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Report

Involvement of TGF-βs/TβRs system in tumor progression of murine mammary adenocarcinomas*

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Summary

We studied the expression of TGF-β/TβR system and its biological role in tumor development, in M3 and MM3 murine mammary adenocarcinomas with different metastasizing capability and in LM3 and LMM3 derived cell lines. All the studied cells secreted TGF-βs and expressed TβRs. While the proliferation of the poorly metastatic M3 cells was significantly inhibited by 4 ng/ml TGF-βs, the highly metastatic MM3 cells were only slightly inhibited in response to the highest dose used. LM3 and LMM3 cells, highly invasive and metastatic, were totally refractory to TGF-β antiproliferative effect. The role of TGF-β in modulating key proteolytic cascades in tumor progression was also studied. TGF-βs enhanced metalloproteinases production in all the studied cells while induced a stimulatory net effect on plasmin system activity only in the more metastatic cells. Our results in this murine mammary tumor lineage support the concept that dissociation of TGF-β regulated growth control versus proteolytic enzyme pathways promotes tumor dissemination.

Introduction

The establishment of tumor metastasis is the final step of a highly selective process that involves uncontrolled extracellular matrix (ECM) invasion and an efficient response to mitogenic signals in the new microenvironment [1].

TGF-βs belong to an evolutionary conserved superfamiliy of versatile cytokines which strongly regulate cell growth and differentiation as well as cellular migration, morphogenesis, angiogenesis and immune responses [2–4]. Three highly homologous mammalian TGF-β isoforms (TGF-β1, -β2 and -β3), encoded by different genes, have been identified and they can exhibit distinct effects in a variety of *in vitro* biological assays [5, 6]. TGF-βs are secreted as latent high molecular weight complexes unable to bind to their specific receptors [7]. The mature and biologically active form of TGF-β is a 24 kDa homodimer protein, released from the precursor by *in vivo* degradation by enzymes such as plasmin or thrombospondin-1 [8] or by various *in vitro* mechanisms such as heat, extreme pH and selected chaotropic agents [9, 10].

TGF-βs exert their effects through binding to high affinity receptors. There have been reported three TGF-β receptors, known as TβRI (53–65 kDa), TβRII (70–85 kDa) and TβRIII (200–400 kDa). Types I and II receptors have been shown to constitute a new superfamily of single pass, transmembrane serine–threonine kinases directly involved in TGF-β signal transduction. TβRIII is a proteoglycan which functions mainly by increasing ligand affinity and presenting it to the catalytic types I and II receptor complex. Moreover, certain evidence correlates TβRII expression with some biological activities of TGF-β [11].

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TGF-β inhibits proliferation of almost all non-neoplastic epithelia in culture while enhances the growth of cells of mesenchymal origin. As with normal cells, the different TGF-β isoforms can exert either positive or negative effects on neoplastic cells [12]. Malignant progression is associated with acquisition of insensitivity to growth inhibition by TGF-β. In addition, overexpression of TGF-β isotypes has been associated with tumor progression in many different human tumors, such as sarcomas, glioblastomas and melanomas, as well as prostate, pancreas and breast cancers [12, 13].

Matrix metalloproteinases (MMPs) consist of a large family of secreted and membrane calcium-dependent endopeptidases which play a key role in ECM remodeling. More than 25 different types of MMPs, including collagenases, gelatinases, stromelysins and membrane-type MMPs (MT-MMPs) are already described [14]. With the exception of the last group, MMPs are always secreted as procollagenases (a catalytic latent form) which are subsequently activated in the extracellular environment [15]. Since several years ago MMPs have been associated with malignancy. Particularly, both type IV collagenases, gelatinase-A (or human 72 kDa) and gelatinase-B (or human 92 kDa), have been closely associated with the invasive and metastatic potential of various tumor cell types, including mammary carcinomas [14]. MMPs activity is specifically controlled by the inhibitory action of the TIMP glycoproteins family [16].

The plasmin system, composed of tissue-type (tPA) and urokinase-type (uPA) plasminogen activators, is another family of enzymes involved in tumorigenesis [17]. uPA is a serine-protease synthesized as a single-chain zymogen (pro-uPA) which can be extracellularly activated by many different mechanisms [18]. Once activated, uPA binds to its specific membrane receptor uPAR and catalyzes the cleavage of the inactive zymogen plasminogen to the active trypsin-like serine-protease plasmin [19–21]. This activity is regulated by the covalent binding of glycoproteic uPA inhibitors, PAI-1 and PAI-2, to uPA/uPAR complex. So, a proteolytic cascade is initiated by action of the plasmin system through ECM degradation, leading to elimination of barriers to cellular migration with the following tumor invasion [22].

It is known that TGF-β is involved in ECM remodeling control by up- or down-regulating many of its components like interstitial collagen, fibronectin, stromelysin and proteoglycans, and components of the plasmin system [23]. Moreover, it has been demonstrated that TGF-β may also regulate the activity of enzymes able to modulate the metastatic phenotype, like MMPs and uPA and their specific inhibitors, and that this regulation is strictly dependent on the cellular type [24–26]. In addition, the role of TGF-β in morphogenetic processes of extracellular matrix formation may also promote the growth and metastatic potential of certain tumor types [12].

The availability of TGF-β can be regulated at every level: production, secretion, activation and clearance. Currently, the mechanisms regulating these TGF-β levels are poorly understood. Although TGF-β can be tumor suppressive, there is increasing evidence that its secretion by tumor cells and/or stromal cells within the peritumoral microenvironment can contribute to tumor maintenance and progression.

Because the precise functions of TGF-β in tumor progression are yet unknown, the aim of the present work was to study the role of the complete TGF-βs/TβRs system in a BALB/c tumor model composed of two closely related mammary adenocarcinomas, M3 and MM3, with different metastatic capabilities, and the derived highly invasive and metastatic LM3 and LMM3 cell lines [27, 28]. In previous works we demonstrated that M3 and MM3 tumor cells were able to respond to purified IGF and EGF upregulating uPA activity but they did not respond to the mitogenic effect of these growth factors [29, 30].

Our results suggest that TGF-β effects on cell proliferation, invasive capability and proteolytic activity of this murine mammary tumor model would be favoring tumor progression.

Materials and methods

Tumors, cell lines and cell cultures

We used primary cultures of two closely related murine mammary adenocarcinomas, M3 and MM3. Only 40% mice inoculated with M3 cells present a low number of lung metastases. MM3 tumor is a highly metastatic variant of M3 that was selected by an *in vivo* procedure [31] and presents a higher incidence (95%) of lung metastases. Both tumors are maintained by subcutaneous trocar implants into BALB/c mice. All animals studies were conducted in accordance with the NIH Guide for the Care and the Use of Laboratory Animals.

Primary cell cultures of M3 and MM3 tumors were obtained by enzymatic digestion of solid frag-

ments with 0.01% Pronase/0.0035% DNAase (Sigma, St. Louis, MO), as previously described [32]. Cells were cultured in medium (MEM 41500, Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS, GEN, Buenos Aires, Argentina), 2 mM L-glutamine and 80 μg/ml gentamicin. M3 and MM3 monolayers were composed primarily of epithelioid polyhedric cells with less than 2% of contaminating fibroblast-like cells [32].

Besides, we used LM3 and LMM3 cell lines, established from successive *in vitro* passages of M3 or MM3 cells, respectively [28]. Cell lines were maintained in MEM with 5% FBS and were harvested with 0.25% trypsin/0.01% EDTA (Sigma) at confluency. LM3 cell line, in contrast to its parental tumor, acquired a highly invasive and metastatic *in vivo* behavior when inoculated into mice, similar to LMM3 cell line.

In bioassays, Mv1Lu mink lung cells (CCL-64 cells, ATCC) were used as TGF-β-like activity reporter cells because of their extremely sensitive response to TGF-β antiproliferative effect [33]. This normal epithelial cell line was maintained in a high-glucose formulation D-MEM medium supplemented with sodium bicarbonate (3.7 g/l) and 10% FBS [33].

MEM added with 5% FBS or with 10% FBS are referred as 'complete medium' for cell lines or for primary cultures, respectively. All cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and were periodically controlled for mycoplasma contamination by Hoescht's technique.

Preparation of conditioned media (CM), cell lysates (CL) and tumor homogenates

To prepare CM, cells were grown to subconfluency (70–90%) in complete culture medium and, after extensive washes with PBS to eliminate serum traces, fresh serum-free MEM was added. After 24 h, CM were collected and cells and debris were removed by centrifugation at 5000 rpm. CL were obtained from subconfluent monolayers collected in a RIPA buffer containing protease-inhibitors (5 mM DTT, 10 μg/ml PMSF, 1 μg/ml aprotinin). Tumor homogenates were obtained by processing of solid tumor fragments in a lysis buffer (20 mM Tris (pH 7.4), 0.1 M NaCl, 5 mM $MgCl_2$, 20 mM NaF, 1% NP40, 0.1 mM β-mercaptoethanol). Then, tumor homogenates were sonicated, ultracentrifuged (45 min, 40,000 rpm, 4°C), and stored at −80°C until use. All other sample were stored at −20°C until use. Protein content of samples was determined by Bradford's method [34].

TGF-β treatments

For TGF-β treatments, cells grown for 24 h in complete culture medium were extensively washed with PBS and further incubated with TGF-β1, TGF-β2 or TGF-β3 (purified human recombinant proteins, Oncogene Research Products, Calbiochem, Cambridge, MA) in MEM with or without FBS, for different time periods. All assays were performed with the three TGF-β isoforms and concentrations used ranged between 0.04 and 40 ng/ml.

Quantification of TGF-β production

Bioassay. To assay for secreted TGF-β activity, Mv1Lu cell line was seeded at 2×10^4 cells/50 μl/well onto a 96-well plate (Corning Costar Corporation, NY) in D-MEM supplemented with 0.4% FBS. An hour later, when cells were attached to the plastic surface, 50 μl of different dilutions of untreated CM were assayed to measure only active TGF-β present in the CM. Cells were also assayed with CM pre-treated with 5N HCl followed by neutralization with NaOH/Hepes [33] or heated 5 min at 80°C [35]. Both treatments activate latent TGF-βs present in the CM, allowing the measurement of total secreted TGF-β (active plus latent), reported here as 'activated CM'. On each assay, some Mv1Lu cells were treated with different concentrations of purified TGF-β1 (0.05–5 ng/ml) to be used as a standard curve. After 20 h incubation, cells were pulsed with 1 μCi/well of methyl-tritiated thymidine ([^{3}H]-thymidine, Dupont NEN Research Products, Boston, MA) and further incubated for 4–8 h. Cells were then lysed with 6 M guanidinium chloride and harvested in glass hollow fibers with a multiwell cell harvester (Nunc, Roskilde, Denmark). Cell-incorporated radioactivity was measured by liquid scintillation counter. To confirm that TGF-β was responsible for Mv1Lu cells growth inhibition, the same experiment was performed by assaying control or heat-activated CM previously incubated (2 h) with 10 μg/ml of a specific anti-TGF-β neutralizing pan-antibody (Anti-TGF-β1,2,3; Genzyme Diagnostics, Cambridge, MA) or with 10 μg/ml normal mouse IgG as control.

Expression of TGF-β proteins. TGF-β protein levels were analyzed in CL by western blot. Samples were diluted in sample buffer (0.5 M Tris (pH 6.8), 10%

SDS, 1 mg/ml bromophenol blue, 5% β-mercaptoethanol), boiled for 5 min and then resolved by 4–15% SDS-PAGE, as described [36]. Electrophoresis gels were transferred (25 V; 60 min) to PVDF membranes (Hybond-P, Amersham Pharmacia Biotech, Buckinghamshire, UK) using a Semidry-transfer method (BioRad, Hercules, CA). Membranes were blocked (1 h) at room temperature with western blot-blocking buffer (PBS, 0.1% Tween 20, 5% skimmed milk) and probed (overnight, 4°C) with 1:200 rabbit polyclonal anti-TGF-β1, anti-TGF-β2 or anti-TGF-β3 (Santa Cruz Biotechnology, Santa Cruz, CA) directed against a region inside the mature TGF-β molecule. After incubation, membranes were washed extensively with washing buffer (PBS, 0.1% Tween 20) and subsequently blotted with 1:1000 anti-rabbit peroxidase-conjugated antibody (Amersham Pharmacia Biotech) for 1 h at room temperature. Then, an enhanced chemiluminescence method (ECL reagent, Amersham Pharmacia Biotech) was used to reveal membrane bands. Electrophoretic band images were analyzed by densitometry (Molecular Analyst^{TM} GS-700, BioRad).

An anti-actin mAb (Santa Cruz Biotechnology) was used as control of the assay. Membranes previously blotted for TGF-β expression were incubated with stripping buffer (62.5 mM Tris, 2% SDS, 100 mM β-mercaptoethanol, pH 6.7) for 40 min at room temperature, blocked and incubated with 1:500 mouse anti-actin antibody and subsequently blotted with 1:2000 anti-mouse peroxidase-conjugated antibody (Vector, Burlingame, CA).

Expression of TGF-β receptors

CL were also analyzed by 4–7.5% SDS-PAGE to determine TGF-β receptors expression. After electrophoresis, membranes were transferred, blocked, and probed with 1:250 rabbit polyclonal anti-TβRI or anti-TβRII (Santa Cruz Biotechnology) and then with 1:1000 dilution of a peroxidase-conjugated anti-rabbit secondary antibody. Membranes were revealed and analyzed as described above. Besides, TβRIII expression at protein level was determined by dot blot. Briefly, the same samples used for TβRI and TβRII detection were seeded onto PVDF membranes. Then, membranes were treated with 1:500 goat polyclonal anti-TβRIII (R&D systems, Minneapolis, MN) and then with 1:2000 peroxidase-conjugated anti-goat secondary antibody (R&D systems). Membranes were revealed as described above.

Cell proliferation assays

[^{3}H]-thymidine incorporation assay. M3 and MM3 primary cultures (1.2×10^4 cells/well) as well as LM3 and LMM3 cells (0.6×10^4 cells/well) were seeded onto 96-well plates in complete medium. Mv1Lu cells were used as positive control. Twenty-four hours later, cultures were down-shifted to medium with 2% FBS (to minimize the effects due to serum-derived TGF-β and other growth factors) and supplied or not with purified TGF-β, as mentioned above. After 24 h treatment, cells were incubated with [^{3}H]-thymidine (1 mCi/ml) for additional 18 h. Cells were then lysed, harvested and analyzed as described above.

The following studies were also performed on LM3 and LMM3 cells.

Cell counting. To measure the effect of TGF-β on cell proliferation, 1.2×10^4 cells/well were seeded onto 24-well plates (Corning) in complete medium. Then, cells were treated with TGF-β for 48, 72 or 96 h in MEM plus 2% FBS, after which they were trypsinized, dyed with Trypan Blue and counted under a microscope. Both live and death cells were recorded.

Cell cycle analysis. To determine if TGF-β were able to modulate any phase of the cell cycle, image analyzing microscopy (Cell Analysis System (CAS)) was employed. Briefly, LM3 and LMM3 cells (1.2×10^4 cells/ml) were seeded onto tissue culture chamber-slides (Lab-Tek, Nunc) in complete medium and incubated until subconfluency. Then, cells were treated 24 or 48 h with 4 ng/ml TGF-β, after which slides were fixed and stained with Feulgen for DNA content evaluation. Two hundred cells of each treatment were analyzed. Besides, we calculated the mitotic index based on the analysis of a thousand cells of each TGF-β treatment or control cells.

Clonogenic assay. Clonal cell growth was studied as previously described [28]. Briefly, monodispersed LM3 or LMM3 cells (1.3×10^3 cells/ml) were seeded onto 6-well plates (Corning) in complete medium, to detect the capability of isolated cells to form cell colonies. After 24 h, cells were treated with 4 ng/ml TGF-β1, -β2 or -β3 in complete medium during 6 days. Fresh MEM with FBS, supplemented or not with TGF-β, was replaced every 72 h. After 7 days, cells were washed, fixed with methanol and dyed with Giemsa. Scanned images of the plates were analyzed by densitometry.

Enzymatic assays

We studied TGF-β effects on the production of the proteolytic enzymes metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), and uPA-specific inhibitor PAI, in 24 h CM and CL from M3 or MM3 primary culture cells and LM3 or LMM3 cell lines, either treated or not with TGF-β.

MMPs activity. The biological activity of MMPs was studied by quantitative gelatin zymography as described [37]. CM were analyzed in 4–9% SDS-PAGE co-polymerized with 0.1% gelatin (Sigma). Gels obtained were incubated at 37°C in a buffer (0.25 M Tris, 1 M NaCl, 0.025 M $CaCl_2$ (pH 7.4)), dyed with 0.1% Coomassie Brilliant Blue G-250, and analyzed by densitometry. Then, OD values were corrected in function of cellular protein content and finally MMP activity was expressed as OD per mg cell protein. To confirm that the activity measured in cell CM was specifically due to MMPs, we also incubated gelatin zymograms with 25 mM EDTA, which inhibits MMP activity, or with 5 mM aprotinin or 5 mM PMSF, which inhibit serine-protease-like activity. MMP enzymatic activity was observed as cleared zones in the blue-stained background.

Plasmin system

(1) *Secreted uPA activity.* Levels of secreted uPA activity were determined by radial caseinolysis assay [38]. Briefly, CM (10 μl) were placed into a 3 mm diameter well punched in a gel composed of 2.5% agarose (Biodynamics SRL, Buenos Aires, Argentina), 33 mg/ml skimmed dry milk as casein source, 2 μg/ml plasminogen (Chromogenix, Mölndal, Sweden) and 0.2 M Tris–HCl (pH 8.0). Human urokinase (Serono, Buenos Aires, Argentina) (0.1–25 IU/ml) was used to construct a standard curve. Plates were incubated overnight at 37°C in a humidified chamber. Diameters of the lytic areas were measured, referred to the standard curve and then data were normalized by cellular protein content, as previously described [38].

(2) *Membrane bound uPA (mb-uPA) activity*. To determine whether any TGF-β isoform was able to modify mb-uPA activity, we performed an assay described by Jänkun et al. [39]. Subconfluent monolayers, treated or not with TGF-β, were treated 5 min with 50 mM glycine-HCl buffer containing 0.1 M NaCl (pH 3.0) (Acid Treatment or AT) and then neutralized with 0.5 M Tris–HCl (pH 7.8), or were treated only with 0.5 M Tris–HCl (pH 7.8) (Neutral Treatment or NT). Cellular AT allows to efficiently detach uPAR-bound uPA at the cell surface. So, all uPA detected after this treatment is intracellular uPA. Then, monolayers were lysed with 1% Triton X-100 and the obtained CL were assayed for uPA activity by radial caseinolysis, as described above. Mb-uPA activity was calculated as the difference between total cell-associated uPA (NT) and intracellular uPA (AT) activities.

(3) *uPAR expression*. To determine protein levels of murine uPA receptor (uPAR), western blot analysis was performed. Briefly, CL were analyzed by 4–10% SDS-PAGE and gels were transferred to PVDF membranes, as described above. Then, membranes were blocked 1 h with PBS, 5% skimmed milk, washed and blotted with 1:1000 rabbit polyclonal anti-uPAR (Ab-2) (Oncogene Research Products). Subsequently membranes were blotted with 1:1000 anti-rabbit peroxidase-conjugated secondary antibody. Bands were revealed as described above and were analyzed by densitometry.

(4) *Plasminogen activator inhibitor (PAI)-like activity*. PAI-like activity was determined by reverse zymography, as previously described [40]. Briefly, CM samples were electrophoresed in 4–10% SDS-PAGE co-polymerized with 33 mg/ml skimmed dry milk and 2 μg/ml plasminogen. Gels were washed (PBS, 1% Triton X-100), rinsed in water and incubated 2 h in a buffer (20 mM Tris, 15 mM EDTA (pH 8.3)) supplemented with 1 IU/ml purified urokinase. Then, gels were dyed with Coomassie Brilliant Blue G-250. PAI-like activity was visualized as blue bands in a lighter background.

Invasion assay

Transwell cell culture chambers (Corning) were used for invasion assays. The filters used (8 μm membrane pores) were previously coated with 0.1% gelatin on the lower side and with a thin layer (250 μg/ml) of reconstituted basement membrane Matrigel (Becton Dickinson Labware, Bedford, MA) on the upper side, and then dried 24 h. Later, 2×10^5 cells in 0.15 ml MEM supplemented with 1% FBS were seeded on the upper face of the chamber. The lower chamber contained human cellular fibronectin (16 μg/ml) (Sigma) in 0.5 ml MEM, as chemoattractant. Cells were treated

20 h with 4 ng/ml TGF-β1, in culture medium. Those cells that invaded Matrigel passed through the pores and reattached on the lower surface of the filter. Only these cells were considered invasive, so cells on the upper surface of the filter were completely removed by wiping them with a cotton swab. Membranes were fixed in Carnoy and stained with Hoescht 33258. The nuclei of those cells that invaded Matrigel were counted in 400× fields under a fluorescence microscope (Eclipse E400, Nikon). Data were expressed as fold of control cells without treatment.

Statistical analysis

Analyses were performed on mean values from replicate experiments, using one-way analysis of variance (ANOVA) and Scheffe's test or Student's *t*-test. For bioassay, ANOVA and Tukey–Kramer's test for multiple comparisons [41] were used. Statistical analysis of dose–response curves were performed using a computer program (ALLFIT) based on a four-parameter logistic equation and *F*-test [42]. Results were considered of biological significance at $p < 0.05$ level.

Results

Production of functional TGF-β

In vitro production of both active and total TGF-β by mammary adenocarcinoma cells was measured by a bioassay using Mv1Lu mink lung cells. We determined that both M3 and MM3 primary culture cells produced TGF-β and secreted it to the conditioned media (CM). As shown in Figure 1(A) and Table 1, TGF-β present in CM was predominantly found in its biologically latent (inactive) form. Approximately only 5–10% of total secreted TGF-β corresponded to active cytokine. It is important to note that although M3 and MM3 cells secreted a similar proportion of inactive TGF-β, the highly metastatic MM3 cells produced ∼2-fold the total TGF-β produced by the poorly metastatic M3 cells. It is noteworthy that acid as well as heat treatment of CM were able to activate TGF-β in a similar way (data not shown).

On the other hand, the same experiment was performed with CM from LM3 and LMM3 cells. As observed with primary culture cells, both derived cell lines produced TGF-β, also predominantly in its biologically latent form. Besides, they secreted similar amounts of total TGF-β than the highly metastatic MM3 cells (data not shown).

Furthermore, to confirm that the inhibitory activity detected on Mv1Lu cells by the bioassay was specifically due to TGF-β action, the different CM prepared from primary culture cells and cell lines were tested following an incubation with a specific anti-TGF-β neutralizing antibody (Anti-TGF-β 1,2,3). We determined that the inhibition of Mv1Lu cell proliferation was completely reverted (almost 100%) by effect of the specific anti-TGF-β antibody (Figure 1(B)), confirming that every cell type analyzed was able to secrete TGF-β, either latent or in its biologically active form. Neither anti-TGF-β alone nor IgG alone modified the growth of Mv1Lu cells compared to control cells (data not shown).

Expression of TGF-β isoforms at protein level

TGF-β expression at protein level was examined by western blot of CL from primary cultures and cell lines. Specific polyclonal antibodies against the different TGF-β isotypes were used. From the analysis of bands obtained under reducing conditions it was shown that all the studied cells expressed the three mammalian TGF-β isoforms, which had the same Mr as the three purified recombinant TGF-βs, employed as positive control of the assay. All the cells analyzed showed detectable levels of the ∼24 kDa dimeric mature form of TGF-β protein (Figure 2(A)). Interestingly, when we compared TGF-β1 expression level we determined that MM3 cells presented always a higher level of expression of any TGF-β isotype than the poorly metastatic M3 cells (e.g., ∼2-fold for TGF-β1 expression). On the other hand, LM3 and LMM3 cells expressed similar TGF-β1 protein levels (Figure 2(A)). Similar results were found when TGF-β2 and TGF-β3 expression levels were analyzed (data not shown).

Expression of TGF-β receptors

The expression of the three major cell surface TGF-β receptor types (TβRs) was studied by western blot of cell lines lysates and tumor homogenates. M3 and MM3 cells as well as LM3 and LMM3 ones constitutively expressed two bands of ∼65 and 80 kDa corresponding to TβRI and TβRII, respectively (Figure 2(B)). Densitometric analysis of bands indicated that the highly metastatic MM3 cells presented the highest expression of both signaling TβRs. On the other hand, by dot blot we determined that both parental tumors and the derived cell lines expressed

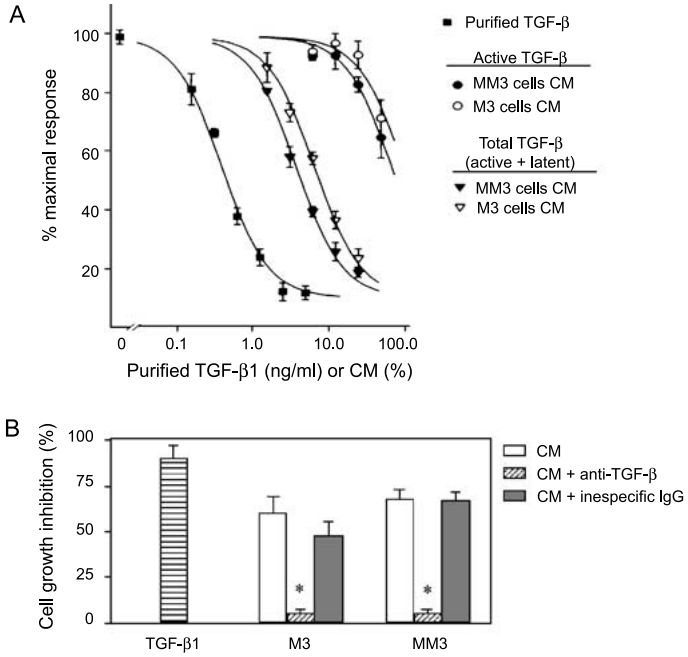

Figure 1. (A) Endogenous TGF-β production measured by a bioassay. Mv1Lu mink lung cells were treated with increasing concentrations of TGF-β1 or with CM from M3 or MM3 primary culture cells. The active form of TGF-β was measured in untreated CM. Total TGF-β was detected in CM pre-activated by heat or acid treatment, as indicated in "Materials and methods". Results are represented as percentage of maximal [^{3}H]-thymidine incorporation respect to control cultures without TGF-β. Values expressed as mean ± SD of triplicates are representative of three independent experiments. (B) Capability of anti-TGF-β to abolish the growth inhibitory effect of TGF-β1 (1 ng/ml). Mv1Lu cells were treated with CM from M3 or MM3 cells in the presence of either an anti-TGF-β neutralizing antibody (10 μg/ml) or a normal mouse IgG (10 μg/ml). Values are mean ± SD of triplicates. * $p < 0.05$ (ANOVA) when compared with the respective controls.

Table 1. TGF-β production by primary culture cells[a]

	Total (active + latent) TGF-β (ng/ml)[b]	Active TGF-β (ng/ml)[b]
M3 cells	5.90 ± 0.40	0.42 ± 0.02
MM3 cells	10.60 ± 0.80*	0.58 ± 0.05

[a] TGF-β bioactivity in 24 h CM was determined by comparison between ID50 in dose–response curves for standard TGF-β and M3 or MM3 cell CM on Mv1Lu cells.
[b] Values expressed as mean ± SD of triplicates were normalized by protein content of CL from which CM were prepared and are representative of three independent experiments.
* $p < 0.05$ versus M3 cells (*F*-test).

TβRIII, without significant differences between them (data not shown).

Effect of TGF-β on the proliferation of murine mammary adenocarcinoma cells

It is well known that TGF-β is a potent growth inhibitor of a wide variety of cell types, especially epithelial ones. By [^{3}H]-thymidine incorporation assay primary cultures of M3 and MM3 adenocarcinomas were tested for their growth response to TGF-β. Mv1Lu cells were used as control of antiproliferative TGF-β action. Treatment with exogenous purified TGF-β1 (Figure 3(A)) and TGF-β2 or -β3 (data not shown) induced a dose-dependent inhibition of cell proliferation on both cell types. However, as shown by the slope of the curves, the highly metastatic MM3 cells were less sensitive to TGF-β antimitogenic effect than the poorly metastatic M3 cells. While M3 cells were inhibited ∼50% with 4–40 ng/ml TGF-β1, the proliferation of MM3 cells decreased only ∼30% with the highest concentration.

Then, TGF-β effect on the proliferation of LM3 and LMM3 cell lines was tested in the same way as described for primary cultures. Interestingly, cell lines proliferation was not modulated by any TGF-β isoform, even at high concentrations (Figure 3(B)).

In any case, no change in cell morphology was observed in TGF-β-treated cells. Besides, to confirm that TGF-β had no effect at all on the growth of LM3 or LMM3 cells, additional assays were performed. TGF-β treatment did not modulate cellular distribution along the different cell cycle phases neither affected

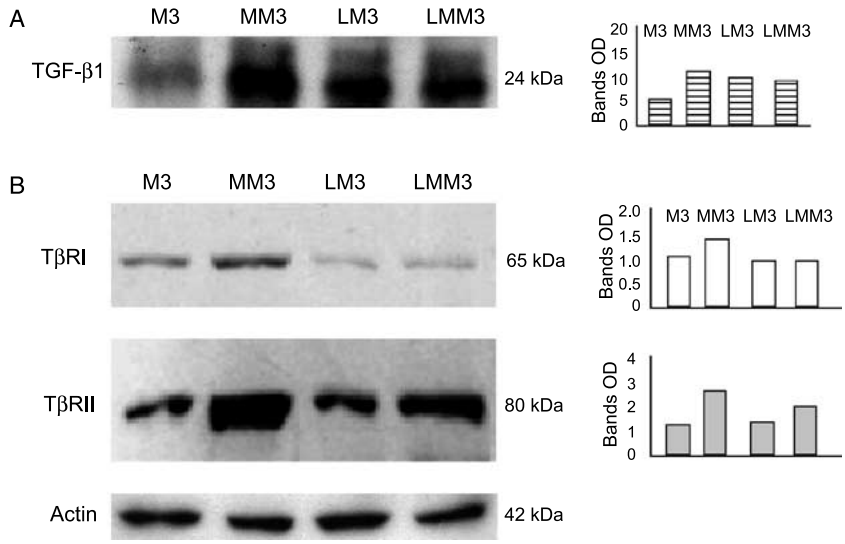

Figure 2. (A) Western blot for TGF-β1 expression in mouse mammary adenocarcinoma CL. Bands were analyzed by densitometry (OD) and are representative of three different experiments. Similar results were obtained for TGF-β2 and TGF-β3 expression (data not shown). (B) Western blot for the expression of TGF-β signaling receptors (TβRs) in mouse mammary adenocarcinoma CL. Bands were analyzed by OD and are representative of two different experiments. The expression of actin in CL was used as control of the assay and a representative blot is shown.

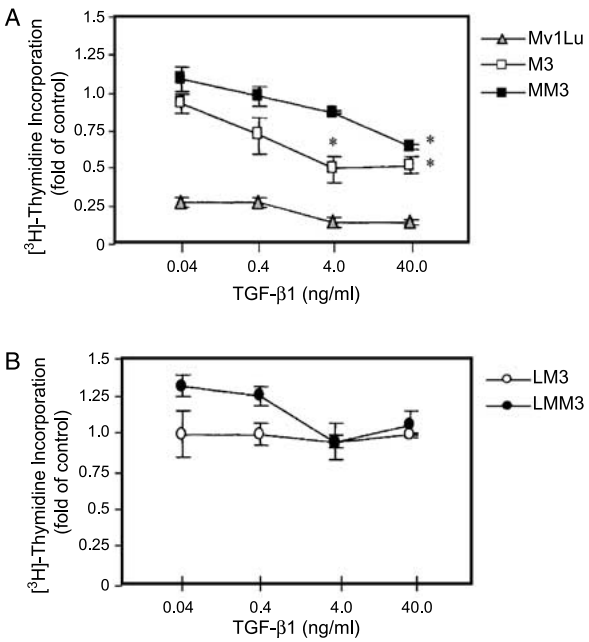

Figure 3. Effect of TGF-β1 on the growth of murine mammary adenocarcinoma cells. The rate of DNA synthesis of primary culture cells (A) or cell lines (B) was assayed by measuring uptake of [^{3}H]-thymidine after 42 h of treatment with a wide range of TGF-β1 concentrations. Mv1Lu cells were used as positive control for growth inhibition by TGF-β. Values expressed as mean ± SD of triplicates are representative of three independent experiments. Similar results were obtained with TGF-β2 or TGF-β3 in all the studied cells (data not shown). * $p < 0.05$ (ANOVA) versus untreated cells.

Table 2. Effect of TGF-β1 on the cell cycle distribution of LM3 cells[a]

TGF-β1 (ng/ml)	Cell cycle phase			
	G0/G1	S	G2/M	Mitotic index (%)
0	36.3 ± 0.2	35.7 ± 4.3	27.0 ± 6.1	10.0
4	35.6 ± 9.7	25.6 ± 2.7	34.8 ± 3.8	11.7

[a] The effect of TGF-β on the cell cycle of LM3 cell line was studied by CAS and by microscopic counting of mitotic images. Two hundred cells treated or not 24 h with TGF-β1 were analyzed by CAS and 1000 cells for mitotic index. Results are expressed as mean ± SD of duplicates and represent the percentage of cells in each cell cycle phase. The same experiments were also performed with TGF-β2 and -β3 with similar results. Similar data were obtained from LMM3 cells under the same conditions (data not shown).

the mitotic index (Table 2), nor the capability of cells to grow at low density in a clonogenic assay (data not shown).

Effect of TGF-β on secretion of proteases by mammary tumor cells

Effect of TGF-β on metalloproteinases (MMP) activity

To detect whether TGF-β was able to modulate MMPs secretion, CM from primary cultures or cell lines treated or not 24 h with TGF-β1, -β2 or -β3 were

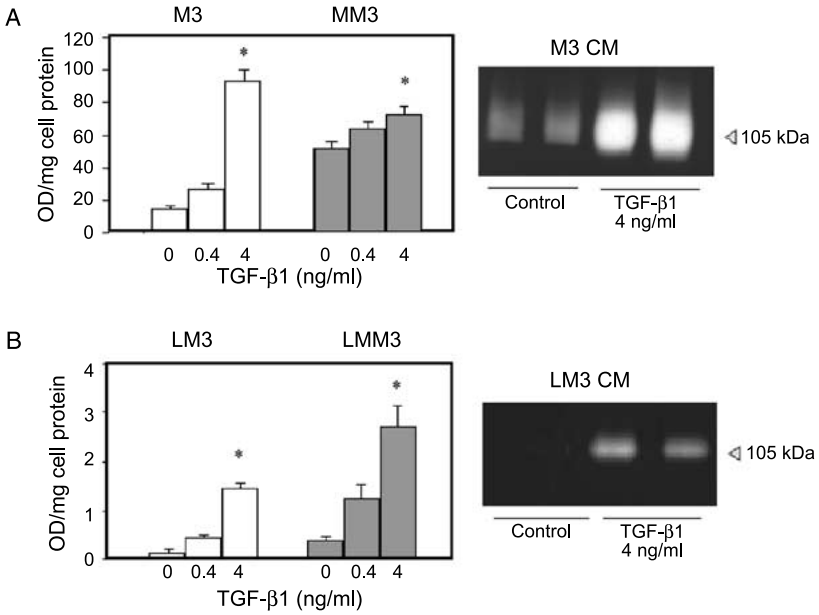

Figure 4. Effect of TGF-β1 on MMP-9 secretion by murine mammary adenocarcinoma cells. The activity of the 105 kDa MMP-9 murine form was analyzed by quantitative zymography of 24 h serum free-CM from primary cultures (A, left panel) or cells lines (B, left panel) treated or not with TGF-β1. Values are expressed as mean ± SD of duplicates. Representative zymograms of duplicate CM from M3 cells (A, right panel) and LM3 cells (B, right panel) are shown. Similar results were obtained in response to TGF-β2 or TGF-β3. * $p < 0.05$ (*T*-test) compared to untreated cells.

analyzed by quantitative zymography. No band was observed in gels incubated with EDTA-containing buffer and the same pattern of bands was shown in gels incubated in the presence of PMSF or aprotinin, confirming that the gelatinolytic activity seen was due to calcium-dependent metalloproteinases. All the studied cells constitutively secreted a 105 kDa MMP-9 (Figure 4). Interestingly, while M3 cells showed a barely detectable constitutive MMP-9 activity, the highly metastatic MM3 cells presented a remarkably higher MMP-9 basal activity (Figure 4(A)). When any exogenous TGF-β was added to cultures, a pronounced and dose-dependent increase in MMP-9 activity was observed both in primary cultures and in cell lines. The three TGF-β isotypes had similar effect on MMP response (data not shown).

Effect of TGF-β on plasmin system

The activity of secreted uPA was evaluated by caseinolytic assay of CM from cells treated or not with the different TGF-β isotypes. TGF-β effect on uPA activity was different according to the cell type tested (Figure 5(A)). Interestingly, TGF-β1 and -β2, but not -β3, induced a dose-dependent inhibition in the activity of uPA secreted by M3 primary culture cells; for example, uPA activity was inhibited ∼50% by 4 ng/ml TGF-β2 and it was completely abolished by the maximum concentration assayed of the cytokine. In contrast, no TGF-β isoform was able to modulate uPA activity either in the highly metastatic MM3 cells or in LM3 cell line. Moreover, the highly invasive and metastatic LMM3 cells showed a significant and dose-dependent increase (up to 2-fold) in enzymatic uPA activity in response to any TGF-β isoform.

To better understand the differences found on secreted uPA activity among the different studied cells, we analyzed the presence of the natural uPA inhibitor PAI. The secretion of PAI-like enzyme was studied by reverse zymography of CM from cells treated or not with TGF-β. A barely detectable basal PAI-like activity was shown in every cell type analyzed. Concentrations of 4 ng/ml or higher of any TGF-β isotype induced a remarkable and dose-dependent increase in PAI-like activity in M3 cells; for example ∼7-fold increase with 4 ng/ml TGF-β2 (Figure 5(B)). A similar but slighter effect was noted in LM3 and LMM3 cells; for example, ∼5-fold increase in PAI-like activity with 4 ng/ml TGF-β2 in LMM3 cells (Figure 5(B)). On the contrary, none TGF-β isoform at any concentration assayed was able to affect PAI-like secretion by the highly metastatic MM3 cells (data not shown).

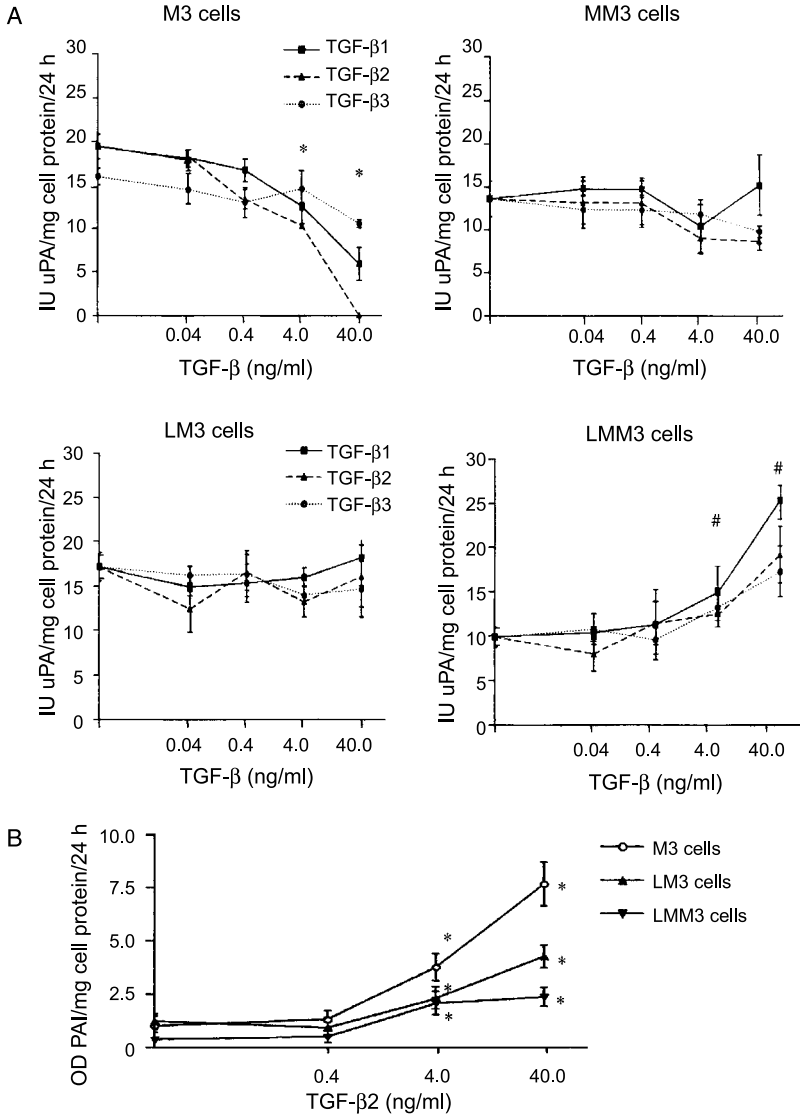

Figure 5. (A) Effect of TGF-β on the activity of secreted uPA. CM from primary cultures and the derived cell lines treated or not with TGF-βs were measured by radial caseinolysis. Mean ± SD of triplicate samples from a representative experiment of four independent ones are shown. * $p < 0.05$ (ANOVA) versus untreated cells, for TGF-β1 and TGF-β2 only; # $p < 0.05$ (ANOVA) versus control cells, for the three TGF-β isotypes. (B) Effect of TGF-β2 on the activity of secreted PAI, as detected by reverse zymography of CM from murine mammary adenocarcinoma cells. Data expressed as mean ± SD of duplicates are representative of three independent experiments. Similar results were obtained with other TGF-β isotypes. * $p < 0.05$ (*T*-test) versus control cells.

It is well known that uPA binding to its membrane receptor uPAR increases and focalizes the invasiveness of tumor cells [43]. Thus, we also studied the effect of the different TGF-β isoforms on mb-uPA activity in CL from the primary cultures and the cell lines. Untreated cells showed only a very low mb-uPA activity. While TGF-β did not modulate mb-uPA activity in the poorly metastatic M3 cells, it significantly increased this activity in the highly metastatic MM3 cells (e.g., ∼10-fold with 4 ng/ml TGF-β1) (Figure 6). Moreover, although total cell-associated uPA activity was also increased by effect of TGF-β in MM3 cells, the level of mb-uPA activity reached ∼30–50% the level of total cell-associated uPA. Besides, TGF-β was not able to modulate either mb-uPA or cell-associated uPA in LM3 and LMM3 cell lines (data not shown).

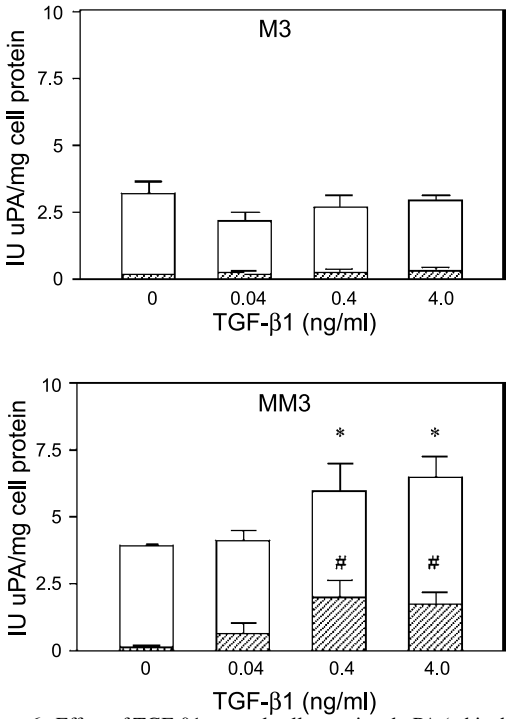

Figure 6. Effect of TGF-β1 on total cell-associated uPA (white bars) and mb-uPA (hatched bars) activities detected in CL from primary culture cells. Assays were performed as described in 'Materials and methods'. Values expressed as mean ± SD of triplicates are representative of three independent experiments. Similar results were obtained with TGF-β2 or TGF-β3. * $p < 0.05$ (ANOVA) for total cell-associated uPA versus untreated cells; # $p < 0.05$ (ANOVA) for mb-uPA versus control cells.

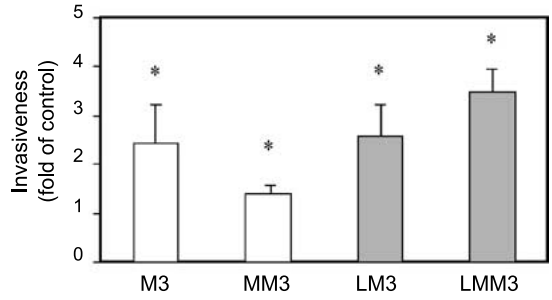

Figure 7. Effect of TGF-β1 (4 ng/ml) on the *in vitro* invasiveness of tumor cells through Matrigel. Data expressed as mean ± SD of triplicates represent the number of invasive cells per field under fluorescence microscope (400×) relative to untreated cells. * $p < 0.05$ (*T*-test) versus control cells.

Finally, the expression of the specific membrane receptor for uPA, uPAR [18], was analyzed by western blot. All the studied cells expressed the cell surface (∼57 kDa) uPAR, which was not modulated by treatment with TGF-β (data not shown).

Effect of TGF-β in cellular invasion

To study whether TGF-β was able to modulate *in vitro* invasiveness of tumor cells we employed an invasion assay through reconstituted basement membrane proteins (Matrigel). Exogenous purified TGF-β1 (4 ng/ml) significantly enhanced the capability to invade Matrigel in all the studied cells (Figure 7).

Discussion

For a century it has been considered that tumor progression is a multistep process not only determined by events intrinsic to tumor cells but also largely influenced by environmental factors that include growth factors and cytokines as well as interactions between tumor cells and ECM molecules and with adjacent normal cells. These multiple interactions may explain the pathogenesis of malignant disease [44].

TGF-β is considered one of the most important components of tumor microenvironment complex network. Because both stromal and tumor cells could be able to produce TGF-β, and it is abundant in the circulation, TGF-β could exert autocrine and/or paracrine effects over these cells along the metastatic cascade [45].

We report here that TGF-β could be promoting tumor progression in our murine model of M3 and MM3 mammary adenocarcinomas and the derived LM3 and LMM3 cell lines, with different invasive and metastasizing capabilities. Besides, our findings may help to explain the pleiotropic features of TGF-βs and their receptors (TβRs) and their association with mammary tumor dissemination.

Any cell type studied until now can possibly express TGF-βs and their specific receptors TβRs [9]. *In vitro*, tumor cells usually secrete TGF-β in its latent form and they are not usually able to activate a significant fraction of secreted TGF-β [46]. However, some tumor cell types are known to secrete active TGF-β [47, 48]. The entire experimental model used in this study, both M3 and MM3 primary cultures as well as LM3 and LMM3 cell lines produce and secrete the three known mammalian TGF-β isoforms and express the three membrane TβRs, so all these cells express the complete TGF-β/TβR system. We found that all the studied cells predominantly secreted TGF-β in its inactive latent form and that a small percentage of active cytokine was present in 24 h conditioned media. However, a different pattern of production of TGF-βs was observed. Through a bioassay we determined

that the highly metastatic MM3 cells produced higher levels of total TGF-β than the poorly metastatic M3 cells and that the highly invasive and metastatic LM3 and LMM3 cell lines reached the level of TGF-β production showed by MM3 cells. So, endogenous TGF-β production seems to be related to the more aggressive phenotype in our experimental model.

It is already known that TGF-β profoundly inhibits epithelial cell proliferation of both normal and some transformed cells, so it is paradoxical that it is also strongly implicated in the development of many cancers of epithelial origin [45]. In our experimental model, whereas any TGF-β isoform showed antimitogenic effect on both M3 and MM3 primary culture cells, the highly metastatic MM3 cells demonstrated to be less sensitive to this effect because only the highest doses of TGF-β used could inhibit their growth, and also the level of inhibition was lower than that observed in M3 cells. Furthermore, LM3 and LMM3 cell lines were insensitive to growth modulation by exogenously added TGF-β. This complete lack of response to TGF-β antiproliferative effect could probably be a consequence of the successive *in vitro* passages along their establishment. So, since the highly metastatic MM3 cells still responded to TGF-β antimitogenic effect but the derived cell lines did not, it seems to be a progressive loss of TGF-β-dependent growth regulation in our experimental model. Many different tumor cell types, including those of mammary epithelial origin, have been shown to exhibit a diverse degree of resistance to TGF-β antiproliferative effect both in human and murine models, and in some cases this response has been correlated with tumorigenesis [49–51]. Our data are in agreement with these reports.

Because the murine mammary adenocarcinoma cells studied in this work secrete the different TGF-β isotypes and express the specific TβRs, we consider that the TGF-β/TβR system is functional and it may be acting by an autocrine mechanism. Besides, because latent TGF-β produced by these tumor cells could be exogenously activated (bioassay), it is feasible to suppose that it could be activated *in vivo* by factors released to tumor microenvironment, either by host cells or by the own tumor cells, as found by other authors [13, 45].

The basic TGF-β signaling engine consists of two serine/threonine kinase receptors (TβRI and TβRII) and their substrates, the Smad proteins, which move into the nucleus and regulate transcription of target genes [6]. Cancer cells can acquire resistance to TGF-β growth inhibitory effect by a number of different mechanisms including inability to secrete and/or to activate the latent TGF-β complex, loss of expression or function of the transmembrane receptors, and disruption of postreceptor signal transduction pathways. Because LM3 and LMM3 cells are able to produce TGF-β and to express the signaling TβRs, and because these cell lines do respond to certain TGF-β biological effects, we suppose that the resistance of these cells to TGF-β antiproliferative effect could be the result of an alteration in some intracellular pathway(s) triggered by TGF-β, and it may represent one of the multiple changes required for tumor progression [13]. In further studies we will analyze cell growth regulation by TGF-β focusing on expression and activation of Smads.

One of the most important steps for the progression of epithelial tumors is the dissolution of ECM that separates epithelial and stromal compartments by the action of serine-proteases like uPA and metalloproteinases like MMP-9, universally expressed during tumor progression and metastasis [23]. A lack of regulation in this process during the different steps of the metastatic cascade is a hallmark of malignancy [14, 52]. In this sense, it is already known that TGF-β is a strong regulator of both ECM components and the enzymes involved in its remodeling [24, 25].

MMPs play a critical role in the proteolytic degradation of basement membranes [16]. In our experimental model, TGF-β markedly enhanced MMP-9 activity in all the studied cells. However, no direct association between the level of MMP-9 stimulation and the metastatic capability of the different cells analyzed has been observed. Interestingly, in the highly metastatic MM3 cells TGF-β induced a smaller increase in MMP-9 activity compared to the other cell types studied, possibly because of an already high basal MMP-9 production.

The importance of the plasmin system in tumor progression results from the balance between activators (both secreted and cell-associated uPA) and their inhibitors, which would ultimately determine the degree of malignancy achievable by tumor cells [53]. In our model, TGF-β induced a broad spectrum of effects on the plasmin system. In the poorly metastatic M3 cells, TGF-β decreased secreted uPA activity at the same time that it markedly increased the activity of the uPA inhibitor PAI. On the other hand, while in the highly metastatic MM3 cells both secreted uPA and PAI activities were not modulated by TGF-β, the activity of membrane-bound and total cell-associated

uPA were significantly enhanced by the cytokine. It has been described that mb-uPA can be ∼40-fold more effective in its proteolytic activity than secreted uPA [54], so TGF-β could be promoting MM3 cells metastatic capability in part through the induction of an enhanced ECM degradation by mb-uPA activity. It is well known that tumor cells secrete inhibitory proteolytic molecules which function controlling degradative processes. We may suppose that in M3 cells, PAI activity could be blocking uPA activity by effect of TGF-β, and this regulation could help to maintain a low metastatic capability.

In the highly invasive and metastatic cell lines, the effects of TGF-β on the plasmin system seem to be more complex, as TGF-β enhanced the activity of both uPA and PAI in LMM3 cells. In this case, TGF-β-induced increase in PAI activity seems not to be enough to modulate either secreted or mb-uPA activities. Certain evidence supports that breast tumor progression may occur even in the presence of high levels of the inhibitor PAI [26], as could be in our tumor model. Moreover, our results suggest that the enzyme and its inhibitor could be modulated by TGF-β in an independent way.

uPA is unique among serine proteases in that it has its own high-affinity cell-surface receptor, uPAR, localized on cell–cell junctions and in the leading edge of invading cells [54]. Besides, there is a continuous interaction between the different components of plasmin system; for example, uPAR can be recycled to cell surface by PAI-induced receptor-bound uPA internalization [55]. All the tumor cells studied here expressed uPAR, but TGF-β seems not to be involved in regulating its synthesis.

We can hypothesize that TGF-β could be also acting indirectly on the plasmin system, perhaps through the novel serine-protease inhibitor maspin, a pleiotropic regulator of the pericellular plasmin system, able to modulate tumor invasion and metastasis [56, 57]. On the other hand, several other biological processes besides a strongly active plasmin system could be responsible for the acquisition of a highly invasive and metastatic phenotype in our tumor model. Further research will help to elucidate these multifactorial mechanisms.

The invasive process requires cellular adhesion and motility and finally ECM components degradation to successfully occur. Irrespective to their metastatic capability, all the studied cells of our model increased their invasiveness through Matrigel in response to TGF-β . Certain evidences suggest that TGF-β production could stimulate *in vitro* invasiveness and that this effect could be understood as an enhancement in the *in vivo* migratory capability of tumor cells during the subsequent steps of the metastatic cascade [46]. So, we may consider that TGF-β could be promoting the capability to invade basement membranes in all the cells studied here, since the full lineage is metastatic in mice.

There is evidence indicating that TGF-β effects on cell growth could be dissociated from its effects on specialized cellular responses, like stimulation of ECM components and motility [13, 58, 59]. It has been recently suggested the existence of different thresholds for distinct cellular responses to TGF-β and that the threshold for resistance to TGF-β antimitogenic effect is lower than that required to loose responses associated with cellular adhesion, invasion and metastasis [13].

When tumor cells loose their sensitivity to TGF-β antiproliferative effect, TGF-β can provide them with particular benefits, like stimulation of migration and angiogenesis, at the time that it could inhibit host immune responses [45]. So, despite it was initially proposed an indirect role of TGF-β on tumor progression by its antiproliferative effect on the surrounding normal tissue, certain data support that TGF-β could also have a direct effect on promoting metastasis of breast tumor cells [59]. Our results are in agreement with these last data since LM3 and LMM3 cells, with a highly malignant phenotype, have lost the growth inhibitory response to TGF-β but their proteolytic modulation of microenvironment as well as their invasive capability are still upregulated by TGF-β.

In conclusion, our findings suggest that TGF-β effects on tumor cell growth and on the pattern of tumor-related enzymes and their inhibitors are mediated through different mechanisms and that these signaling pathways may be differentially compromised in response to the acquisition of a highly aggressive phenotype. Furthermore, the balance between the different effects of this pleiotropic cytokine suggests that TGF-β would be favoring tumor progression in our mammary adenocarcinoma model.

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