The expression of an Ets1 transcription factor lacking its activation domain decreases uPA proteolytic activity and cell motility, and impairs normal tubulogenesis and cancerous scattering in mammary epithelial cells

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INTRODUCTION

Epithelial cells have the potential to develop motile and invasive properties in a transient or more persistent manner. These cell properties play a crucial role during early embryonic development and organogenesis or during pathological development and metastasis. Cell migration and invasion involve modifications of the architecture of the cells, changes in cell adhesion and remodeling of the extracellular matrix, which in turn can affect cell behaviour. These events require the coordinate expression of several sets of genes (Jiang et al., 1994). It is of major importance to identify the molecular actors mediating the transcriptional regulation of these genes.

The proto-oncogene c-ets1 is the cellular progenitor of the viral oncogene v-ets and the founder of the Ets transcription factor family. These proteins share a highly conserved 83 amino acids domain, the DNA binding domain, which recognises specific nucleotide sequences with a GGAA/T core (Wasylyk et al., 1993). Analysis of Ets family gene expression in developing embryos revealed a broad pattern of expression, which suggests that these genes may serve multiple and overlapping functions. Of particular interest, expression of several members of the Ets family was observed in situations involving extensive cell migration and tissue remodelling. Expression of c-ets1 was detected at a very early stage of gastrulation in migrating mesodermal cells (Quéva et al., 1993). At later stages, c-ets1 mRNAs are found during epithelial-mesenchymal transitions in emigrating neural crest cells and in sclerotomal cells of the somites (Fafeur et al., 1997), and in endothelial cells during angiogenesis (Pardanaud and Dieterlen-Liévre, 1993; Vandenbunder et al., 1989). During embryogenesis, the expression patterns of c-ets1 and erg, but not of ets2 were similar in forming blood vessels, in migrating neural crest cells and in dissociating somites (Dhordain et al., 1995; Maroulakou et al., 1994). In Xenopus embryos, Xi-fli and Xi-ets2 mRNAs are preferentially expressed in regions undergoing important reorganisation, in particular in migrating cells (Meyer et al., 1995; Remy et al., 1995).
During organogenesis of kidney, lung and salivary gland, three genes of the *pea-3* group within the *Ets* family are expressed either in epithelial or mesenchymal cells while tissue reorganisation is occurring (Chotteau-LeLievre et al., 1997). During mammary gland development, transcripts for *c-ets1* are first detected in the mesenchymal component at the onset of primary epithelial bud formation. Later, *c-ets1* as well as the three *pea-3* members transcripts are detected in the epithelial component at the invading extremities of epithelial ducts (Chotteau-LeLievre et al., 1997; Delannoy-Courdent et al., 1996). *c-ets1* expression also correlates with invasive processes during tumour development; *c-ets1* transcripts are detected in stromal cells surrounding invasive carcinomas, as well as in endothelial cells of the new blood vessels neighbouring the tumour (Wernert et al., 1992, 1994). These data suggest that several *Ets* family members can take part in the control of cell migration and invasion during normal or pathological development.

Cell migration is facilitated by the proteolytic degradation of extracellular matrix components. Plasminogen activators (PAs) play a key role in this degradation process by their ability to induce a cascade of proteolytic events. Indeed, PAs are serine proteases that convert plasminogen into plasmin, which in turn activates several proteases capable of degrading extracellular matrix components (Murphy et al., 1992). Both urokinase-type and tissue-type PAs (tPA and uPA, respectively) have been identified; whereas tPA is thought to be predominantly involved in vascular fibrinolysis, uPA is thought to participate in the extracellular proteolysis accompanying tissue remodeling and cellular invasion (Schnitt et al., 1997).

During mouse embryogenesis, uPA expression is detected in the maternal decidua during implantation and placentation (Grévin et al., 1993), but is not detected in the embryo proper until E15.5 after the onset of organogenesis (Grévin et al., 1993; Sappino et al., 1991). In the normal mammary gland, uPA gene expression is transcriptionally regulated both during embryogenesis (Delannoy-Courdent et al., 1996) and during the succession of lactation and involution stages occurring in female adult life (Busso et al., 1989; Ossowski et al., 1979). Overexpression of uPA or of *uPA-receptor* in mammary cancer cells correlates with increased invasiveness in vitro or metastatic ability in rats (Holst-Hansen et al., 1996; Xing and Rabbani, 1996).

Several lines of evidence indicate that *Ets* transcription factors can regulate the transcription of proteinase genes, including the *uPA* gene. Functional *Ets* binding sites have been identified in the promoters or enhancers of various genes encoding extracellular proteinases, such as *uPA* (Rorth et al., 1990), stromelysin 1 (Wasylyk et al., 1991), collagenase 1 (Gutman and Wasylyk, 1990). E1A-F, a *pea-3* homologous factor, can activate the promoters of proteinases such as collagenase 1, stromelysin 1, and 92 kDa gelatinase B (Higashino et al., 1995). Ets2 and Erg differentially regulate collagenase 1, stromelysin 1, and 92 kDa gelatinase B (Gutman and Wasylyk, 1990). E1A-F, a *pea-3* homologous factor, can activate the promoters of proteinases such as collagenase 1, stromelysin 1, and 92 kDa gelatinase B (Higashino et al., 1995).

**MATERIALS AND METHODS**

**Cell types and culture conditions**

The MMT epithelial cell line, established from mouse mammary tumour (ATCC), was routinely cultured in Dulbecco’s modified Eagle medium (DMEM, Gibco) containing antibiotics, 4.5 g/l glucose, 1% non essential amino acids and 10% fetal calf serum (FCS, Eurobio). The NMuMG epithelial cell line, derived from normal mouse mammary glandular tissue (ATCC), was cultured in the same medium supplemented with 2 µg/ml insulin (Endopancrin). Non-infected cells, i.e. wild-type cells, will be referred to as WT MMT cells and WT NMuMG cells.

Experimental assays were performed using type I collagen solutions prepared from rat tails as described by Montesano et al. (1991). For each test, cells were seeded at the appropriate density, either on plastic dishes coated for three hours with 0.5 mg/ml collagen solution for two-dimensional cultures or in 1.5 mg/ml collagen gels for three-dimensional cultures.

The fibroblast-conditioned medium was obtained by collecting the supernatant of sub-confluent NIH-3T3 fibroblasts grown for two days in serum-free DMEM. It was then stored at –80°C until use. Fibroblast-conditioned medium was used in two-dimensional cultures to stimulate cell migration and invasion, and in three-dimensional cultures to induce tubulogenesis or to enhance scattering of mammary epithelial cells, as previously demonstrated (Delannoy-Courdent et al., 1996; Montesano et al., 1991).

Inhibition of plasminogen activator activity in non infected cells was performed using aprotinin (200 µg/ml, Sigma) a serine proteinase inhibitor. In similar experimental conditions, it has been shown to inhibit plasminogen activator activity and basal membrane degradation in the MDA-MB-231 cancerous epithelial mammary cell line (Stonelake et al., 1997).

**Viral constructions and production**

Virus were constructed and produced as described by V. Mattot et al. (unpublished). A schematic representation of the virus constructs is shown in Fig. 1. Briefly, the cDNA sequence encoding the mouse Ets1 DNA-binding domain (Ets1-DB) and corresponding to amino acids 306-423 was obtained by PCR amplification of mouse Ets1 cDNA
The Ets1-DB sequence was cloned in front of an IRES and Neo sequence in order to translate both the Ets1-DB and neomycin-inducing resistance proteins from a single viral mRNA. A second plasmid carrying only the IRES and Neo sequences was constructed and used as control in the entire study. These Ets1-DB- IRES-Neo and IRES-Neo sequences were inserted into the genome of a MFG retroviral vector (Dranoff et al., 1993). For production of the virus, the packaging GP+E86 cell line was transfected by lipofection with these MFG retroviral vectors and the GP+E86-virus producing cell lines were then obtained following selection in the presence of 0.8 mg/ml Geneticin (Gibco). After an overnight incubation of sub-confluent cells with fresh medium, the supernatant containing viral particles were collected, filtered and used for infection. Viral titration was performed by counting the number of genetin-resistant clones after infection and selection of NIH-3T3 cells. The titer was found to be higher than 10^6 cfu/ml in the supernatant of each virus-producing cell line.

Epithelial cell infection and characterisation
NMuMG and MMT cells (150,000 cells/100 mm dish) were incubated for 6 hours with 1 ml of virus-producing GP+E86 cell supernatant containing 4 µg/ml of polybrene (Aldrich). Selection started two days after, by the addition into the culture medium of 0.8 mg/ml genetin. After 10 days, cell colonies were pooled. The resulting infected cell lines were maintained in their respective specific media, supplemented with 0.8 mg/ml genetin. These infected cells are referred to as Neo MMT, DB MMT, Neo NMuMG and DB NMuMG cells.

The expression of the Ets1-DB protein was assessed by immunoprecipitation. For metabolic labelling, epithelial cells (150,000 cells/100 mm) were cultured for 24 hours in DMEM-10% FCS. The medium was replaced for three hours in methionine- and cysteine-free MEM containing 250 µCi/3 ml of [35S]methionine and -cysteine (Trans 35S-Label, 1,066 Ci/mmol, ICN). At the end of the experiments, cells were lysed and processed for immunoprecipitation as described by Gilles et al. (1996). Briefly, cell extracts containing identical amounts of TCA-precipitable [35S]-labelled material were incubated in the presence of an anti-Ets1-DB rabbit polyclonal antibody (Gilles et al., 1996) and immunoadsorbed onto Protein-A-agarose beads. Proteins were then separated onto 15% SDS-polyacrylamide gels and the gels were fixed, treated with Amplify (Amersham) and exposed to Hyperfilm MP (Amersham).

Transient transfection with uPA reporter vector
Neo and DB NMuMG cells (22,000 cells/well) and Neo and DB MMT cells (16,000 cells/well) were seeded in 12-well dishes. The next day, the cells were transiently transfected with a uPA reporter vector (uPA-Luc, pGL99, containing a functional Ets-binding site (Stacey et al., 1995). This reporter contains a fragment of the mouse uPA promoter (−2446, −2356) linked to the proximal uPA promoter (−114, +398) and driving the luciferase reporter gene. For each well, 1 µg uPA reporter vector was mixed with 10 µg lipofectamine (Gibco) in OPTI MEM (Gibco) (total volume 110 µl) and incubated for 30 minutes at room temperature. Meanwhile, the cells were washed and incubated in 445 µl OPTI MEM. The DNA/lipofectamine mixture was added to the wells for 6 hours and cells were then incubated overnight following addition of 555 µl of OPTI MEM-20% FCS. The following day, medium was replaced by 2 ml of fibroblast-conditioned medium for 24 hours. Cells were rinsed and lysed in reporter lysis buffer (Promega). The lysates were centrifuged (14,000 rpm for 10 minutes) and pellets were used to quantify DNA in each sample. Luciferase activities in the supernatant were measured in a Berthold Lumat LB 9501 luminometer and values obtained were normalised to the DNA content of the cell extract. Transfection efficiencies were monitored in parallel by assay of β-galactosidase activity, following transfection of a pSG5-Lac Z plasmid. These efficiencies were identical in each experiment for Neo and DB MMT cells (routinely 5-10%) and for Neo and DB NMuMG cells (routinely 10-15%).

Immunocytochemistry
WT, Neo and DB epithelial cells were seeded in the wells of collagen-coated 8-well slides (Lab Tech, Nunc) at a density of 31,000 NMuMG cells/well and 10,000 MMT cells/well. The next day, cells were treated with fibroblast-conditioned medium for 24 hours. Cells were then fixed with methanol and stored at −20°C until use. Cells were rehydrated 5 minutes in phosphate buffered saline (PBS). Blockage and endogenous peroxidase saturation were performed by incubation with 0.3% H2O2 and 1.5% goat serum in PBS. Cells were rinsed 5 minutes in PBS and then incubated with an anti-uPA rabbit polyclonal antibody (Chemicon) diluted 1:100 (v/v) in PBS containing 0.5% Tween-20 (v/v) and 0.5% BSA (w/v). Slides were incubated overnight at 4°C, rinsed in PBS and incubated at room temperature for 30 minutes in goat anti-rabbit biotin-conjugated antibody (Sigma) diluted 1:20 (v/v) in PBS, 0.5% Tween-20, 0.5% BSA. Cells were rinsed in PBS and incubated at room temperature for 30 minutes in Extravidin peroxidase (Sigma) diluted 1:20 (v/v) in PBS, 0.5% Tween, 0.5% BSA. Cells were rinsed in PBS and color development was done with DAB (Sigma) according to the manufacturer’s indications. Cells were counter stained with hematoxylin and slides were mounted in Xam (DBH Laboratory).

uPA activity assays
uPA activity was measured as described by Reinartz et al. (1993). Briefly, following conversion of plasminogen into plasmin by the uPA contained in cell extracts, the quantification of uPA activity was obtained by measuring the formation of a yellow precipitate resulting from the specific cleavage of a synthetic substrate, D-Val-Leu-Lys-paranitroaniline dihydrochloride (S-2251, Sigma) by the produced plasmin. Cells were seeded in 35 mm collagen-coated dishes, at a density of 90,000 cells for WT, Neo and DB NMuMG cells, and 60,000 cells for WT, Neo and DB MMT cells. The next day, the cells were treated with fibroblast-conditioned medium for 24 hours. Cells were rinsed and 750 µl DMEM without Phenol Red (to avoid interference with the colorimetric reaction of the test) was added. The following day, cells were lysed on ice in 200 µl of extraction buffer (100 mM Tris-HCl, pH 7.6, 2 mM EDTA, 0.4% Triton X-100, v/v) and the cell extracts were centrifuged (14,000 rpm for 15 minutes). The pellets were used for DNA quantification and supernatants stored at −20°C until uPA activity measurement. In each well of a 96-well plate, 50 µl of supernatant was added to 50 µl of substrate (0.45 mM, Sigma) and 50 µl of plasminogen (60 µg/mL, Sigma), both diluted in 0.1 M phosphate monosodic, 10 mM EDTA, 0.01% NaN3, 0.01% Triton X-100, pH 7.3. The plate was incubated at 37°C for 5 hours to allow the reaction to take place. Absorbance at 405 nm was measured in a microplate reader (MR 700, Dynatech).

Migration tests
WT, Neo and DB cells were seeded in collagen-coated dishes, at a density of 40,000 NMuMG cells/35 mm dish and 30,000 MMT cells/35 mm dish. After 24 hours of incubation at 37°C, 1.5 ml of fibroblast-conditioned medium and 0.75 ml of Leibovitz medium (L15; Sigma) were added. When appropriate, 200 µg/ml of aprotinin, a serine proteinase inhibitor, was added to WT cell cultures. Each dish was placed in a humidified chamber maintained at 37°C on the stage of an inverted Olympus microscope IMT-2 fitted with a Sony-CCD-IRIS camera and a time lapse video recorder (HS-5300, Mitsubishi). A field containing 6 to 10 cells was chosen and cell migration was recorded overnight. From the time lapse video tape, the migration tract of each individual cell was determined by marking the centre of the nucleus every 30 minutes, beginning within 2 hours after addition of the conditioned medium. Migration speed was obtained by dividing the covered distance by the time of migration.
**Invasion tests**

These tests were performed using 12 mm Transwell chambers containing a 12 μm pore polycarbonate membrane (Costar). The lower and upper sides of the membrane were coated for 3 hours with 0.5 mg/ml of collagen solution. A cell suspension (100,000 cells/0.4 ml) of each cell line (WT, Neo, DB NMuMG cells and MMT cells) was added to the upper chamber and 0.8 ml of NIH3T3-conditioned medium was added to the lower chamber. In addition, aprotinin (200 μg/ml) was added or not to WT NMuMG and MMT cultures. The Transwell chambers were incubated for 18 hours at 37°C. Cells which did not cross the membrane were scraped off the upper side of the membrane with a cotton swab. Cells which had migrated to the lower side were fixed in methanol at –20°C and stained with the intercalating dye, Hoechst 33258. We found by independent cell counting that after 24 hours of culture, in these experimental conditions, the proliferation rate was equivalent for each cell line. The membrane was excised from its support and mounted on a glass slide with Glycergel (Dako). Cells were counted using an Olympus BH2 microscope.

**Three-dimensional cultures in type I collagen gel**

Collagen gels were prepared according to the method of Montesano et al. (1991). Briefly, 8 volumes of a 2 mg/ml collagen solution were mixed at 4°C with one volume of 10× MEM and one volume of 22.2 g/l sodium bicarbonate. For the low density cultures, 300 μl of collagen gel were dispensed in 16 mm wells of a 24-well plate. After 30 minutes at 37°C, a gel was obtained and a second layer was dispensed containing 1,200 cells/300 μl collagen gel. After gel formation, 0.5 ml of appropriate standard medium (containing 10% FCS) was added. These cultures were maintained for 5 days. The culture medium was then replaced by fibroblast-conditioned standard medium-5% FCS (standard medium/fibroblast-conditioned medium, v/v) and cells were further cultured for 4 days. As indicated, some WT cultures were treated with aprotinin (200 μg/ml) during these last 4 days. For the high density cultures, 300 μl of collagen gel were dispensed in 16 mm wells of a 24-well plate. Then, an 8 μl drop containing 24,000 cells was laid onto the centre of the collagen gel surface. Epithelial cells were allowed to attach to the substrate for approximately one hour at 37°C. Then, 300 μl of the collagen gel were gently applied to cover the cells and the plate was incubated for 30 minutes at 37°C. As described, 300 μl of either control medium-5% FCS (standard medium/fibroblast-conditioned medium, v/v) or of conditioned medium-5% FCS (standard medium/fibroblast-conditioned medium, v/v) were added. Cultures were further cultured for 5 days.

At the end of the experiments, both types of three-dimensional cultures were stained with Neutral Red (0.5%, w/v), fixed at 4°C for 16 hours in PBS containing 4% paraformaldehyde, washed in PBS and dehydrated. Whole-mount pictures were taken using either a Nikon SMZ-U binocular for high density cultures or an Olympus BH 2 microscope for the low density cultures. Gels were embedded in paraffin and 7 μm thick sections were serially obtained. Serial sections were transferred to Superfrost/Plus slides (Kindler, Polylabo) and allowed to attach at 37°C for 5 days. The slides were stored at 4°C until their processing for histological analysis and/or in situ hybridization, as described below.

**Histological and in situ hybridization analysis of three-dimensional cultures**

Slides were deparaffinised and rehydrated. For histological analysis, the slides were stained with Nuclear Red for 10 minutes and Picro-Indigo Carmine for 10 seconds, rinsed using tap water, dehydrated and dipped in toluene. Slides were mounted with Xam (DBH Laboratory) and observed using an Olympus BH 2 photo-microscope equipped with a Nomarski lens.

In situ hybridization on slides was performed as described byQuéva et al. (1992). The antisense and sense 35S RNA probes were transcribed from the following cDNA fragments: the 1.6 kb SacI/KpnI

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**Fig. 1.** Ets1-DB protein expression in infected epithelial cell lines. (A) Schematic representation of the viral constructs. The 78 amino acids Ets1-DB protein contains the DNA binding domain of Ets1 and the presumed nuclear transport signal, without the C-terminal inhibitory region of c-Ets1. The cDNA encoding this mutant was cloned in front of the IRES-Neo sequences and inserted into the genome of a MFG virus (MFG-DB). The control virus was obtained from the same construction, lacking the Ets1-DB domain (MFG-Neo). ψ+· SD and SA are the packaging, splice donor, and acceptor sites, respectively. (B) Expression of the mutant Ets1-DB protein. Expression of the protein was analysed in NMuMG and MMT cells by immunoprecipitation using wild-type cells (WT), MFG-Neo (Neo) and MFG-DB (DB) infected cells. The arrow indicates the presence of the Ets1-DB protein (~13 kDa). Molecular mass markers are indicated on the right.
fragment of mouse c-ets1 cDNA (Chen, 1990) cloned into the Bluescript KS (Stratagen); the 660 bp PstI/HindIII fragment of mouse uPA cDNA (Belin et al., 1985) cloned into pSP64 and pSP65 (Promega). Since this c-ets1 antisense probe can hybridise the Ets1-DB sequence, this probe was not used to detect c-ets1 expression in DB cells. After development, cells were stained by Hoechst 33258, mounted in Glycergel and observed under a double illumination using an Olympus BH2 photo-microscope with epifluorescence for Hoechst staining and a dark-field condenser for silver grain detection.

RESULTS

Epithelial cell infection and expression of the Ets1-DB protein

Packaging GP+E86 cells were transfected with either the MFG DB or the MFG Neo retroviral constructs in order to produce recombinant ecotropic retroviruses. The supernatant of these cells containing viral particles was used to infect NMuMG and MMT epithelial cells (Fig. 1A). After selection in geneticin for 10 days, the colonies were pooled and the expression of the Ets1-DB protein was determined by immunoprecipitation (Fig. 1B). In DB NMuMG and DB MMT cells, the anti-Ets1 antibody recognised a protein migrating at 13 kDa, the expected size for the Ets1-DB protein.

The Ets1-DB mutant inhibits uPA expression and activity

Constitutive expression of the Ets1-DB protein in the infected epithelial cells was expected to cause inhibition of transactivation of promoters in which functional Ets-binding sites have been identified, such as the uPA promoter (Fafeur et al., 1997; Rorth et al., 1990; Stacey et al., 1995). We examined
this possibility by transfecting transiently a uPA-Luc reporter vector in the infected cell lines (Fig. 2A). By using a pSG5-Lac Z control plasmid, transfection efficiencies were found to be identical for Neo and DB cells (see Materials and Methods). In DB NMuMG cells, the activation of the uPA-Luc reporter was reduced to 80% compared with Neo NMuMG cells. A 45% inhibition was found in DB MMT cells compared with Neo MMT cells.

We next examined whether expression of the uPA protein was modified in the DB cells (Fig. 2C). WT and Neo NMuMG cells grew mainly as cell clusters, with few cells presenting a fusiform shape at the periphery of the cell clusters. The presence of uPA was detected by immunocytochemistry in these NMuMG cells, in particular at the periphery of cell clusters. In contrast, DB NMuMG cells formed less compact cell clusters; rather they appeared to spread on the substrate. These DB NMuMG cells displayed a faint uPA signal. MMT cell lines did not grow as cell clusters; rather they presented a scattered homogeneous distribution onto the substrate. The uPA protein was detected in WT and Neo MMT cells, while it was faintly detectable in DB MMT cells. In addition, uPA activity was measured in these NMuMG and MMT DB cell lines; the expression of Ets1-DB mutant induced a 2-fold decrease in uPA activity compared with their respective control cell lines (Fig. 2B).

The Ets1-DB mutant reduces cell migration and invasion in two-dimensional cultures

The migration ability of the infected cell lines was estimated by measuring the speed of individual cells (Fig. 3A). In parallel, experiments were conducted using non infected cell lines treated with 200 µg/ml of aprotinin, a serine proteinase inhibitor which was found to inhibit uPA activity (data not shown) as previously demonstrated in the MDA-MB-231 cancerous epithelial cell line (Stonelake et al., 1997). WT and Neo NMuMG cells migrated with equivalent speeds (29 and 33 µm/hour, respectively) whereas DB NMuMG cells migrated at 12 µm/hour, which represents a 60% inhibition compared with NMuMG control cells. Similarly, WT NMuMG cells treated with aprotinin migrated at 14 µm/hour which corresponds to a 50% inhibition. WT and Neo MMT cells migrated with similar speeds of 36 and 33 µm/hour, respectively. DB MMT cells migrated at 15 µm/hour and WT MMT cells treated with aprotinin migrated at 19 µm/hour, corresponding to a 50% and 40% inhibition compared with MMT control cell lines, respectively.

The invasion ability of the epithelial cell lines was estimated by measuring their ability to migrate through a collagen-coated membrane in Transwell chamber assays (Fig. 3B). Invasion ability was found to be similar for WT and Neo NMuMG cells. It was reduced by approximately 20% both in WT NMuMG cells treated with aprotinin and in DB NMuMG cells compared with the control cells. WT and Neo MMT cells which derive from a mammary tumor showed the same invasiveness rate, and were twofold more invasive than WT and Neo NMuMG cells, which derive from normal mammary gland. The invasion ability was reduced by 80% both in WT MMT cells treated with aprotinin and in DB MMT cells, compared with MMT control cells.

The Ets1-DB mutant impairs tubulogenesis and scattering in low density three-dimensional cultures

WT cells in the absence or presence of aprotinin, Neo and DB cells were cultured at low density in three-dimensional collagen gels. WT and Neo NMuMG cells underwent branching and tubular morphogenesis which resemble normal morphogenesis occurring in the mammary gland in vivo, with tubules showing terminal end buds at their extremities (Fig. 4A,E,B,F, arrows). Serial sections in the WT and Neo NMuMG cultures (Fig. 4I and J) confirmed the presence of extending tubules (Fig. 4I, arrowhead) presenting lumen (Fig. 4J, arrows). WT NMuMG cells treated with aprotinin and DB NMuMG cells formed more spiky and bushy masses than WT and Neo NMuMG cells (Fig. 4C,G,D,H). Sections revealed heaps of cells from which cell alignments extended without luminal structures (Fig. 4K,L), confirming the absence of tubular organisation.

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**Fig. 3.** Effects of the ETS1-DB mutant and aprotinin on cell motility and invasion. (A) The migration of cells plated onto type I collagen-coated wells was recorded using a time lapse video, within a period of 18 hours following addition of fibroblast-conditioned medium, containing or not aprotinin. Grey bar: WT cells (WT), white bar: Neo cells (Neo), black bar: DB cells, lined bar: WT cells treated with aprotinin (Aprot). Results are the means ± s.d., of 12 individual migration speeds from two separate experiments. (B) The invasion ability of the cells was investigated by seeding the cells in the upper chamber of a Transwell chamber containing a type I collagen-coated membrane, with the lower chamber containing fibroblast-conditioned medium. Aprotinin was added or not into the culture medium of the upper chamber. After 18 hours of culture, the cells present on the under side of the membrane were fixed, coloured with Hoechst 33258 and the number of cells across two membrane diameters were counted. Grey bar: WT cells (WT), white bar: Neo cells (Neo), black bar: DB cells, lined bar: WT cells treated with aprotinin (Aprot). Each value is the mean ± s.d. of duplicate samples.
WT and Neo MMT cells generated bushy cell masses from which spindle-shaped cells scattered to the periphery (Fig. 5A,E,B,F). This scattering effect was evidenced in serial sections by the presence of numerous isolated cells invading the gel, presenting a stellate configuration (Fig. 5I,J). In contrast, WT MMT cells treated with aprotinin and DB MMT cells form cell masses with cell alignments, forming cord-like structures (Fig. 5C,G,D,H). Sections showed no lumen in these cord-like structures, but only cell alignments (Fig. 5K,L). These data demonstrate that the expression of Ets1-DB mutant or treatment with aprotinin induce similar morphotypical changes in three-dimensional cultures, i.e. the inhibition of tubular morphogenesis for NMuMG cells and a reduced scattering for MMT cells.

The Ets1-DB mutant impairs tubulogenesis and scattering in high density three-dimensional cultures

High density cultures in collagen gels were initiated by laying a drop containing 25,000 epithelial cells between two collagen gels. These culture conditions allow the rapid formation of a cell mass that can organise or not into an empty vesicle according to the normal or cancerous origin of the cells (Delannoy-Courdent et al., 1996). In control medium, WT and Neo NMuMG cultures showed identical aspects: the cell mass extended laterally between the two gel layers, with short peaks delimiting the invasion front (Fig. 6A,E). Sections of these gels revealed that the seeded zone reorganised into an empty vesicle delimited by two continuous cell layers (Fig. 6C,G). The addition of fibroblast-conditioned medium resulted in the formation of tubules budding from these layers (Fig. 6B,F, arrows). They formed a three-dimensional network infiltrating the gel and extending from the initial deposit to the periphery. Sections revealed the presence of a lumen (Fig. 6D, arrow) or extending tubules (Fig. 6H, arrow). In contrast DB NMuMG cells, both in the presence or absence of fibroblast-conditioned medium, showed only short peaks at the invasion front and never developed into tubules infiltrating the gels (Fig. 6I,J). Sections of these cultures revealed only empty vesicles (Fig. 6K,L).

Fig. 4. Three-dimensional cultures of NMuMG cells at low density. NMuMG cells suspended in collagen gels were cultured for 5 days, then the medium was replaced by fibroblast-conditioned medium and the cells were cultured for an additional period of 4 days. At the end of the experiments, cells were stained with Neutral Red. Pictures of the whole-mounts were made using two magnifications. Bars: 100 μm (A,B,C,D); 50 μm (E,F,G,H); 25 μm (I,J,K,L). Serial sections (I,J,K,L) were stained with Nuclear Red and Picro-Indigo Carmine and observed using a Nomarski system. (A,E,I) WT cells (WT); (B,F,J) Neo cells (Neo); (C,G,K) DB cells (DB); (D,H,L) WT cells treated with aprotinin during the last 4 days (Ap). See Results for further information.

WT and Neo MMT cells generated bushy cell masses from which spindle-shaped cells scattered to the periphery (Fig. 5A,E,B,F). This scattering effect was evidenced in serial sections by the presence of numerous isolated cells invading the gel, presenting a stellate configuration (Fig. 5I,J). In contrast, WT MMT cells treated with aprotinin and DB MMT cells form cell masses with cell alignments, forming cord-like structures (Fig. 5C,G,D,H). Sections showed no lumen in these cord-like structures, but only cell alignments (Fig. 5K,L).

These data demonstrate that the expression of Ets1-DB mutant or treatment with aprotinin induce similar morphotypical changes in three-dimensional cultures, i.e. the inhibition of tubular morphogenesis for NMuMG cells and a reduced scattering for MMT cells.
WT and Neo MMT cells were able to migrate and invade the gel around the initial cell deposit, producing a three-dimensional network of spindle-shaped cells and showing an irregular and indented invasion front. These effects were observed both with control and fibroblast-conditioned medium (Fig. 7A,B,E,F). Sections revealed that in control medium, MMT cells did not form empty vesicles delimited by two cell layers, as observed with NMuMG cell lines (compare Fig. 6C,G and Fig. 7C,G). Rather, they form a single layer, with isolated cells scattering from the margin of this layer corresponding to the initial seeded zone (Fig. 6C,G). Fibroblast-conditioned medium increased the dispersion of these cells, which did not form tubules but got away from the periphery of the central area (Fig. 7D,H). In both culture conditions, the invasion front of DB MMT cells was more regular than the one of WT and Neo MMT cells (Fig. 7I,J, arrowheads, and compare IJ and A,B,E,F). Sections showed that the cells did not scatter and grew as a compact layer (Fig. 7K,L).

In agreement with the results obtained with the low density cultures, in high density cultures we found that the expression of the Ets1-DB mutant resulted in the impairment of the tubulogenic property of NMuMG cells and of the scattering and invasive properties of MMT cells.

The inhibition of tubulogenesis and scattering elicited by the Ets1-DB mutant correlates with inhibition of uPA expression

On sections of the high density cultures, we performed in situ hybridization analysis, to investigate the expression of c-ets1 and uPA mRNAs. In WT and Neo NMuMG cells cultured in control medium, both c-ets1 and uPA signals were detected (Fig. 8A,D,G,J). The intensity of these signals was low compared with those detected in cells cultured in fibroblast-conditioned medium (Fig. 8B,C,E,F,H,I,K). In the presence of fibroblast-conditioned medium the expression of c-ets1 and uPA was detected in the tubules extending from the two cell layers (Fig. 8B,C,E,F,H,I,K, arrows). In DB NMuMG cells, both in control and in fibroblast-conditioned medium, no uPA signal was detected (Fig. 8L,M).
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The c-ets1 and uPA mRNAs were also observed in WT and Neo MMT cells (Fig. 9). Overall, the intensity of the signals was found to be higher in control MMT cells than in control NMuMG cells (compare Figs 8 and 9). In control medium, isolated cells which infiltrated the surrounding gel expressed c-ets1 and uPA mRNAs (Fig. 9A,C,E,G). The intensity of the c-ets1 and uPA signals, in cells scattering from the periphery of the seeded zone was higher in fibroblast-conditioned medium than in control medium (compare Fig. 9B,D,F,H and A,C,E,G). The uPA signal was low in DB MMT cells growing as a compact layer, both in the control and in fibroblast-conditioned medium (Fig. 9I,J).

These data show a co-expression of c-ets1 and uPA mRNAs occurring during tubulogenesis of NMuMG cells and scattering of MMT cells. The expression of the Ets1-DB mutant in these cell lines resulted in the impairment of uPA mRNA expression, which correlated with a perturbation of their morphogenetic and/or invasive properties.

**DISCUSSION**

We have previously shown that expression of c-ets1 and uPA genes is associated with mammary epithelial cell tubulogenesis or neoplastic scattering (Delannoy-Courdent et al., 1996). To address the question of the functional implication of c-Ets1 and
Ets family members during these processes, we examined the effect of the expression of the DNA-binding domain of c-Ets1 in cellular situations in which we could measure epithelial cell motility and invasion. This Ets1-DB mutant was able to inhibit uPA gene expression and activity, and to reduce the migrating and invasive properties of mammary epithelial cells, leading to the impairment of normal tubular morphogenesis and of cancerous invasive properties.

As previously shown with human mammary epithelial cells (Delannoy-Courdent et al., 1996), we demonstrate here with murine mammary epithelial cells, issued from normal or cancerous tissue, that the expression of c-ets1 and uPA mRNAs correlates with the occurrence of invasive processes in collagen gels. Following addition of fibroblast-conditioned medium, NMuMG cells undergo branching and tubular morphogenesis; c-ets1 and uPA mRNAs were detected in the tips of the forming tubules. MMT cells which scatter and invade the collagen gels both in basal and fibroblast-conditioned medium, without forming tubular morphogenesis, were found to express both c-ets1 and uPA mRNAs.

The gene expression and activity of uPA were reduced both in NMuMG and MMT cell lines expressing the Ets1-DB mutant. In two-dimensional cultures, when we examined the whole cell population, inhibition of uPA expression or activity did not exceed 50%. Although this inhibition might reflect a partial ability of the Ets1-DB mutant to inhibit uPA expression,
it is possible that other transcription factors, which do not belong to the Ets family, take part in the transcriptional activation of the uPA gene. In contrast, a drastic decrease in uPA transcripts was found at the cellular level in DB cells grown in three-dimensional cultures. This result suggests that Ets family members play a crucial role in the transcriptional activation of uPA gene when epithelial cells are cultured in collagen gels.

The fact that the Ets1-DB mutant was able to decrease uPA gene expression and activity in mammary epithelial cells, led us to investigate their migrating and invasive properties. Cell migration and invasion has been associated with the activation of extracellular matrix degradation. This degradation involves serine proteinases (uPA, plasmin), metalloproteinases (collagenases, stromelysins) and lysosomal proteinases (cathepsins). The relative importance of these proteinases in the degradation of basement membrane by MDA-MB-231 breast cancer cells has been investigated using a variety of inhibitors (Stonelake et al., 1997). Aspartic and cysteine proteinase inhibitors were found to have little effect, whereas aprotinin, a serine proteinase inhibitor, demonstrated that the plasmin system plays a major role. Addition of an anti-uPA antibody inhibited cell migration from arterial smooth muscle explants, whereas the addition of plasminogen increased this migration (Kenagy et al., 1996). Migration of endocardial-derived mesenchymal cells was also found to be inhibited by...
the addition of uPA antisense oligonucleotides (McGuire and Alexander, 1993). In mouse models, injection of uPA synthetic inhibitors or of anti-uPA antibodies was found to cause inhibition of local invasion and metastasis (Alonso et al., 1996; Ossowski et al., 1979).

MMT and NMuMG cells expressing the Ets1-DB mutant, migrated with a lower speed than WT and Neo cells. A similar reduction of migration speed was also obtained by treating non infected cells with aprotinin. In addition, invasion through collagen I coated membranes was reduced both in the DB cells and in the aprotinin-treated WT cells. Taken together, these results demonstrated that the Ets1-DB mutant can decrease cell migration and invasion, most likely by its ability to inhibit uPA expression and activity. These data are consistent with the work of Chen et al. (1997) showing that c-ets1 antisense oligonucleotides reduce the levels of uPA mRNAs and inhibit VEGF-induced invasiveness of endothelial cells through Matrigel-coated membranes. In three-dimensional cultures in collagen gels, expression of the Ets1-DB mutant impaired the ability of NMuMG cells to form tubular structures and of MMT cells to invade the gels as scattered structures. Inhibition of serine proteinase activity by aprotinin in non infected cells gave similar results. These data, together with the lack of expression of uPA in the same experimental culture conditions, suggest that the down regulation of the uPA proteolytic activity by the Ets1-DB mutant leads to different morphological changes according to the normal or tumoral origin of the cells.

Beside extracellular matrix degradation by uPA, these morphological effects can also be related to a modification of adhesion properties. In two-dimensional cultures, DB NMuMG cells appeared to spread more than control NMuMG cells. In three-dimensional cultures, histological analysis demonstrated that DB MMT cells had an impaired ability to form scattered structures; rather in high density cultures they grow as a compact sheet, corresponding to the initial deposit of cells. This suggests that the expression and/or activity of molecules involved in cell-cell and cell-matrix association are also modified in infected NMuMG and MMT cells.

Adhesion molecules and cytoskeleton elements are involved in normal and pathological remodelling (Adams and Watt, 1993; Gille and Swerlick, 1996). Integrins are cell surface receptors mediating interactions of the cells with the extracellular matrix. Decreased expression of α2 integrin, a collagen and laminin receptor, in mammary cells was found to be associated with increased cell motility (Keely et al., 1995). The presence of Ets binding sites in the αV integrin gene promoter suggests that Ets transcription factors can regulate expression of this gene (Donahue et al., 1994; Zutter et al., 1994). Vimentin is a cytoskeleton molecule, which belongs to intermediate filaments and can interact with integrins (Biddle and Spandau, 1996). Invasive mammary carcinoma cells were found to specifically express vimentin. In these cells, c-ets1 expression correlated with vimentin expression (Gilles et al., 1997). These data suggest that the Ets transcription factors can also regulate cellular adhesive properties. In addition, it is interesting to point out that the cell adhesion system can collaborate with the urokinase proteolytic system. For example, αVβ3 integrin, a vitronectin receptor can mediate cell migration when the uPA/uPA-receptor activation system is induced (Yebra et al., 1996). It is therefore possible that the inhibition of the uPA proteolytic activity by the Ets1-DB mutant is related in a more complex manner with cell adhesion properties. This hypothesis will require a more detailed study of the cellular genes modified by the Ets1-DB mutant.

In addition to c-Ets1, other members of the Ets family can play a role in mammary epithelial cell migration and invasion. In three-dimensional collagen gels, NMuMG cells expressed c-ets1 as well as pca-3, erm and er-81 during tubular morphogenesis; MMT cells constitutively expressed these
genes (A. Chotteau-Lelièvre et al., unpublished observations). The expression of the pea-3 group genes is associated with the metastatic ability of epithelial mammary cells in mouse (Trimble et al., 1993) and with the invasive capacity of human cancerous mammary epithelial cells (Baert et al., 1997). Moreover, expression of E1A-F, a pea-3 homologous gene, induces the 92 kDa gelatinase B expression in non invasive MCF-7 cancerous mammary cells and increases their invasive ability after implantation in nude mouse (Kaya et al., 1996). These observations indicate that several members of the Ets family, distinct from c-Ets1, can be involved in migration and invasion of epithelial mammary cells. The DNA binding domain of different members of the Ets family has been shown to specifically inhibit transcription from promoters containing Ets binding sites (Galang et al., 1994; Langer et al., 1992; Wasylyk et al., 1994). Since Ets proteins have similar overlapping DNA binding specificity, it is likely that the DNA binding domain of c-Ets1 interferes not only with the function of c-Ets1, but also with the function of other Ets family members present in NMuMG and MMT cells.

In conclusion, we demonstrated that the expression of the DNA-binding domain of the c-Ets1 transcription factor decreased the expression of uPA gene and the activity of this serine proteinase in mammary NMuMG and MMT epithelial cells. These effects correlated with reduced cell migration and invasion in two-dimensional cultures and impairment of tubulogenesis of NMuMG and scattering of MMT cells in three-dimensional cultures. Similar results were obtained when non infected epithelial cells were treated with aprotinin, a serine protease inhibitor. We propose that the biological effects of Ets1-DB expression are mediated by the inhibition of uPA which results in a reduction of extracellular matrix degradation. This does not exclude the possibility that adhesion and cytoskeleton molecules can play a role in these effects. Thus, the Ets1-DB mutant demonstrates the involvement of Ets transcription factors in the control of mammary epithelial cell migration, invasion and morphogenesis.

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