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LAMININ AND ESTRADIOL REGULATION OF THE PLASMINOGEN-ACTIVATOR SYSTEM IN MCF-7 BREAST-CARCINOMA CELLS

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We have investigated the effects of laminin, on the plasminogen-activator system of MCF-7 breast-carcinoma cells. MCF-7 cells were incubated on plastic or laminin-coated wells, and medium and cell lysate aliquots were assayed for tissue-type (tPA) and urokinase-type plasminogen activator (uPA) by a chromogenic assay in combination with anti-uPA antibodies. Cells cultured on laminin displayed a 5-fold increase in tPA activity and a 2-fold decrease in uPA activity relative to cells on plastic. These effects could be mimicked by laminin fragment P1 but not by collagen I or fibronectin. tPA activity of cells treated with estradiol (10 nM) was 3-fold higher, that of cells on laminin treated with estradiol was 15-fold higher, than that of control. Northern-blot analysis showed that tPA mRNA levels were up-regulated by estradiol and laminin, whereas PAI-1 mRNA levels were down-regulated by laminin and not affected by E2. Concomitant treatment with laminin and estradiol, decreased PAI-1 mRNA and increased tPA mRNA levels, accounting for the synergistic increase in tPA activity. Laminin exerted only a modest (approx. 2-fold) inhibitory effect on uPA mRNA levels. In the breast-carcinoma cell line MDA-MB-231, down-regulation of PAI-1 and uPA mRNA by laminin was not observed. Adhesion assays indicated that α2β1 is the predominant receptor for laminin in MCF-7 cells. MDA-MB-231 cells expressed α2 (54%) but this integrin is not used as a laminin receptor. These results support a role for α2β1 in mediating interactions of MCF-7 with LN. ***Int. J. Cancer*** **76:77–85, 1998.**
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Degradation of the extracellular matrix (ECM) is a key feature of malignant-cell invasion, requiring the concerted action of a number of proteolytic enzymes. One such enzyme is plasminogen activator (PA), a serine protease that catalyzes the conversion of plasminogen to plasmin, which in turn catalyzes the degradation of major ECM components, including fibronectin, laminin, proteoglycans and fibrin, and may indirectly control collagen breakdown. Two types of PA have been identified: urokinase-type (uPA) and tissue-type PA (tPA). The enzyme generally implicated in tumor invasion is uPA, whose activity is localized to the leading edge of invasive cells by specific uPA surface receptors. PA expression and activity are regulated both at transcriptional and at post-transcriptional level, and indirectly through the regulated expression of specific PA inhibitors, PAI-1 and PAI-2 (reviewed by Mignatti and Rifkin, 1993).

It has been shown that in addition to hormones, cytokines and growth factors, the expression of matrix-degrading proteases can be regulated by ECM components. Thus, fibronectin, tenascin and osteonectin (SPARC) have been shown to be physiological regulators of metalloproteinase expression (Damsky and Werb, 1992), and laminin (LN) has been shown to regulate tPA activity in MCF-7 breast-cancer cells (Pourreau-Schneider *et al.,* 1989). Regulation of the PA system by LN is of particular interest, since attachment to a LN substratum is essential for mammary-cell differentiation (Streuli *et al.,* 1995), and since mammary proteolytic (including PA) activity is tightly regulated during lactation and involution. Thus, proteolytic activity is repressed during lactation and de-repressed at the onset of involution, leading to ECM breakdown and mammary-cell apoptosis (Busso *et al.,* 1989). It is conceivable, therefore, that LN regulates the PA system to maintain it in check during the course of differentiation. In the present study, we investigated the effect of LN on tPA and uPA activities, and on tPA, uPA and PAI-1 mRNA steady-state levels in the MCF-7 breast-cancer cell model. The effects of LN were compared with those of estradiol (E2), which is known to up-regulate tPA mRNA in MCF-7 cells (Davis *et al.,* 1995). In addition, the capacity of LN to modulate tPA, uPA and PAI-1 mRNAs expression was assessed in the estrogen-insensitive MDA-MB-231 breast-carcinoma cells.

Cell adhesion to LN is mediated by specific surface receptors, most of which belong to the integrin family of structurally related glycoproteins. Several integrins have been shown to function as receptors for LN (Mercurio, 1995). In the present study we also examined the expression and the functional role of integrins in the adhesion of both breast-cancer cells to LN.

MATERIAL AND METHODS

Cells

The MCF-7 human mammary-carcinoma cell line was obtained from the Michigan Cancer Foundation (Detroit, MI) and was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 mM HEPES, gentamicin (40 µg/ml), streptomicin (50 µg/ml), 7.5% calf serum (CS) and bovine insulin (5 µg/ml) at 37°C, in an atmosphere of 5% CO_2, 95% air. MDA-MB-231 cells were purchased from the ATCC (Rockville, MD) and grown in RPMI-1640 containing 5% fetal bovine serum (FBS), 10 mM HEPES and antibiotics as defined above.

Extracellular-matrix proteins

Laminin was purified from EHS tumors, fibronectin from human plasma and type-I collagen from rat-tail tendons as described (Noel *et al.,* 1988; Pourreau-Schneider *et al.,* 1989). Laminin fragment P1 was obtained after pepsin digestion as described (Rao *et al.,* 1982). The purity of fibronectin and LN was monitored by sodium-dodecyl-sulphate-polyacrilamide-slab-gel electrophoresis. Each matrix protein was solubilized in PBS filtered through a 0.45-µm nitrocellulose membrane, and diluted with sterile PBS to concentrations ranging from 3 to 48 µg/ml. To triplicate wells of 24-well Falcon plates (Becton Dickinson, Plymouth, UK) were then added 0.5 ml of each preparation (for a range of 1.5 to 24 µg/well or approx. 1.8 cm^2) and protein adsorption allowed during 1 hr of incubation at 37°C. The plates were then washed twice with PBS and used immediately or stored at 5°C. When coating 75-cm^2 flasks, we used an amount of LN equivalent to 3 µg/cm^2 (approx. 250 µg) was used. For cell-adhesion assays, the LN-coating protocol was slightly modified by diluting LN in 0.05 M Tris.HCl, pH 7.4, containing 2% BSA and 2 mM $CaCl_2$ and the wells were coated overnight at 20°C, followed by 3 washes with PBS to remove unbound material.

Cell treatments

To study the effect of the culture substratum on PA, approximately 7×10^4 MCF-7 cells were seeded onto regular plastic or

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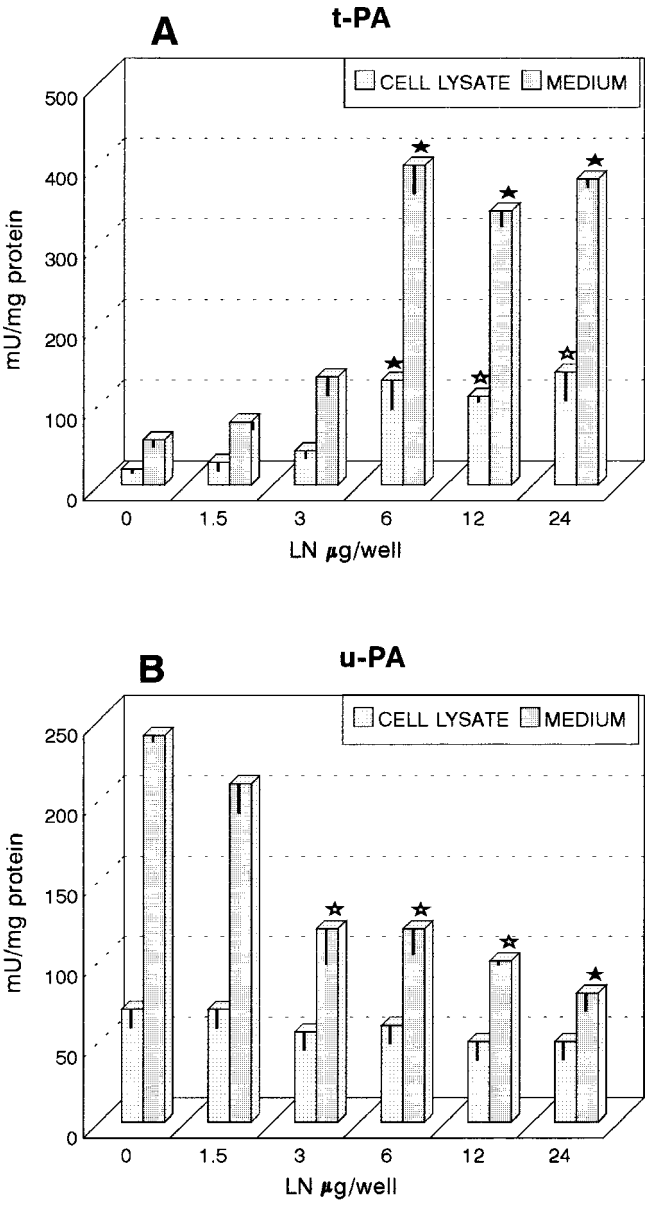


FIGURE 1 – Effect of laminin on tPA and uPA activity. MCF-7 cells (7×10^4) were seeded on LN-coated wells overnight, switched to serum-free medium and incubated for 48 hr with one medium change. Aliquots of conditioned medium corresponding to the last 24 hr of incubation and cell lysates were then assayed for tPA *(a)* and uPA *(b)* by a chromogenic assay. See ''Material and Methods'' for details. Values represent the mean of at least 2 independent experiments (each performed in duplicate). Bars show standard errors. Student's *t*-test on unpaired values was used to determine significant differences between values for cells grown in the presence of LN and values from cultures on plastic. $^{\bigstar}p < 0.01$; $^{\star}p < 0.05$.

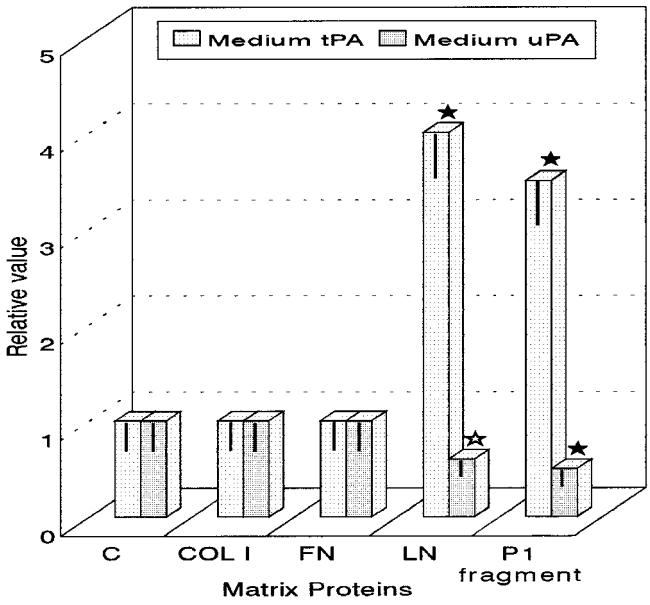


FIGURE 2 – Effect of extracellular-matrix proteins on tPA and uPA activity. MCF-7 cells were seeded onto wells coated with collagen I (COL I), fibronectin (FN), LN (each at 6 µg/well) or LN P1-fragment (12 µg/well), and aliquots of serum-free conditioned medium (as in Fig. 1) assayed for uPA and tPA (data normalized to control values). See ''Material and Methods'' for details. Values represent the mean of at least 2 independent experiments (each performed in duplicate), with error bars indicating the standard error between individual determinations. Student's *t*-test on unpaired values was used to determine significant differences between values for cells grown on matrix components and values for corresponding controls. $^{\bigstar}p < 0.01$; $^{\star}p < 0.05$.

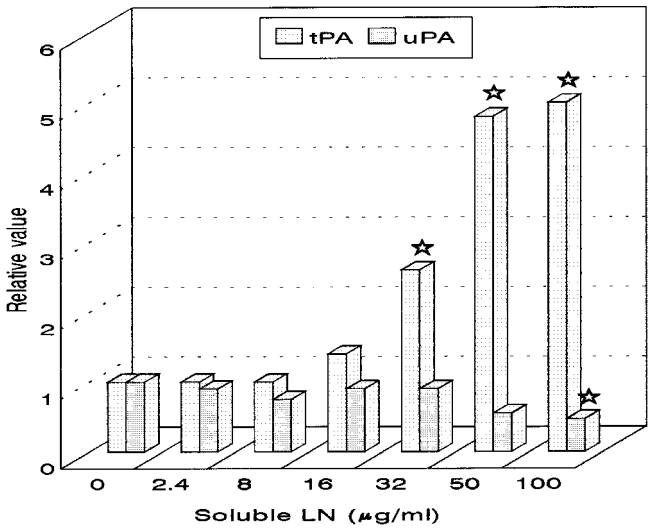


FIGURE 3 – Effect of laminin in solution. MCF-7 cells were seeded overnight on uncoated wells, then switched to serum-free medium containing the indicated concentrations of soluble LN, and incubated for 48 hr with one medium change. Aliquots of conditioned medium corresponding to the last 24 hr of incubation were then assayed for tPA and uPA (data normalized to control values). Standard error between duplicates of 2 experiments was: $< \pm$ 10%, control; $< \pm$ 5%, 2.4 µg; $< \pm$ 5%, 8 µg; $< \pm$ 5%, 16 µg; $< \pm$ 17%, 32 µg; $< \pm$ 20%, 50 µg and 100 µg. Student's *t*-test on unpaired values was used to determine significant differences between values for cells grown in the presence of LN and values from cultures on plastic. $^{\star}p < 0.05$ (Student's *t*-test).

LN-coated wells of 24-well plates and allowed to attach overnight. In the absence of serum, MCF-7 cells do not attach to plastic, as was observed by Noel *et al.* (1988). Since cells seeded on plastic in the presence of serum were the control cells of our experiments, serum was added to MCF-7 cells plated on LN, to keep treatment uniformity. Relatively long incubation time is needed for MCF-7 to achieve reasonable levels of attachment either to plastic or to immobilized LN. After attachment, the medium was removed and the cells were rinsed and cultured for more than 48 hr in serum-free medium containing 0.05% BSA and 5 µg/ml insulin, with one

TABLE I – EFFECT OF β-ESTRADIOL AND LAMININ SUBSTRATUM ON tPA AND uPA ACTIVITY

	tPA (mU/mg)	uPA (mU/mg)
Control	55.0 ± 3.0☆	240.0 ± 9.0
βE2	153.0 ± 9.0☆	230.0 ± 8.5
LN	272.0 ±10.0★	140.0 ± 5.0☆
LN + βE2	763.0 ± 28.0★	140.0 ± 5.0☆

MCF-7 cells were seeded on uncoated or LN-coated culture wells (6 µg/well) and subsequently treated without or with 10 nM β-estradiol for 24 hr. tPA and uPA activity was assayed in aliquots of conditioned medium. (See ''Material and Methods'' for details). Results are mean ± SE of 2 independent experiments in duplicate. Variations between experiments were < ± 10% (Student's *t*-test as compared with corresponding control value). ★$p < 0.01$; ☆$p < 0.05$.

medium change. Aliquots of the last conditioned medium and of cell lysates obtained by lysis with 0.5 ml of 0.1 M Tris.HCl, 0.5% Triton X-100 (pH 8.1) at 37°C/1 hr, and cleared by centrifugation, were assayed for PA as described below.

To study the effect of estradiol (E2), MCF-7 cells were allowed to attach overnight as described, then the medium was changed to serum-free medium supplemented with 0.05% BSA and 10 nM of the anti-estrogenic compound LY117018, generously provided by Ely Lilly (Indianapolis, IN). After 24 hr, the medium was replaced with the same serum-free medium with or without 10 nM E2, obtained from Sigma (St. Louis, MO). Conditioned medium and cell-lysate aliquots were then assayed for PA.

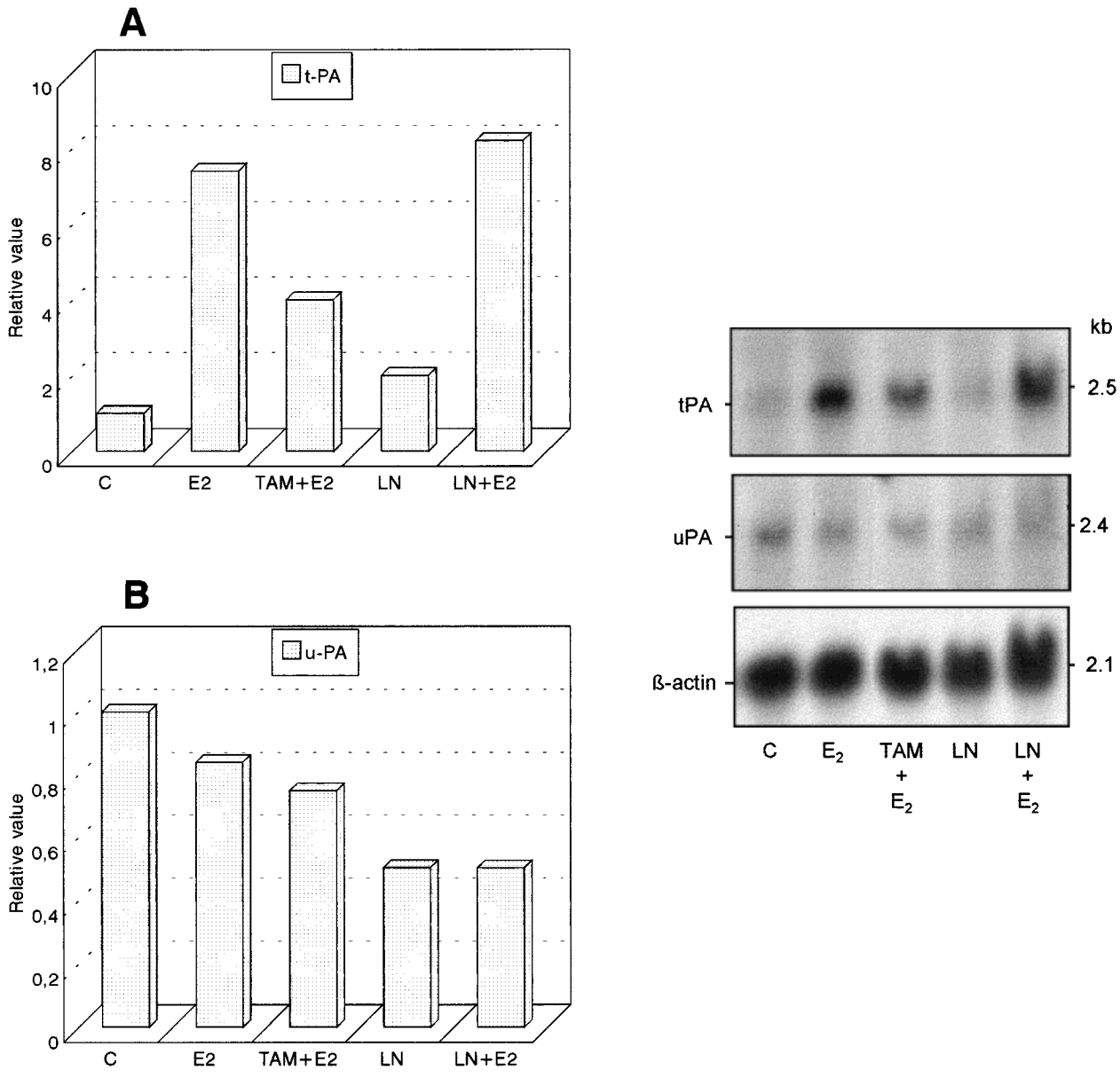


FIGURE 4 – Effects of LN and E2 on tPA and uPA mRNA. MCF-7 cells were seeded on uncoated (control) or LN-coated flasks, grown to sub-confluence, then incubated for 72 hr with or without medium containing 10 nM E2. Cells seeded on uncoated plastic were also incubated in medium containing 10 nM E2 plus 1 µM tamoxifen (TAM). Total cellular RNA (35 µg) was Northern-blotted and sequentially hybridized with specific tPA and uPA cDNA probes. The filter was subsequently hybridized with a probe for β-actin as a control for RNA loading. The bands corresponding to the mRNA were scanned by densitometer and results were expressed as the ratio of each mRNA/β-actin mRNA, normalized to control. See ''Material and Methods'' for details.

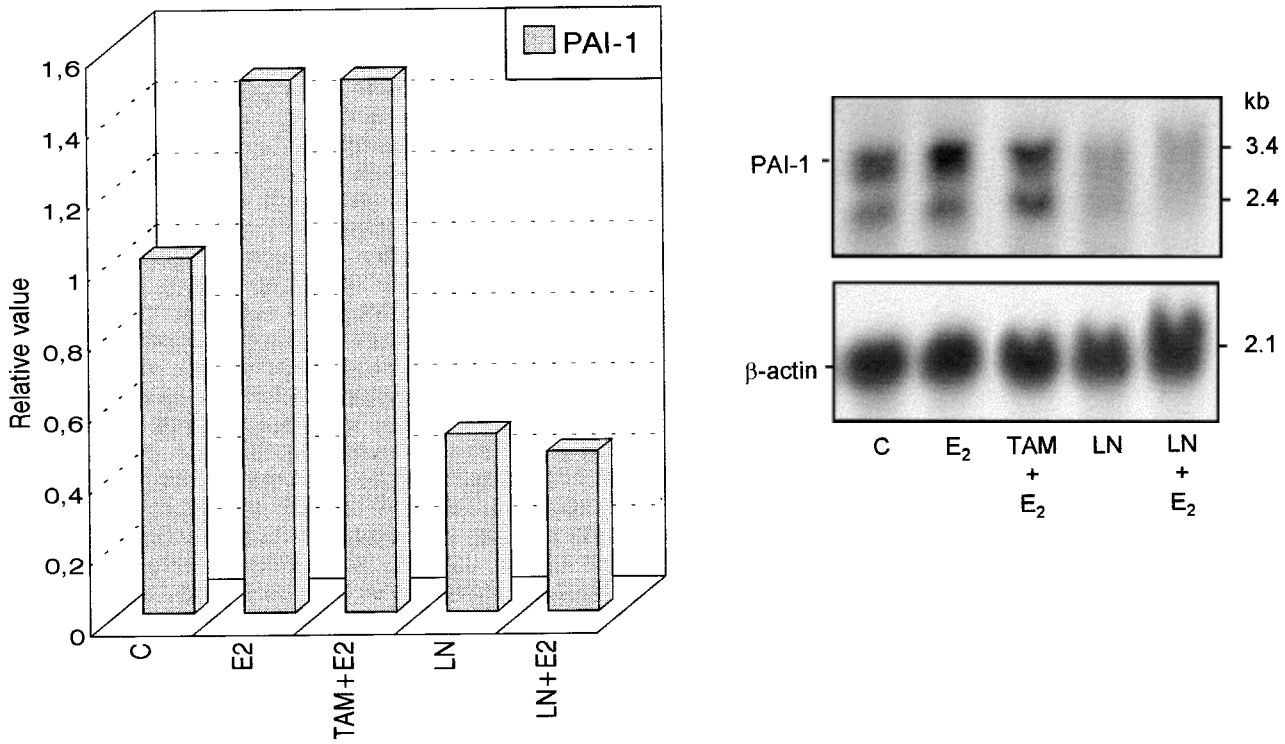


FIGURE 5 – Effect of LN on PAI-1 mRNA. MCF-7 cells were cultured as in Figure 4, and Northern-blot analysis was carried out using PAI-1 and β-actin probes. Note the presence of 2 PAI-1 mRNA described by Quax *et al.* (1990). Values were normalized to control.

Alternatively, to obtain sufficient cells for RNA isolation, MCF-7 cells were grown for 24 hr either in uncoated or in LN-coated 75-cm^2 flasks in phenol-red-free RPMI-1640 medium supplemented with 5% dextran-charcoal-stripped serum, and then treated as above with or without 10 nM E2. Cells seeded on uncoated flasks were also incubated in medium containing both 10 nM E2 and 1 µM tamoxifen (TAM). Medium was changed 48 hr later, and cells were harvested after another 24 hr (72 hr of E2 or E2/TAM treatment). Tamoxifen was a gift from Imperial Chemical (London, UK). MDA-MB-231 cells were grown as described above for 72 hr in the presence or the absence of LN. E2, TAM and LY117018 were dissolved in absolute ethanol and further diluted with culture medium (ethanol in the culture medium never exceeded 0.02%). Controls received the vehicle alone.

PA determination

PA assays were performed in 96-well plates essentially as described (Mira-y-Lopez *et al.*, 1991). Briefly, 10 µl of conditioned medium or cell lysate was pre-incubated for 1 hr at 5°C with or without an optimal concentration of an anti-uPA monoclonal antibody (MAb) kindly provided by L. Ossowski (Mount Sinai School of Medicine, NY) (Ossowski and Reich, 1983). To each well were then added 120 µl of 0.1 M Tris.HCl, pH 8.1, containing 0.1% Triton X-100, 24 µg/ml human plasminogen (American Diagnostica, Greenwich, CT), and 28 µg/ml fibrinogen fragment. After 2 hr incubation at 37°C, 15 µl of 1 mg/ml plasmin chromogenic substrate (American Diagnostica) were added. Absorbance at 405 nm was read at several time intervals. All assays were made in duplicate and included appropriate controls (no uPA source or no plasminogen). Control experiments using a uPA standard confirmed that a saturating antibody concentration was used (all uPA activity was inhibited), and additional control experiments using a specific tPA-blocking antibody (a gift from Dr. E. Wilson, New York) confirmed that all residual activity was indeed tPA activity (Wilson *et al.*, 1982). The activity obtained without anti-uPA antibody pre-treatment represented total PA activity (tPA + uPA), whereas the activity remaining after antibody pre-treatment represented tPA activity; uPA activity was calculated by subtraction (total PA activity − tPA activity). Both the uPA and the tPA activities were converted to Ploug mU based on a uPA standard included in every assay.

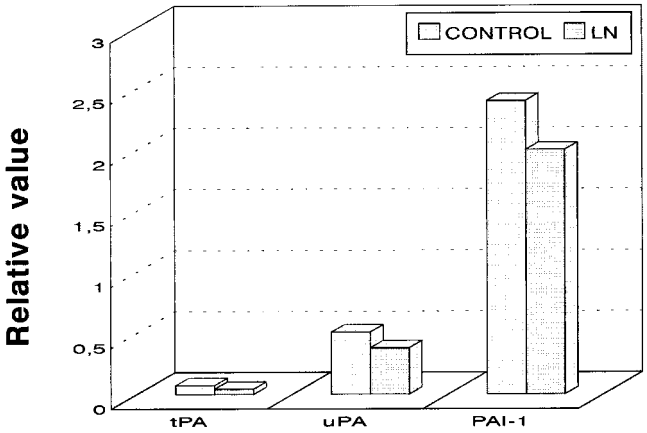
Northern-blot analysis

The human α6 cDNA in pT2B (1.2 kb) (Tamura *et al.*, 1990) and the human α2 cDNA (1.8 kb EcoRI fragment; Takada and Hemler, 1989) were donated by Dr. R.N. Tamura (La Jolla, CA); the PAI-1 cDNA probe (2.2 kb BamHI) was a gift from Dr. P. Andreasen (Copenhagen, Denmark; Andreasen *et al.*, 1986). Human uPA (ATCC 57328) and tPA (ATCC 57410) mRNA expression were analyzed with 1.5 kb PstI cDNA fragment and 1.6 kb EcoRI cDNA respectively. Both were purchased from the ATCC. Cells grown in uncoated or in LN-coated 75-cm^2 flasks were washed twice with ice-cold PBS, and total RNA was isolated by solubilization in guanidine monothiocyanate and centrifugation in a cesium-chloride gradient. Electrophoresis of total RNA (30 µg) was performed using 1% agarose-formaldehyde gel, transferred to a nylon Hybond-N membrane (Amersham, Arlington Heights, IL), and hybridized for 16 to 22 hr at 42°C in the presence of 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 10% dextran sulfate, 100 µg/ml denatured-salmon-sperm DNA and 0.5% SDS with 1 to 2 × 10^6 cpm/ml of random-primed DNA probes (random-primer DNA-labeling kit from GIBCO-BRL (Gaithersburg, MD). Filters were washed twice in 2 × SSC/0.1% SDS at room temperature for 15 min and then twice in 0.1 × SSC/0.1% SDS at 52°C for 30 min. After stringent washing, the membranes were exposed to Kodak XAR-5 X-ray film and intensifying screens at −70°C.

Cytofluorometric analysis

Flow cytometry was carried out by incubating 1 × 10^6 cells with specific MAbs in pre-tested concentration ranges, for 60 min at 4°C. The cells were then washed 3 times in PBS containing 1%

BSA and incubated with the secondary fluorescein-labeled antibodies for 45 min at 4°C. After 3 washes, cells were re-suspended in PBS and fluorescence was analyzed using a Coulter Epics-Profile II (Coulter, Hialeah, FL). MAbs recognizing human integrin sub-units α1 (CD49a), α2 (CD49b), α3 (CD49c), α6 (CD49f) and β1 (CD29) were purchased from Immunotech (Marseille, France). β4 (CD104) was donated by Drs. R. Pasqualini and M. Hemler (Boston, MA). FITC-conjugated sheep anti-mouse IgG (1:100) and goat anti-rat IgG (1:300) were bought from Sigma and Immunotech respectively. Cells were gated for analyses using forward- and octogonal-scatter parameters. At least 5000 cells were analyzed for each test. The percentage of positive cells was determined by subtracting background fluorescence of cells defined in the absence of specific MAb and the presence of FITC-labelled secondary antibodies.



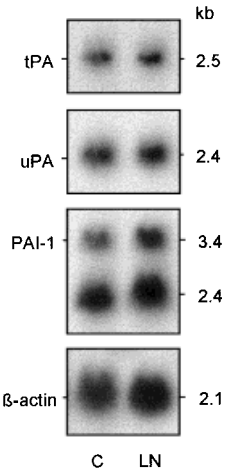


FIGURE 6 – Effect of LN on tPA, uPA and PAI-1 mRNA in MDA-MB-231 cells. Total cellular RNA was extracted either from control or from MDA-MB-231 cells seeded on LN, cultured as described in ''Material and Methods.'' RNA (30 μg) was separated by electrophoresis and transferred to a nitrocellulose filter. The filters were then hybridized with α6, β1, α2 and β-actin cDNA probes. Results were expressed as the ratio of each mRNA/β-actin mRNA.

Cell-attachment assay

To measure cell attachment to LN-coated surfaces, cells were metabolically labeled with L-[4, 5-3H] leucine (156 Ci/mmol, Amersham), harvested by brief 0.2% trypsin/EDTA 1 mM treatment and pre-incubated in DMEM containing calf serum 7.5%, 10 mM HEPES, pH 7.3, in a humidified atmosphere of 5% CO_2 in air for 2 hr. The cells were then washed with the same medium containing 2% BSA instead of serum, and re-suspended at 10^5 cells/ml (approx. 15,000 to 20,000 cpm/ml). The cell suspension was incubated with or without MAbs against α2, α3, α6 and β1 for 1 hr at 4°C, and 1 ml of cell suspension added to each pre-coated well (10 μg/well) for 2 hr in a humidified atmosphere of 5% CO_2 in air. After 2 washings with PBS, attached cells were solubilized with 0.5 N NaOH/1% SDS, and the radioactivity was counted in a beta-radiation counter (Beckman LS-400, Palo Alto, CA).

RESULTS

Effects of laminin on tPA and uPA

Adhesion of MCF-7 cells to a LN substratum was followed by an increase in tPA activity and a concomitant decrease in uPA activity relative to cells on plastic. These reciprocal changes were dose-dependent, leveling-off at 6 to 12 μg LN/well, and affected both cell-associated and extracellular PA activity (Fig. 1). These changes had slow kinetics, being first detected at 6 hr post-adhesion to LN, and increasing gradually over 72 hr (data not shown). The effect on tPA was more marked than that on uPA, with 5-fold increases in tPA activity routinely observed at 72 hr, as compared with approximately 2-fold decreases in medium uPA activity (cell-associated uPA showed a modest decrease). LN P1 fragment (corresponding to the cross region of LN) mimicked the effects of intact LN on uPA and tPA activities. For cells grown on fibronectin and collagen, tPA and uPA levels remained equivalent to those for cells grown on plastic (Fig. 2).

To determine whether cell attachment to a LN substratum was essential for tPA/uPA regulation and whether soluble LN was equally effective, we tested a wide range of soluble LN concentrations. As shown in Figure 3, large quantities of soluble LN were required to obtain 4-fold induction of tPA or 40% decrease of uPA over control. The induced activity achieved with 100 μg of soluble LN is equivalent to that resulting from adhesion to dishes coated with 6 μg of laminin; in this last condition fewer LN remain tightly bound to the dish after washing.

Synergism between laminin and estradiol

Estradiol has been shown to up-regulate tPA activity in MCF-7 cells (Ryan *et al.*, 1984). To test whether E2 and LN activated tPA synergistically, MCF-7 cells seeded on plastic or LN (6 μg/well) were cultured in E2-free medium for 2 days, then challenged without or with E2. Compared with control cells (untreated), tPA activity increased 3-fold in cells treated with E2, 5-fold in cells

TABLE II – PERCENTAGE OF EXPRESSION OF INTEGRIN SUB-UNITS ON BREAST-CANCER CELLS

Cells	Integrin sub-units				
	α1	α2	α3	α6	β1 integrin
MCF-7	2.5 ± 3.0	74.0 ± 5.0	3.5 ± 1.1	10.0 ± 2.7	84.0 ± 5.0
MDA-MB231	4.6 ± 1.4	53.5 ± 7.0	79.0 ± 7.0	47.0 ± 5.8	73.5 ± 9.0

Cytofluorimetric analysis of MCF-7 and MDA-MB-231 cells with a panel of MAbs directed against α and β sub-units of integrins. The results indicate the percentage of positive cells. Data shown are mean values ± SE of 3 independent experiments done in duplicate.

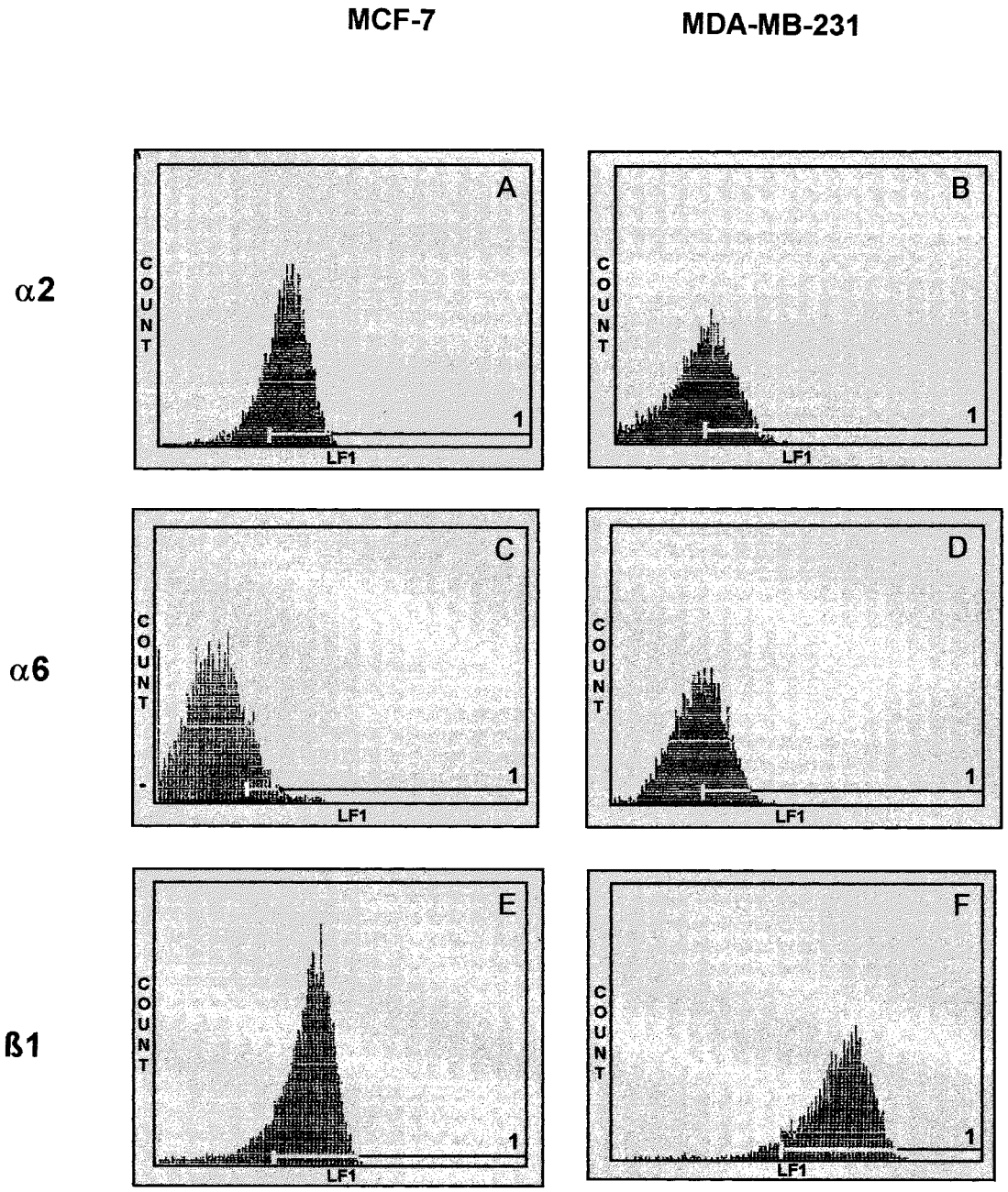


FIGURE 7 – Surface expression of integrin sub-units on MCF-7 and MDA-MB-231 cells. Cells were analyzed by flow cytometry, using optimal concentrations of MAbs specific for the indicated integrin sub-units, followed by incubation with fluorescein-isothiocyanate-labeled goat anti-mouse for α2 and β1 or goat anti rabbit for α6. MCF-7: α2 *(a)*, α6 *(c)*; β1 *(e)*; MDA-MB-231: α2 *(b)*, α6 *(d)* and β1 *(f)*. Background fluorescence was defined in the absence of specific MAb and in the presence of FITC-labelled secondary antibody. Regions excluded from region 1 on the histograms correspond to areas where blackground fluorescence occurred.

treated with LN, and 15-fold in cells treated with both LN and E2, demonstrating that these 2 signals acted synergistically, in agreement with a Pourreau-Schneider *et al.* (1989). On the other hand, E2 had no effect on MCF-7 uPA activity, and the decrease in uPA activity induced by LN was not altered by the addition of E2 (Table I).

Steady-state levels of tPA, uPA and PAI-1 mRNA

The observed LN-induced changes in tPA and uPA activity could be a direct consequence of LN regulation of tPA and uPA mRNA metabolism or could be secondary to LN regulation of PAI-1 expression. To distinguish between these possibilities, and to address the mechanism of the synergistic interaction between LN and E2, Northern-blot analyses of uPA, tPA and PAI-1 mRNAs were performed.

Laminin had a modest effect on tPA mRNA steady-state levels in MCF-7 (Fig. 4), but visibly decreased PAI-1 mRNA steady-state levels (Fig. 5), suggesting that the LN-dependent increase in tPA activity was secondary to a decrease in PAI-1 synthesis. In addition, LN caused a modest decrease in uPA mRNA levels (Fig. 4), which may in part account for the decrease in uPA activity (see ''Discussion''). In MDA-MB-231 cells, levels of t-PA, u-PA and PAI-1

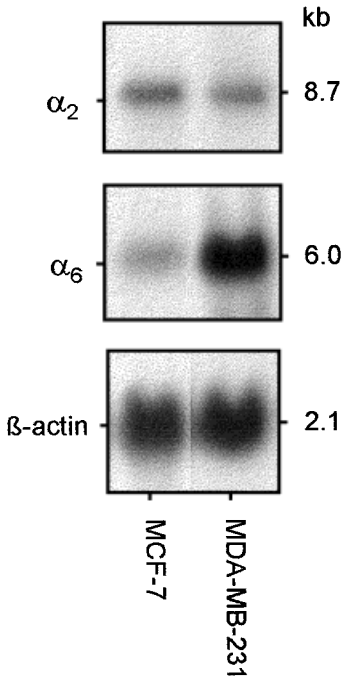


FIGURE 8 – α2 and α6 mRNA expression in MCF-7 and MDA-MB-231 cells. Total RNA was extracted from cells cultured as described in ''Material and Methods.'' RNA (30 µg) was loaded and transferred onto nitrocellulose membranes; Northern-blot analysis was carried out using α2, α6 and β-actin probes.

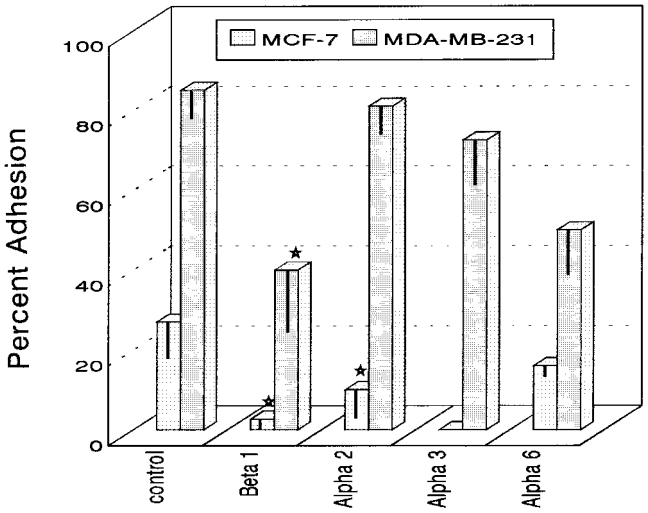


FIGURE 9 – Effects of anti-α2, anti-α3, anti-α6 and anti-β1 blocking antibodies on MCF-7- and MDA-MB-231-cell attachment to laminin. Cells were pre-incubated with antibodies to specific integrin sub-units before addition to LN-coated wells. Optimal concentrations of the blocking antibodies were pre-determined by adhesion assays. After incubation at 37°C, non-adherent cells were removed by washing, and adherent cells were quantitated as described in ''Material and Methods.'' The ability of antibodies to inhibit MCF-7-cell adhesion to plate-bound laminin (10 µg/well) was determined. Control cells show adhesion in the absence of antibodies. Data are presented as mean values ± SE of 3 independent experiments done in duplicate, compared with the corresponding control value. ☆$p < 0.05$ (Student's *t*-test).

mRNAs remained constant when cells were cultured on LN (Fig. 6).

As reported by Davis *et al.* (1995), E2 up-regulated tPA mRNA levels in MCF-7 cells, and this effect could be partially blocked by TAM (Fig. 4). No significant modification of uPA mRNA levels was observed when control MCF-7 cells were cultured with E2 or E2 plus TAM (Fig. 4). The small increase in PAI-1 mRNA after E2 treatment was not reversed by TAM (Fig. 5). The different mechanisms of tPA regulation by LN (via PAI-1) as opposed to E2 (direct tPA regulation) suggested that the synergistic increase in tPA activity observed when cells on LN were treated with E2 was due to a simultaneous increase in tPA mRNA and decrease in PAI-1 mRNA levels. Northern-blot analysis confirmed that this was the case.

Expression of integrin receptors

The expression of α1, α2, α3, α6, β1 and β4 integrin sub-units in MCF-7 and MDA-MB-231 cells was analyzed by flow cytometry. The results are summarized in Table II and Figure 7. Both cells expressed high levels of α2 and β1. The α6 sub-unit was expressed at borderline levels (10% of the cells were positive) in MCF-7 cells, whereas it was elevated in MDA-MB-231 cells (50%); β4 was not expressed in any cells. Northern-blot analysis confirmed that the level of α6 in MCF-7 is lower than in MDA-MB-231 (Fig. 8). Laminin did not alter the level of expression of α2 and α6 mRNA (data not shown).

Adhesion to laminin

To assess whether α2β1 and α6β1 participate in mediating laminin interactions, adhesion assays were performed in the presence of inhibitory antibodies specific for each of the integrin sub-units. MCF-7 and MDA-MB-231 cells were metabolically radiolabeled, pre-incubated with the antibodies that might potentially block adhesion to LN, and seeded on a LN substrate (10 µg/well). The percent of attachment-competent cells that adhere to LN in the presence of each antibody is shown in Figure 9. In this 2-hr short-term adhesion assay, only 20 to 40% of control MCF-7 cells (N = 7) were adherent to LN in the experimental conditions defined in ''Material and Methods.'' On the other hand, 85 ± 5% of MDA-MB-231 (N = 7) cells were attached and spread within 1 hr. Antibodies that recognized the β1 sub-unit inhibited almost completely the adhesion of MCF-7 cells to LN ($p < 0.05$), whereas the antibody specific for the α2 sub-unit had an inhibitory effect of 60% ($p < 0.05$), and inhibition by the antibody specific for α6 was not significant. Antibodies directed against α2 and α3 failed to block binding of MDA-MB-231 to LN, anti-β1 blocked 50% of adhesion ($p < 0.05$), and inhibition by anti-α6 was marginally significant ($p < 0.055$).

DISCUSSION

The ECM exists in a dynamic steady state that may be set at different levels depending, among other factors, on the expression of matrix-degrading proteases such as PA. In turn, specific ECM components can exert regulatory feedback and modulate the expression of certain cellular genes, including protease genes. The experiments described here show that attachment of MCF-7 breast-carcinoma cells to a LN substratum induces several changes in PA profile relative to identically treated cells on a plastic substratum. We started out by confirming the findings of Pourreau-Schneider *et al.* (1989), who have demonstrated, in MCF-7 cells, a marked increase in tPA activity by estradiol in synergy with LN. We have extended their studies, showing by Northern-blot analysis that tPA activity increase occurs without a comparable increase in tPA mRNA levels, but accompanied by a 2-fold decrease in PAI-1 mRNA abundance, suggesting that the increase in tPA activity was chiefly secondary to decreased PAI-1 expression. Comparison of the effect of LN with that of E2 enhances the plausibility of this hypothesis. Thus, in contrast to LN, the lesser E2-induced increase in tPA activity (3-fold as compared with 5-fold for LN) was accompanied by a large (7-fold) increase in tPA mRNA (Fig. 4). As

might be expected, in view of their different mode of action (PAI-1 mRNA decrease by LN, tPA mRNA increase by E2), LN and E2 interacted synergistically, causing a 15-fold increase in tPA activity (Table I), while LN had the opposite effect on uPA activity, which decreased despite a decrease in PAI-1 expression, leading us to anticipate a much reduced level of uPA mRNA. Instead, we observed a modest reduction, suggesting that LN regulated uPA activity post-translationally. For instance, LN may alter the phosphorylation state of uPA, which is known to interfere with the inhibitory action of PAI-1 (Franco *et al.,* 1992). uPA and PAI-1 mRNA expression are strongly correlated in breast-cancer biopsies (Grondahl-Hansen *et al.*, 1993) and co-regulation of uPA and PAI-1 is often seen in cell lines (Quax *et al.,* 1990). Our results demonstrating concomitant down-regulation of uPA and PAI-1 mRNA by LN agree well with these observations. Moreover, such down-regulation is consistent with the hypothesis that LN keeps the PA system in check to maintain the mammary gland in its differentiated state (see introductory section).

The dose-dependent and saturable effect of the LN substratum on MCF-7 tPA activity, the ability of P1 fragment, but not fibronectin or collagen type-I, to mimic this effect, and the observation of an opposite effect on uPA, all attest to the specificity of tPA regulation by LN. The effect of LN on PAI-1 mRNA abundance, which most likely accounts for the increase in tPA activity, was itself specific, since LN had no comparable effect on tPA mRNA, and since it was not seen in response to a different regulator, *i.e.,* E2 (Fig. 5). Although local regulation of PAI-inhibitory activity by components of ECM has been described by Waltz and Chapman (1994), our study shows an effect of LN on PAI-1 mRNA steady-state levels. In addition, in contrast to most instances of ECM-induced gene regulation studied to date, which involved positive regulation (Damsky and Werb, 1992), the effect of LN on PAI-1 is an example of negative gene regulation. Taken together with the moderate negative effect of LN on uPA mRNA levels (Fig. 4), these observations suggest that matrix-induced regulation of proteolysis is somewhat complex, with both proteases and their inhibitors susceptible to positive or negative regulation.

The induction of tPA by E2, a growth-promoting hormone in breast cancer, may at first appear to implicate tPA in disease progression. However, it is well established that tPA expression is inversely correlated with disease progression (Duffy *et al.,* 1988). This apparent discrepancy can be reconciled if tPA expression is regarded as a differentiated breast function, which is consistent with the idea of a role of tPA in mammary-duct patency (Busso *et al.,* 1989) and with the up-regulation of its activity by LN. Estradiol treatment did not alter uPA and PAI-1 mRNA, in agreement with earlier findings on uPA activity and PAI-1 levels (Ryan *et al.,* 1984; Davis *et al.,* 1995).

From the repertoire of β1-laminin receptors analyzed, we have verified that MCF-7 cells expressed α2 (74%) and α6 (~10%) sub-units, whereas MDA-MB-231 cells expressed lower α2 levels (53.5%) and higher quantities of α6 (47%) and α3 (79%). The levels of β1 were comparable in both cell types. These results agree well with those of other investigators (Berdichevsky *et al.,* 1994; Maemura *et al.,* 1995; Yao *et al.,* 1996). Our results showed that soluble LN produced effects similar to, but much smaller than, those of immobilized laminin. Arcangeli *et al.* (1996) have suggested the engagement of β1-integrin receptors with either bound or soluble LN.

Expression of an integrin receptor on the cell surface does not always correlate with functional activity (Mercurio, 1995). Adhesion assays in the presence of inhibitory antibodies to β1, α6 and α2 indicated that the attachment of MCF-7 cells to a LN substratum is via β1 integrin and that α2 is the dominant LN receptor in those cells. Since α2β1 binds to a cross-region of LN represented by the short arm (Yao *et al.,* 1996), the involvement of α2β1 was strengthened by the observation that the P1-fragment mimics the effect of intact LN (Fig. 2). Conversely, in MDA-MB-231 cells which do not use α2 as a receptor for LN, uPA- and PAI-1-mRNA down-regulation was not observed.

These results support a role of α2β1 in mediating interactions of MCF-7 with LN, although the mechanistic link between LN binding and the regulation of the PA system remains to be determined.

In view of evidence for a predominant role of the stroma in tumor PA production, including breast cancer (Nielsen *et al.,* 1996), the physiological relevance of the MCF-7 model used here is unclear. Nevertheless, the data shown suggest that cell attachment to LN initiates a signaling cascade culminating in down-regulation of PAI-1 mRNA and, to a lesser extent, uPA mRNA. Whether or not such a signaling pathway targets uPA/PAI-1 expression in other producer cells, such as stromal fibroblasts, remains an open question.

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