with the initiation of tumor invasion would represent an obvious improvement in the pursuit of rational targets for the onset of metastasis. This knowledge would also be a valuable tool for the molecular stratification of patients because myometrial affection determines an increase in the rate of recurrence after a first surgical treatment and a decrease in survival at the 5-year follow-up.

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References


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