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Hormonal and extracellular matrix regulation of plasminogen activator in a bovine mammary epithelial cell line

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The effects of lactogenic hormones on urokinase plasminogen activator (u-PA) produced by bovine mammary epithelial cells (MAC-T) were examined. High levels of u-PA activity were detected in growth arrested cells cultured on plastic. This suggests that high levels of PA activity alone are not sufficient to induce proliferation of bovine mammary epithelial cells. Cells were cultured on various extracellular matrices: plastic, fibronectin, collagen, matrigel, and laminin. Basal levels of u-PA activity in media from MAC-T cells cultured on matrigel were 1.6-, 2-, 2- and 3.5-fold higher than that of cells cultured on plastic, fibronectin, collagen, and laminin, respectively. Insulin increased ($P<0.01$) mammary epithelial u-PA activity on a per cell basis, an effect observed irrespectively of the type of extracellular matrix onto which cells were cultured. Not unexpectedly, insulin-like growth factor (IGF)-I, Des(1-3) IGF-I and IGF-II increased mammary epithelial u-PA activity on a per cell basis and u-PA mRNA levels, thus, mimicking the effect of insulin. Dexamethasone suppressed ($P<0.01$) u-PA activity but was unable to suppress the insulin-induced increase in u-PA activity of cells cultured on various extracellular matrices. These data indicate that u-PA activity is modulated by both lactogenic hormones and the type of extracellular matrix.

Keywords: Plasminogen activator; extracellular matrix; mammary epithelial cells

Introduction

In mammals, the zymogen plasminogen can be activated by two specific serine proteases, known as plasminogen activators (PA). There are two types of PA: urokinase-PA (u-PA) and tissue-PA (t-PA). These enzymes are products of two distinct genes (Saksela & Rifkin, 1988). Several studies have implicated t-PA in the maintenance of the fluidity of the extracellular milieu and in particular in thrombolysis, while u-PA is mainly involved in the proteolytic events that accompany tissue remodelling events such as involution of the mammary gland (for a review, Saksela & Rifkin, 1988). Bovine mammary epithelial cells produce u-PA (Heegard *et al.*, 1994).

A relationship exists between expression of u-PA and the growth state of cells. Many of the factors regulating PA activity *in vitro* are among the most powerful growth regulators of cultured cells. In fact, a growing body of evidence attests to a prominent role of PA in cell proliferation, possibly by breaking cell-matrix interactions during cell division (for a review, Saksela & Rifkin, 1988).

The endocrine control of mammary function is complex but some hormonal conditions have been clearly defined. Estrogen, hydrocortisone, prolactin and insulin are thought to be important modulators of mammogenesis and galactopoiesis (Akers, 1990). More recently, several growth factors have been implicated in affecting growth and differentiation of mammary epithelial cells and include: insulin-like growth factors (IGFs), epidermal growth factor (EGF) and the transforming growth factor family (TGFs) (Akers, 1990).

Prolactin and hydrocortisone, hormones important for mammary epithelial cell differentiation, blocked PA while insulin that enhances cell proliferation produced parallel effects on PA production by mouse mammary explants (Ossowski *et al.*, 1979). However, the origin of PA (epithelial versus non epithelial cells) was not established. A comprehensive study is required to evaluate the effects of the entire family of insulin and IGFs on PA production by normal mammary epithelial cells.

The objective of this study was to determine the effect of various hormones and growth factors on PA produced by bovine mammary epithelial cells, using the recently developed MAC-T cell line as the model system. The MAC-T cells deviate from normal cells, yet they retain many of the specialized functions normally seen in normal cells. These functions include: 1) production of casein, in a hormonally regulated manner, and 2) responsiveness to various growth factors and growth inhibitors. Furthermore, MAC-T cells are not tumorigenic (Huynh *et al.*, 1991).

Results

Kinetics of PA production

u-PA activity detected in the culture medium of MAC-T cells increased with increasing incubation time (Figure 1A). Following an initial 24 h incubation phase, the increase in u-PA activity was linear for up to 96 h. An increase in u-PA activity can be due to an increase in cell numbers and/or an increase in u-PA biosynthetic activity per cell. Cell numbers were maintained for up to 96 h of incubation in DMEM alone (Figure 1B). A further increase of the incubation time to 144 h led to a 27% decrease ($P<0.01$) in cell numbers. Thus, the linear increase in activity with increasing incubation time is suggestive of constitutive expression of u-PA by non proliferating cells. Mammary epithelial cells produce very low levels of PAI-1 (Heegard *et al.*, 1994).

Effects of insulin and IGFs on PA production

The effect of insulin, IGF-I, Des(1-3)IGF-I, and IGF-II on PA activity was examined. Insulin increased u-PA activity in all concentrations used (0.01, 0.1 or 1 μg/ml) (Table 1). IGF-I and Des(1-3)IGF-I also increased PA activity, mimicking the effect of insulin. Interestingly, IGF-II mimicked the effect of insulin but only at the high concentration (50 ng/ml) (Table 1). The effect of insulin on PA activity, using the modified protocol (test medium changed every 24 h), was examined. Insulin increased u-PA activity by 2- to 6-fold during the first, second or third 24 h in culture (Table 2). The effect of insulin became more pronounced during the second and third 24 h in culture.

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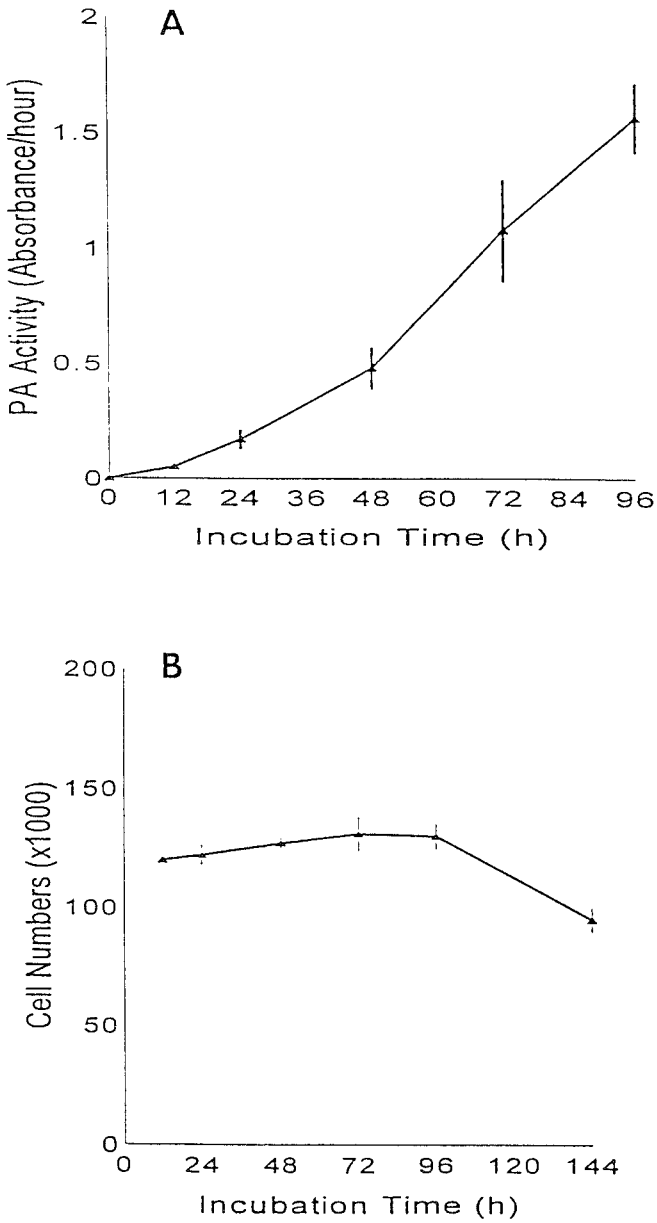

Figure 1 Time-course changes of u-PA activity detected in the culture medium (**A**) and cell numbers (**B**) of MAC-T bovine mammary epithelial cells. Cells were cultured in serum-free Dulbecco's Modified Eagle's Medium for up to 72 h. Values are means + S.D. of four independent samples for each data point assayed in duplicate

Effect of dexamethasone on PA production

There were no differences in u-PA activity detected in the medium of cells cultured for 12 h in the presence or absence of dexamethasone (Figure 2). However, dexamethasone (25 nM) suppressed ($P<0.01$) u-PA activity by 53, 30 and 39% following incubation for 24, 48 and 72 h, respectively. Dexamethasone at 100 or 1000 nM suppressed ($P<0.01$) PA activity by 59, 58 and 40% following incubation for 24, 48 and 72 h, respectively. Both concentrations inhibited PA activity equally well. Companion experiments showed that dexamethasone had no effect on DNA content of cultures (data not shown). The effect of dexamethasone on u-PA activity, using a slightly different experimental design, was examined. In this experiment, the test medium was changed every 24 h. Dexamethasone suppressed u-PA activity during the first, second, but not during the third 24 h in culture (Table 3).

Table 1 Effect of insulin, IGF-I, IGF-II, on mammary epithelial plasminogen activator (PA) activity

Treatment	*PA activity (absorbance/h)/μg DNA*
DMEM	0.049 + 0.01[a]
Insulin dose (μg/ml)	
0.01	0.091 + 0.01[b]
0.1	0.091 + 0.01[b]
1	0.088 + 0.01[b]
IGF-I dose (ng/ml)	
1	0.092 + 0.01[b]
10	0.089 + 0.01[b]
50	0.098 + 0.02[b]
Des (1–3) IGF-I dose (ng/ml)	
1	0.101 + 0.01[b]
10	0.094 + 0.01[b]
50	0.092 + 0.01[b]
IGF-II dose (ng/ml)	
1	0.071 + 0.007[c]
10	0.075 + 0.005[c]
50	0.093 + 0.024[b]

Cells were cultured in serum-free Dulbecco's Modified Eagle's Medium (DMEM) for 72 h in the absence or presence of IGF-I, des (1–3)IGF-I or IGF-II. Values are means from four independent samples at each hormone concentration assayed twice. At the end of the incubation period the medium was recovered for determination of PA activity, cells were harvested, and total DNA (μg/well) was determined. Values within a column with different superscripts were significantly ($P<0.05$) different by Duncan's multiple comparison test

Table 2 Effect of insulin on mammary epithelial plasminogen activator (PA) activity

Incubation time (h)	*PA activity* (absorbance/h/10^5 cells) *Control*	*Insulin*
First 24	0.20 + 0.02	0.42 + 0.02*
Second 24	0.17 + 0.10	0.90 + 0.38*
Third 24	0.15 + 0.07	0.77 + 0.25*

Cells were cultured in serum-free Dulbecco's Modified Eagle's Medium (DMEM) or DMEM containing 1 μg/ml insulin for up to 72 h and the test medium was changed every 24 h. At the end of the incubation period the medium was recovered for determination of PA activity, cells were trypsinized and counted. Values are means from six independent samples at each time point. Values with different superscripts were significantly ($P<0.05$) different from the control by Duncan's multiple comparison test

Effect of insulin, IGF-I and dexamethasone on u-PA mRNA

Steady state concentrations of u-PA mRNA were examined. Total RNA obtained from MAC-T cells was hybridized with a bovine u-PA cDNA probe and the results are presented in Figure 3A. Relative amounts of u-PA mRNA were initially determined by scanning the above autoradiogram. Then, the blot was stripped, hybridized with a 18s rRNA probe (Figure 3B), and scanned for a second time to estimate the amount of total RNA in each lane. Values obtained for uPA mRNA levels from the first scanning were then divided by the corresponding 18s rRNA values from the second scanning, therefore, accounting for variability in the amount of RNA loaded to each lane and transfer efficiency. Insulin and IGF-I increased u-PA mRNA levels by 50-120% compared to control values after 24 or 48 h of incubation. On the contrary, dexamethasone had no effect on uPA mRNA levels.

Hormonal and extracellular matrix regulation of PA production

There were differences in u-PA activity in media from MAC-T cells cultured on various extracellular matrices in DMEM alone (Table 4). MAC-T cells cultured on matrigel produced 1.6-fold, 2-fold, 2-fold and 3.5-fold more u-PA activity ($P<0.05$) on a per cell basis than cells cultured on plastic,

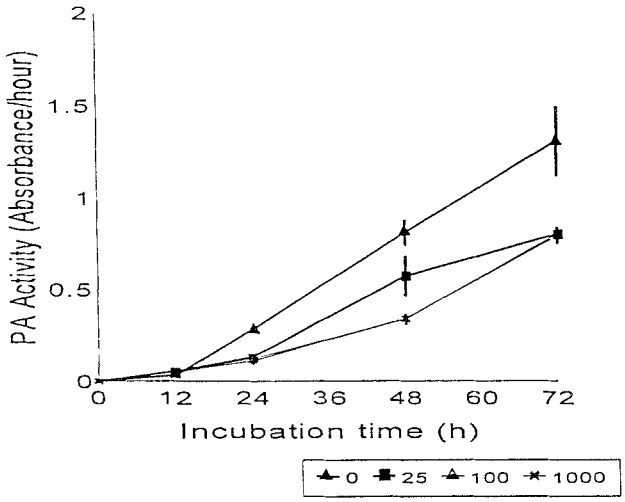

Figure 2 Effect of dexamethasone on u-PA activity detected in the culture medium of MAC-T mammary epithelial cells. MAC-T cells were cultured for various times in serum-free Dulbecco's Modified Eagle's Medium in the absence (Δ) or presence of 25 (■), 100 (+), or 1000 (*) nM of dexamethasone. Values are means + S.D. of four independent samples for each data point assayed in duplicate

Table 3 Effect of dexamethasone on mammary epithelial plasminogen activator (PA) activity

Incubation time (h)	*PA activity* (absorbance/h/10^5 cells) *Control*	*Dexamethasone*
First 24	0.24 + 0.07	0.09 + 0.03*
Second 24	0.17 + 0.03	0.10 + 0.03*
Third 24	0.18 + 0.07	0.14 + 0.02

Cells were cultured in serum-free Dulbecco's Modified Eagle's Medium (DMEM) or DMEM containing 100 nM dexamethasone for up to 72 h and the test medium was changed every 24 h. At the end of the incubation period the medium was recovered for determination of PA activity, cells were trypsinized and counted. Values are means from six independent samples at each time point. Values with different superscripts were significantly ($P<0.05$) different from the control by Duncan's multiple comparison test

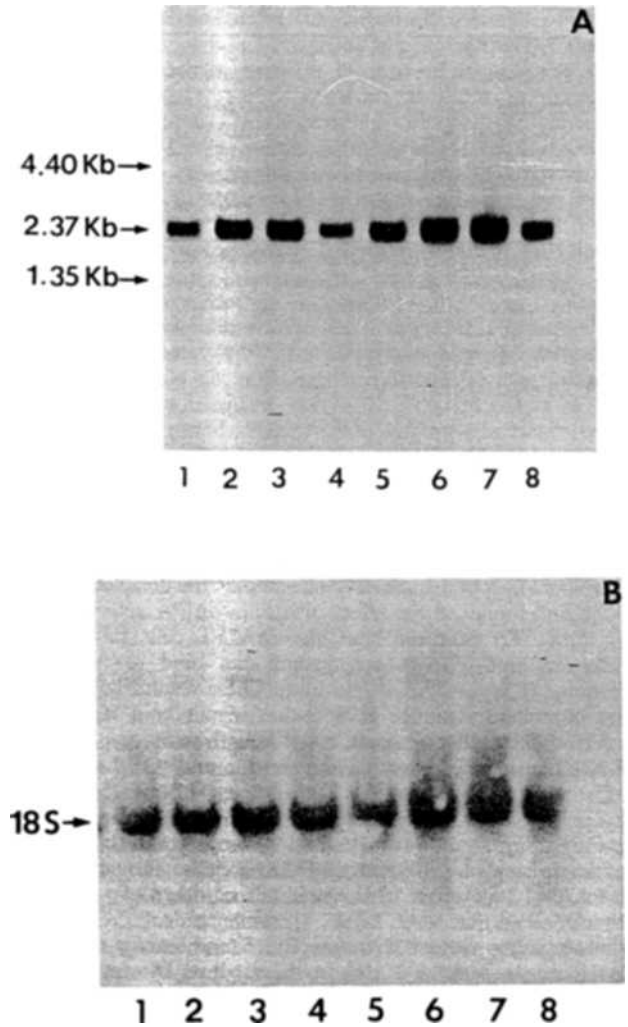

Figure 3 Expression of u-PA mRNA (A) and 18s rRNA (B) in MAC-T mammary epithelial cells cultured in the presence of various hormones. Cells were cultured in the presence of serum free Dulbecco's Modified Eagle's Medium (DMEM) (lanes 1 and 5), 1 μg/ml insulin (lanes 2 and 6), 50 ng/ml IGF-I (lanes 3 and 7), or 100 nM dexamethasone (lanes 4 and 8). Following incubation for 24 h (lanes 1–4) or 48 h (lanes 5–8), total RNA (15 μg) was extracted, electrophoresed on a agarose gel and transferred to membrane, and the filter was probed with nick-translated u-PA (**A**) or 18s rRNA (**B**) probes. Migration of markers (RNA ladder) is indicated on the left

collagen, fibronectin, and laminin, respectively. Insulin increased ($P<0.05$) u-PA production by mammary epithelial cells cultured on all extracellular matrices. Dexamethasone suppressed ($P<0.05$) PA activity by cells cultured on all extracellular matrices except matrigel and laminin (Table 4). The effect of prolactin was less consistent across extracellular matrices. There was an increase ($P<0.05$) in u-PA activity for cells cultured on matrigel and laminin, no effect for cells cultured on fibronectin or collagen and a decrease ($P<0.05$) in u-PA activity for cells cultured on plastic. Dexamethasone added in combination with prolactin suppressed ($P<0.05$) u-PA activity for MAC-T cells cultured on plastic, collagen and laminin (Table 4). Dexamethasone added in combination with prolactin and insulin was unable to suppress the insulin-induced increase in u-PA activity. In fact, the combination of the three hormones increased u-PA activity more than insulin alone in three types of extracellular matrices: plastic, collagen, and laminin (Table 4).

Discussion

The first finding emerging from this study was that non proliferating MAC-T mammary epithelial cells produce u-PA (Figure 1). Our previous work has shown that under these conditions approximately 90–95% of the MAC-T cells are in G_1/G_0 phase of the cell cycle (Zavizion *et al.*, 1993). The increase in PA activity over time without a concomitant increase in cell numbers (Figure 1) suggest that growth arrested cells, in G_1/G_0 phase of the cell cycle, produce high levels of u-PA. The linear increase in activity over time is suggestive of constitutive expression of u-PA by non proliferating, stationary cells.

Saksela and Rifkin (1988) in their review paper suggested that high PA production occurs at the G_1/G_0 transition point and there is a strong correlation between stimulated proliferation and PA activity. Our data indicating high levels of PA activity in growth arrested cells without concomitant cell proliferation argue against this hypothesis and clearly suggest that an increase in PA activity alone is not sufficient to induce cell proliferation of bovine mammary epithelial cells.

The second finding emerging from this study was that insulin increased u-PA activity and mRNA levels. PA activity values were adjusted for increases in cell numbers by insulin. The increase in PA activity taken together with the increase

Table 4 Hormonal and extracellular matrix regulation of mammary epithelial plasminogen activator (PA) activity

	PA activity (absorbance/h/μg of DNA)				
Treatment	*Plastic*	*Fibronectin*	*Collagen*	*Matrigel*	*Laminin*
DMEM	0.049[a]	0.037[a]	0.039[a]	0.078[a]	0.022[a]
DMEM + I	0.084[b]	0.077[b]	0.057[b]	0.138[b]	0.083[b]
DMEM + DEX	0.024[c]	0.023[c]	0.019[c]	0.104[c]	0.020[a]
DMEM + PRL	0.030[d]	0.034[a]	0.040[a]	0.141[b]	0.042[c]
DMEM + PRL + DEX	0.023[d]	0.034[a]	0.030[a]	0.144[b]	0.047[c]
DMEM + I + PRL + DEX	0.111[e]	0.077[b]	0.109[d]	0.115[c]	0.110[d]

Cells were cultured in serum-free Dulbecco's Modified Eagle's Medium (DMEM) in the presence of 1 μg/ml insulin, (I), 1 μg/ml prolactin (PRL), 100 nM dexamethasone (DEX) or their combinations for 72 h. At the end of the incubation period the medium was recovered for determination of PA activity, cells were harvested, and total DNA (μg/well) was determined. Values are means from four independent samples at each time point. Values within a column with different superscripts were significantly ($P<0.05$) different by Duncan's multiple comparison test

in u-PA mRNA levels suggest that insulin alters the u-PA biosynthetic capacity of MAC-T cells. Similarly, insulin increased PA production by mouse mammary epithelial cells in an organ explant system (Ossowski *et al.*, 1979). Not unexpectedly IGF-I and its analog increased u-PA, thus, mimicking the effect of insulin. IGF-II only at the concentration of 50 ng/ml mimicked the effect of insulin on PA activity. Zhao *et al.* (1991) reported that the MAC-T cells have IGF-I receptors and IGF-II and insulin can bind to the IGF-I receptor (IGF-I>IGF-II>insulin). These results, collectively, are consistent with the notion that insulin and IGF-I may exercise their effects through the IGF-I receptor.

We considered it possible that insulin and IGF-I may have affected PAI-1 expression. Two pieces of evidence argue against this possibility. Heegard *et al.* (1994), using regular zymography, found no u-PA/PAI-1 complexes in the medium of stationary or proliferating MAC-T cells. They found the u-PA/PAI-1 complex in the medium of other two cell lines MACT-UV1 and MACT-UV2 (positive control). White *et al.* (1995), using reverse zymography, found barely detectable levels of the 50 kDa PAI-1 in the medium of stationary or proliferating MAC-T cells.

The third finding emerging from this study was that dexamethasone suppressed u-PA activity of bovine mammary epithelial cells in culture. However, there were no differences in u-PA activity of MAC-T cells cultured in the presence or absence of dexamethasone for the first 12 h. Therefore, there is a time-delay for dexamethasone to take its effect. There are two possible explanations for this observation. First, cells during the time of dexamethasone addition are abruptly switched from a serum supplemented to a serum-free medium. It is possible that during the unavoidable adaptation period, dexamethasone can not exercise its PA suppressing effects. Second, the time delay may mean that synthesis of a compound (protein?) is required for suppression of PA activity. Our study can not discriminate between these two possibilities. It is interesting that dexamethasone was unable to suppress u-PA mRNA levels. This indicates that dexamethasone affected u-PA mRNA stability or u-PA protein translation.

Glucocorticoids generally suppress proteolytic activity in various established, primary cultures, and organ cultures of various cell types (Saksela & Rifkin, 1988). It appears that dexamethasone suppresses u-PA much more effectively than t-PA production (Saksela & Rifkin, 1988). Other studies showed that the suppression of proteolytic activity by dexamethasone can be attributed to an increase in PA inhibitor (PAI) biosynthesis (Andreasen *et al.*, 1986) or a combination of decreased PA and increased PAI biosynthesis (Pearson *et al.*, 1987). However, Heegard *et al.* (1994) and White *et al.* (1995) found minimal production of PAI-1 by MAC-T mammary epithelial cells.

The fourth finding emerging from this study was that extracellular matrix modulates PA activity. Numerous studies have documented that the type of extracellular matrices affect the function of mammary epithelial cells in many ways other than PA production (Akers, 1990; Hyunh *et al.*, 1991). For example, mammary epithelial cells cultured on plastic are usually poorly differentiated and produce no appreciable levels of milk caseins. On the other hand, mammary epithelial cells cultured on collagen differentiate and produce casein.

Various lactogenic hormones affected PA activity in a variety of extracellular matrices. Insulin increased PA activity on a per cell basis for MAC-T cells cultured on all extracellular matrices. The effect of prolactin was less consistent. Prolactin which had no effect on cell proliferation, increased PA activity of MAC-T cells cultured on matrigel, and laminin and decreased PA activity of MAC-T cells cultured on plastic. Prolactin suppressed PA production by mouse mammary tissue explants (Ossowski *et al.*, 1979). Dexamethasone suppressed PA activity of MAC-T cells cultured on plastic, fibronectin and collagen. On the contrary, dexamethasone increased PA activity of cells cultured on matrigel. Two possible explanations for this inconsistency include: (1) matrigel contains growth factors (Vukisevic *et al.*, 1992), and (2) some sequestering of PA in the matrigel. On no occasion dexamethasone did suppressed PA activity when added in combination with prolactin and insulin.

Laminin is the main component of matrigel. It was interesting to note that PA activities were different in matrigel and laminin substrates. Once again, the most reasonable explanation for this observation is that matrigel contains various growth factors that may have affected PA activity.

High levels of milk PA have been observed during late lactation in dairy cattle (Politis *et al.*, 1989). The levels of insulin increase as lactation advances (Tucker, 1985). It is tempting to speculate based on the results of the present study that insulin may be responsible for the increase in PA activity during late lactation. It remains, however, to be determined whether the insulin-induced increase in PA activity by mammary epithelial cells occurs *in vivo*.

In summary, the present study demonstrated that lactogenic hormones and extracellular matrix affected u-PA activity and u-PA mRNA levels. Insulin increased u-PA activity, while dexamethasone decreased u-PA activity. However, dexamethasone was unable to suppress the insulin-induced increase in u-PA activity. Further studies will discriminate between effects on PA versus PAI.

Materials and methods

Cell culture

The origin of the MAC-T mammary epithelial cell line was described previously (Huynh *et al.*, 1991). Briefly, these cells were produced from primary bovine mammary epithelial cells by stable transfection with a plasmid bearing the DNA sequence for SV-40 large T-antigen. The MAC-T cells can differentiate when plated on collagen and produce casein in

the presence of prolactin (Huynh *et al.*, 1991). Frozen aliquots of cells in their 20th passage were transported to our laboratories (University of Vermont and Virginia Tech) from McGill University and subsequently utilized in the experiments described below.

Cells were routinely cultivated in plastic petri dishes (Corning Glass, Corning, NY, USA) in Dulbecco's Modified Eagle's medium (DMEM; Sigma Chemical Co., St Louis, MO) supplemented with 10% fetal calf serum (FCS; Sigma), penicillin (100 units/ml; Sigma) and streptomycin (100 μg/ml; Sigma). Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

Experimental protocols

The first experiment examined the effects of various concentrations of insulin, IGF-I, Des(1-3)IGF-I, IGF-II on PA activity. Cells (2×10^5) were seeded in each well of a 24-well tissue culture plastic plates in DMEM containing 10% FCS. After 24 h, media were removed, cells were washed twice with Hank's balanced salt solution (HBSS) and test medium was added for 72 h. Test medium consisted of serum-free DMEM alone, serum-free DMEM containing various levels of insulin (0.01, 0.1, 1 μg/ml; Sigma), serum-free DMEM containing various concentrations of IGF-I, IGF-II, or Des(1-3)IGF-I (1, 10, 50 ng/ml; Gropep, Adelaide, Australia). Media were recovered for determination of PA activity. Cells were removed by complete trypsinization (10 min at 37°C) (Zavizion *et al.*, 1993) and total cells were counted using a hemocytometer. For other experiments, proliferative responses were based on determination of DNA content of harvested cells using the method of Labarca and Paigen (1980).

A second experiment examined the effect of dexamethasone on PA activity and cell numbers. Approximately, 2×10^5 cells were seeded in 24-well tissue culture plates in DMEM containing 10% FCS. After 24 h, media were removed and cells were washed twice with HBSS and test medium was added. Test medium consisted of serum-free DMEM alone or serum-free DMEM containing dexamethasone (25, 100 or 1000 nM; Sigma). Cells were incubated in the presence or absence of dexamethasone for various times up to 6 days. Media were recovered for determination of PA activity (see later). Cell trypsinization, enumeration, and determination of DNA content were performed as described above.

A third experiment examined time-course changes effects of dexamethasone or insulin, using a modified protocol, on PA activity. The major difference in this experiment, compared with the first experiment, was that the serum-free DMEM medium with dexamethasone (100 nM) or insulin (1 μg/ml) was changed every 24 h, and new test medium was added. Other details are as described above.

A fourth experiment examined the effect of various hormones on PA production by culturing MAC-T cells in 24-well plastic plates for 72 h in the presence of DMEM alone, DMEM supplemented with dexamethasone (100 nM), insulin (1 μg/ml), or prolactin (1 μg/ml; National Hormone and Pituitary Program, Baltimore, MD), or various combinations of these hormones. Media were collected for determination of PA activity and cells were harvested for measurement of DNA content. To investigate whether production of PA was regulated by hormones and the extracellular matrix, the experiment was repeated utilizing four additional matrices. These were matrigel, collagen (type I, rat tail), fibronectin, and mouse laminin. Precoated dishes (Bio-coat^R cellware) were purchased from Collaborative Biomedical Products Inc. (Two Oak Park, Bedford, MA, USA) and were used according to the directions provided by the manufacturer.

Determination of PA activity in culture medium

A colorimetric assay, previously used to determine PA activity in milk somatic cells (Zachos *et al.*, 1992) was validated to measure PA activity present in serum-free DMEM culture medium. The assay system utilizes PA that is present in the culture medium to convert exogenously supplied plasminogen to active plasmin. Plasmin, so produced, is subsequently allowed to attack the chromogenic substrate Valine-Leucine-Lysine-*p*-nitroaniline adjacent to Lysine and liberate the free chromophore *p*-nitroaniline (V 7221). In this system, changes in color are directly related to plasmin levels, and therefore indirectly to PA activity.

Assays were performed in 250 μl of 100 mM Tris buffer (pH 8.0) containing plasminogen (50 μg/ml), 0.6 mM Val-Leu-Lys-*p*-nitroanilide and 1 to 5 μl of culture medium. Preliminary experiments indicated that PA activity was maximum at 0.6 mM of V 7221, 50 μg/ml of exogenous plasminogen and pH 8.0 and these conditions were maintained throughout all subsequent assays. The reaction mixture was incubated for 3 h and absorbance at 405 nm was measured at 30 min intervals using a microtiter plate (Bio-Tec Instr., Burlington, VT). The rate of *p*-nitroaniline formation was calculated from the linear part of the absorbance versus time curve. A sample without plasminogen served as a control. Preliminary experiments indicated that PA activity was linear for up to 3 h of incubation and between 1 and 5 μl of sample volume.

Effects of hormones on u-PA mRNA levels

Mammary epithelial cells (MAC-T) were plated in 100 mm tissue culture plates in DMEM containing 10% FCS. When cells were at 85% confluency, media were removed, and cells were washed with HBSS. Cells were cultured in serum-free DMEM containing insulin (1 μg/ml), IGF-I (50 ng/ml), or dexamethasone (100 nM). Following incubation for 24 or 48 h, cells were trypsinized and total RNA was extracted using a guanidinium thiocyanate isolation procedure (Chomczynski & Sacchi, 1987). Total RNA (15 μg) RNA were separated by electrophoresis on a 1.2% agarose gel containing 6% formaldehyde, washed and the RNA was transferred to Zeta probe membranes (Bio-Rad) by capillary blotting with 20 × SSC for 20 h (Politis *et al.*, 1992). Following transfer, the membrane was placed in a plastic bag containing 8 ml of prehybridization buffer (50% formamide, 4 × SSPE (0.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM Ethylenediamine.tetracetic acid, pH 7.4), 1% SDS, 200 μg/ml of salmon sperm DNA and 5 mg/ml of skim milk powder) for 20 h at 42°C. Membranes were hybridized in the same solution containing 4×10^5 cpm/ml of ^{32}P-labelled u-PA cDNA (Ravn *et al.*, 1995), labeled using nick-translation (Politis *et al.*, 1992). The membranes were washed and then exposed to Kodak XAR-S X ray film at −72°C for 24 h using two intensifying screens. Relative radioactivity was estimated from the autoradiograms by densitometry. Membranes were stripped with 50 mM NaOH at room temperature for 30 min and were probed for a second time with a bovine 18s rRNA probe to estimate the amount of total RNA in each lane and, therefore, to normalize for variability in RNA loaded to each well and transfer efficiency.

Statistical analysis

Data are presented as means + S.D. Effects of incubation time on PA activity and cell numbers were evaluated with one-way analysis of variance using the General Linear Model procedure (SAS, 1985). Effects of hormones and extracellular matrices were evaluated by analysis of variance using hormonal treatment and type of extracellular matrix as main effects, and their interaction. Means comparisons were done utilizing Duncan's multiple range test with $P < 0.05$ selected as the level of significance.

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