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Inhibition of Urokinase Activity by the Antiangiogenic Factor 16K Prolactin: Activation of Plasminogen Activator Inhibitor 1 Expression

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ABSTRACT

The N-terminal fragment of PRL (16K PRL) is an antiangiogenic factor that, *in vitro*, inhibits several components of angiogenesis including basic fibroblast growth factor (bFGF)-induced cell division, migration, and organization of capillary endothelial cells. An essential step in the regulation of angiogenesis is the activation of urokinase (urokinase type plasminogen activator, uPA), which in turn activates a cascade of proteases that play essential roles in endothelial cell migration and tissue remodeling. Treatment of bovine capillary endothelial cells (BBEC) with 16K PRL inhibited bFGF-stimulated urokinase activity in BBEC as detected by plasminogen substrate gel assay. 16K PRL did not appear to be acting via an effect on uPA expression because no change in messenger RNA levels were observed. However, protein levels of plasminogen activator inhibitor-1 (PAI-1), a specific inhibitor of urokinase, were increased by 16K PRL independent of the action of bFGF. The 16K PRL-induced increase in PAI-1 protein levels appear to be the result of increased expression of the PAI-1 gene. Increased production of PAI-1 induced by 16K PRL results in the formation of inactive PAI-1/uPA complexes, consistent with the observed decrease in uPA activity.

THE FORMATION of new blood vessels (angiogenesis) is a precisely regulated process throughout life. Angiogenesis appears to be controlled by a series of stimulatory factors, angiogenic, and inhibitory factors, antiangiogenic. In most instances, the balance of angiogenic and antiangiogenic activity is balanced resulting in the maintenance of a slowly turning over population of vascular endothelial cells (1, 2). However, the balance is changed and the growth of the microvasculature is stimulated during tissue repair (wound healing), tissue remodeling (formation of the corpus luteum), or various disease processes (solid tumor growth). New vessels are formed from existing capillaries, which necessitates the proliferation of vascular endothelial cells and their migration and organization into new vessels. Known factors with angiogenic activity include bFGF (3), vascular endothelial cell growth factor (4), and angiogenin (5), whereas factors with antiangiogenic activity include thrombospondin (6), 16K PRL (7), platelet factor-4 (8), and angiostatin (9).

Both the 16 kDa N-terminal fragment of rat PRL (16K rPRL) (7) and human PRL (16K hPRL) are potent antiangiogenic factors *in vivo* in the chicken chorioallantoic membrane assay (10). The antiangiogenic action of 16K PRL appears to be mediated at multiple steps in the formation of new vessels. 16K PRL inhibits bFGF and VEGF-induced cell proliferation of cultured capillary endothelial cells from bovine and hu-man (*7*,10). Furthermore, 16K PRL inhibits the organization of BBEC cultured in type 1 collagen gels into capillary-like structures (10), and the migration of BBEC through the pores of collagen coated filters in Boyden chambers (data not shown). These observations led us to address the mechanisms by which 16K PRL activated cell invasiveness and migration.

Cellular invasiveness in various biological processes, including angiogenesis, requires the activation of proteases capable of degrading extracellular matrixes (11). Urokinase (urokinase type plasminogen activator, uPA) appears to be a key modulator in the neoplastic invasive process (12, 13). Urokinase converts widely distributed and inactive plasminogen into plasmin, a tryptic protease capable of degrading certain matrix components, and activating other matrix degrading enzymes like the metalloprotease, collagenase (14). Additionally, urokinase has also been demonstrated to be important in the migration of the capillary endothelial cells through the interstitial matrix (15-17).

Urokinase activity is regulated by the rate of its synthesis, conversion of the proenzyme to the active form of the enzyme, and the presence of the specific inhibitors of the enzyme activity (21). Plasminogen activator inhibitor-1 (PAI-1), a major endothelial cell derived component of the extracellular matrix, is believed to protect

extracellular matrix proteins from excessive plasminogen activator catalyzed proteolysis (18-21). PAI-1 irreversibly binds to and inactivates uPA (22). A balance between urokinase and PAI-1 levels has been proposed to be important in the regulation of angiogenesis. Consistent with an important role in the control of angiogenesis, PAI-1 gene expression is highly regulated, *e.g. in vitro* TGF-*β* stimulates PAI-1 expression and is a potent antiangiogenic factor (23).

We hypothesized that the antiangiogenic actions of 16K PRL on cell invasiveness and migration could be mediated via the regulation of uPA activity. Data from *in vitro* experiments with BBEC showed that 16K PRL inhibits uPA activity apparently via stimulation of the expression of PAI-1. The stimulatory effect of 16K PRL on PAI-1 is at the transcriptional levels and is not dependent on the action of bFGF.

Materials and Methods

Preparation of 16K PRL

16K *hPRL.* Recombinant human 16K PRL (16K hPRL) was prepared as previously described (24). A Pt7L plasmid containing human 23K PRL complementary DNA (cDNA) was site-directed mutagenized as reported. Briefly, Cys-58 (TGG) of the construct was mutated to serine (TCC) to prevent the formation of incorrect disulfide bonds, and Glu-140 (GAA) was mutated to TAA to generate a premature stop codon. The recombinant 16K hPRL was prepared from inclusion bodies of E. *coli* as described. Purity was >90%, and endotoxin levels were < 0.01 EU/160 ng·

16K rPRL. Rat 16K PRL (16KrPRL)was generated by enzymatic cleavage of the intact, 23 kDa rat PRL (NIH, B-6), as previously described (7). Briefly, rPRL was cleaved by incubation with a particulate fraction from rat mammary gland homogenates, reduced in 2-mercaptoethanol, and the 16 kDa N-terminal fragment separated by gel filtration on Sephadex G-50. To prevent reformation of disulfide bonds, the 16K rPRL was carboidomethylated. 16K rPRL was reduced with DTT (20-fold molar excess over the disulfide bond molecular content) in the presence of 4 M guanidine HC1 in 0.1 M NH_4HCO_3, pH 8.5, and subsequently alkylated with a 40 M excess of iodoacetamide over the DTT concentration. The sample was dialyzed, and its purity and concentration were assessed by SDS-PAGE and densitometry.

Cell culture

BBEC were isolated as previously described (25). The cells were grown and serially passaged in low glucose DMEM supplemented with 10% calf serum, 2 mM L-glutamine, and antibiotics (100 U penicillin/ streptomycin per ml and 2.5 mg of fungizone per ml). Basic FGF (bFGF, Promega) was added (1 ng/ml) to the cultures every 2 days. Experiments were initiated with confluent cells between passages 5 and 13.

Cell stimulation and preparation of cell extracts

Confluent culture of BBEC were enzymatically dispersed and plated at a density of $5 \times l0^5$ cells per well in 6-well plates in 1 ml of BBEC media. Twenty-four hours after plating, cells were transferred to MEM containing low serum for 24 h (0.5% calf serum). Cells were treated with bFGF or 16K PRL alone or together for 16 h. The incubation was terminated by aspiration of the media and addition of 200 µl of the lysis buffer (0.1 M Tris, pH 8.1, and 0.5% Triton X-100) shake at 4 C for 20 min. The insoluble fraction was removed by centrifugation at 4 C for 10 min at 14,000 × *g*, and the protein concentrations of the soluble fraction were determined by BCA assay kit (Pierce, Rockford, IL).

Plasminogen activator substrate gel

Plasminogen activator (PA) substrate gels were performed according to the method described by Blei *et al.* (26) with minor modification. Briefly, aliquotes of conditioned medium and cell extracts were separated under nonreducing conditions by SDS PAGE using a 4% stacking and 10% resolving gel. The SDS gel was washed three times (20 min each) in 2.5% Triton X-100 to remove the SDS and overlaid on a fibrin-agar indicator gel as described above. The gel complex was incubated overnight at 4 C to enable proteins in the SDS-PAGE gel to diffuse into the substrate gel. Zones of lysis that developed following incubation at 37 C (1-5 h) indicated PA activity. The reaction was terminated by incubation with 10% acetic acid/40% methanol for 20 min. The gel was stained in 1% amido black in 10% acetic acid/40% methanol for 10 min and destained in 10% acetic acid/40% methanol with several changes until the solution was clear. The stained gels were dried and photographed on a light box. The bands were scanned and quantified by the Scan Analysis Program.

RNA preparation

For the extraction of total RNA BBEC cultured on 10-mm dishes, cells were lysed in guanidinium solution (4 M guanidinium thiocyanate/25 mM sodium citrate, pH 7.0/0.5% sarcosyl/0.1 M 2-mercaptoethanol). The resulting lysates were layered on 5.7 M CsCl and centrifuged at 40,000 rpm for 12 h. The RNA pellets were dissolved in DEPC treated H_2O and integrity of the RNA was assessed by electrophoresis on agarose gels. The RNA concentrations were determined by spectrophotometry.

Northern blot analysis

For Northern blot analysis, RNA samples were electrophoresed in 1% agarose gels containing 2.2 M formaldehyde, 20 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS, Fisher Scientific, Pittsburgh, PA), 8 mM sodium acetate, and 1 mM EDTA. RNA was transferred onto nylon membranes (*N*-Hybond, Amersham) and covalently cross-linked by irradiation with 120 mj of UV light. 1535 bp of EcoRI HinDIII fragment of Bovine urokinase cDNA (kindly provided by Dr. W. D. Schleuning, Research Laboratory of Schering AG, Berlin, Germany), 600 bp of P*sf*l fragment of PAI-1 cDNA (kindly provided by Dr. M. J. Pepper, University Medical Center, Geneva, Switzerland) and 110 bp of *Pstl* fragment of cyclophillin cDNA (kindly provided by Dr. M. Skinner) were labeled by [^{3}-P]dCTP with oligolabeling kit (Pharmacia, Piscataway, NJ) and purified by Quick Spin columns (Boehringer Mannheim, Indianapolis, IN). The hybridization reactions were performed at 68 C for 1 h in Quickhyb solution (Stratagene, La Jolla, CA). The hybridized blots were washed twice with 2 × SSC/0.1% SDS at RT for 15 min and once with 0.1 × SSC/0.1% SDS at 65 C for 30 min. The resulting blots were subjected to autoradiography. Quantification of the messenger RNA (mRNA) levels was performed by densitometric scanning of the auto-radiograms of cyclophillin or methylene blue staining of 28S and 18S ribosomal RNA as an internal control.

RNase protection assay for bovine urokinase

RNase protection assays were performed according to the protocols of Werner *et al.* (27). The 445 bp *Eco*RI-*Bgl*II fragment of the bovine urokinase cDNA was transcribed with ^{32}P uridine triphosphate using T7 promoter to generate a radiolabeled riboprobe. Ten micrograms of the RNA isolated from BBEC were hybridized with 2.5 × l0^5 cpm of the riboprobes at 42 C for 12 h, digested with RNase A and Tl, and resolved on 8 M urea: 5% polyacrylamide gels. Gels were dried and exposed to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) for 24-72 h with intensifying screen to visualize the protected fragments.

Western blot analysis for bovine PAI-1

Cell homogenates or conditioned media from BBEC were resolved by SDS/PAGE (4-10%) and transferred to nitrocellulose membrane using a semidry transfer apparatus. The transfer blots were stained with Ponceau Red for 1 min to visualize the even transfer of the proteins. The blots were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h and incubated with antibovine PAI-1 mouse monoclonal antibody at a 1:2,000 dilution for 2 h (Gibco BRL, Life Technologies, Gaithersburg, MD). The antigen-antibody complexes were detected with horseradish peroxidase conjugated secondary antibody and the enhanced chemiluminescence system (ECL, Amersham Life Science, Arlington Heights, IL). The blots were exposed on reflection NEF films (DuPont NEN, Boston, MA) to visualize the bands.

Immunoprecipitation of bovine PAI-1

Subconfluent cultures of BBEC grown on 60-mm Petri dishes were incubated in MEM contain 0.5% calf serum for 24 h Cells were treated with bFGF (5ng/ml) or 16K PRL (10 nM) alone or together for 16 h. The medium were removed, and the treated cells were washed 3 times with methionine free MEM. To metabolically label the protein pool, the washed cells were incubated in 500 μl of the methionine free MEM and 50 µCi of the L-[^{35}S]methionine (Dupont NEN) was added to the cells for 4 h. Conditioned media were collected. Cell extracts were prepared by dissolving the cells in 1 ml of 0.1 M Tris-HCl, pH 7.5 containing 0.5% Triton X-100, 0.1% SDS, 0.05% Tween 80, 0.15 M NaCl, and protease inhibitors (0.14 U aprotinin, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 µM leupeptin, final concentrations) at 4 C for 20 min with shaking. The insoluble fraction was removed by centrifugation at 4 C for 20 min at 14,000 × *g*. Before Immunoprecipitation, the samples were diluted 1:1 with 0.1 M Tris-HCl, pH 7.5, and precleared with 40 μl of 50% (vol/vol) protein A Sepharose (Sigma) for 2 h at 4 C. The resulting lysates were incubated with 5 μg of the antibovine PAI-1 (Gibco BRL, Life Technologies) or 5 µg normal mouse IgG at 4 C for 1 h Fifty microliters of the 50% protein A Sepharose were added, and the samples were incubated at 4 C for another hour. The immunoprecipitates were

washed 3 times with 0.1 M Tris-HCl, pH 7.5/0.5%, Triton X-100, and finally dissolved in reducing Laemmli sample buffer. The immunoprecipitated proteins were analyzed by discontinuous 4-10% PAGE, and the gel dried and subjected to autoradiography.

RNA half-life determination

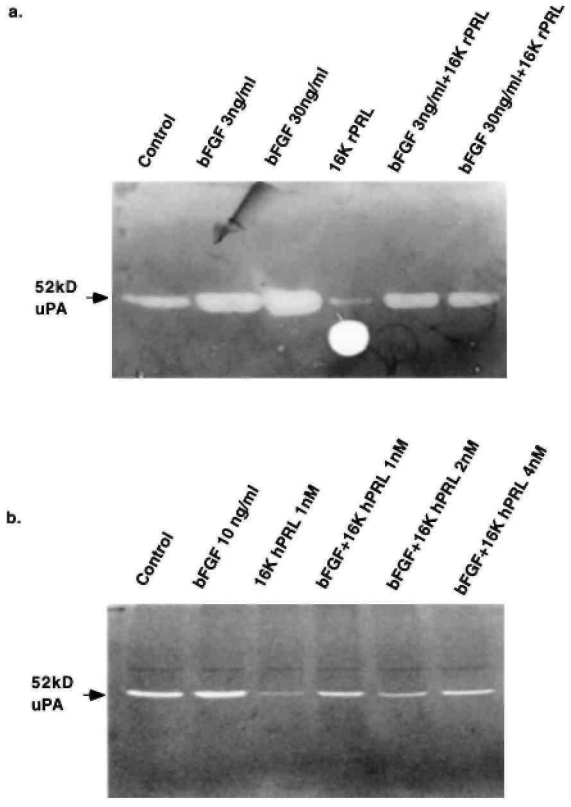
BBEC were initially treated with 10 nM 16K hPRL for 6 h to stimulate PAI-1 mRNA expression. The treated BBEC were then washed and fresh medium containing actinomycin D (3 μg/ml) added to arrest transcription. Cells were harvested 0, 1, 4, and 8 h after actinomycin D treatment. RNA samples were collected and studied by Northern blot analysis.

Statistical analysis

Data are presented as the mean ± SE. Data were statistically analyzed by one-way ANOVA followed by Fisher's protected least significant difference (StatView, Abacus Concepts, Berkeley, CA). A P value of < 0.05 was considered statistically significant.

Results

FIG. 1. *Effect of bFGF and 16K PRL on uPA activity. BBEC uPA activity was determined on plasminogen substrate gels, a, Treatment with 3 ng/ml and 30 ng/ml of bFGF treatment stimulated a band of PA activity migrating at 52 kDa, the expected size for bovine uPA. 16K rPRL (40 nM) inhibited both basal and bFGF stimulated uPA activity, b, 16K hPRL (1 nM) also inhibited basal uPA activity and decreased bFGF stimulated uPA activity in a graded fashion.*

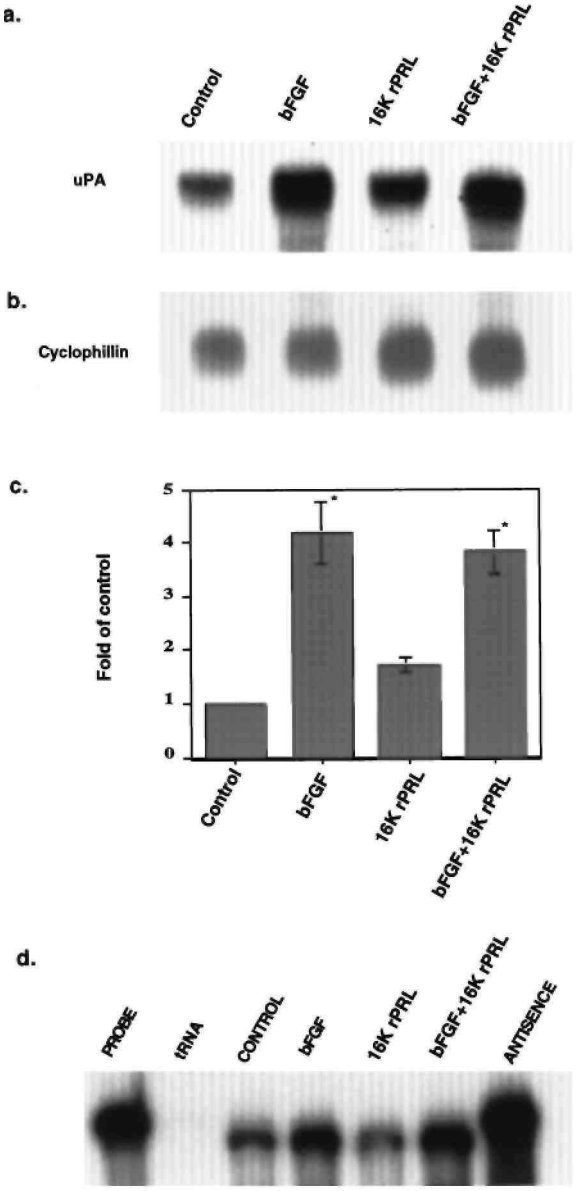


Inhibition of urokinase activity by 16K PRL

To determine the effect of bFGF and 16K PRL treatment on BBEC-associated uPA and/or tPA activity, substrate gel assays were performed using the SDS-PAGE substrate gel method, which determines plasminogen activator activity relative to molecular weight. Treatment of BBEC for 16 h with both 3 ng/ml and 30 ng/ml of bFGF resulted in a large increase in the size of the lysis zone fromed by proteins in the cell associated fraction migrating at 52 kDa, the expected size for uPA (Fig. la). This observation is consistent with earlier reports that uPA is the predominant plasminogen activator in bovine capillary endothelial cells (26, 28). No observable lysis band was seen at 70 kDa, the expected size for tPA activity. Rat 16K PRL (40 nM) almost totally inhibited the bFGF-induced increase in urokinase activity. At 1 nM concentration, 16K hPRL inhibited both basal and bFGF-

stim-ulated uPA activity (Fig. lb). These observations were repeated in three independent experiments with 16K rPRL and 3 times with 16K hPRL. Similar observations were made in the secreted fraction (conditioned media) from treated cells (data not shown). Importantly, 16K PRL inhibited basal urokinase activity to almost undetectable levels. The increased potency of 16K hPRL compared with 16K rPRL is consistent with differences seen on the inhibition of mitogen-induced BBEC proliferation (10).

FIG. 2. *Effect of 16KPRL on urokinase mRNA levels, a, Northern blot analysis for bovine uPA mRNA of total RNA harvested from BBEC treated for 16 h with bFGF (3 ng/ml), 16K rPRL (40 DM), or the two combined. b, Blots were stripped and reprobed with a bovine cyclo-phillin probe to correct for loading, c, Densitometric quantitation of relative uPA mRNA levels. Data are the mean ± SE of three experiments. Significant difference from the control are indicated by * (P < 0.05). d, RNase protection assay of bovine uPA in BBEC treated with bFGF (3 ng/ml), 16K rPRL (40 nM) or the two combined. The tRNA lane was added to control for nonspecific background, and the anti-sense lane to identify the size of the protected fragment.*



Effect of 16K PRL on uPA mRNA levels

The next obvious question was whether treatment with 16K PRL affected the level of uPA in the cell extracts. Because no reagents were available to perform Western blotting or immunoprecipitation studies for bovine uPA, and because changes in uPA levels are usually associated with changes in mRNA levels (29, 30), we measured uPA mRNA levels by both Northern blot analysis and an RNase protection assays. Treatment of BBEC with bFGF (3 ng/ml) stimulated uPA mRNA levels 4-fold as estimated by Northern blot analysis, and to a similar degree when estimated by RNase protection assays (Fig. 2), a-d. Treatment with 40 nM 16K rPRL had no effect

on the stimulation of uPA mRNA levels by bFGF nor on basal levels (Fig. 2, a-d). Similar results were obtained in three independent Northern blot analyses and two RNase protection assays. These findings suggested that the blockage in uPA activity was not dependent on changes in the level of uPA mRNA.

FIG. 3. *Effect of 16K hPRL on PAI-1 levels. Conditioned media (secretory) or cell lysates (cell associated) from BBEC treated with bFGF (10 ng/ml), 16K hPRL (10 DM), or the two combined were immuno-blotted using an antibovine PAI-1 monoclonal antibody (1:2000) and visualized by ECL. Treatment with 16K hPRL and bFGF, to a lesser degree, stimulated the appearance of a stained band at 50 kDa, consistent with the expected size of PAI-1. The stimulation by bFGF and 16K hPRL appeared additive.*

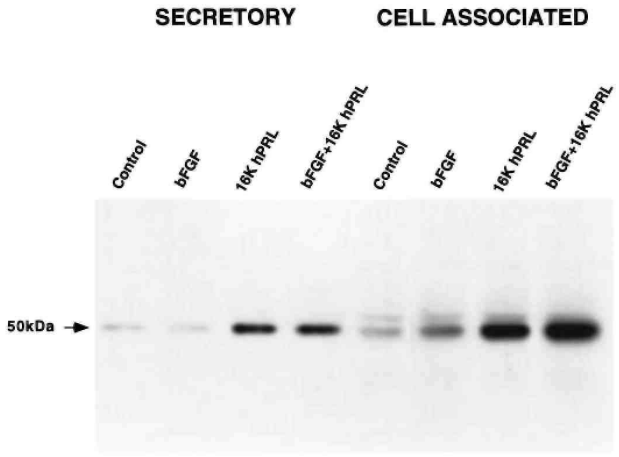
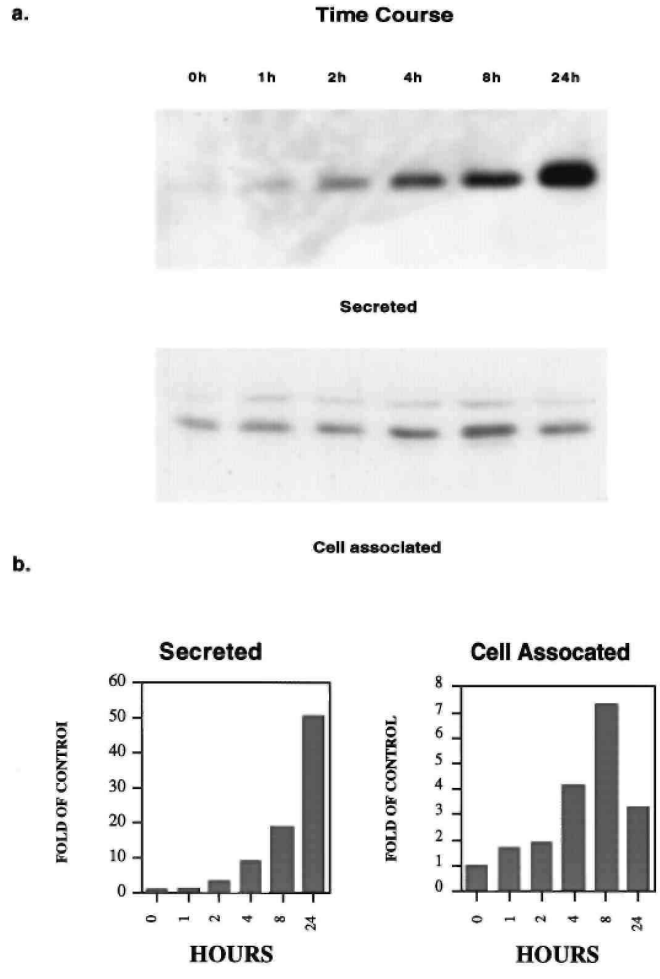


FIG. 4. *Time course of PAI-1 stimulation by 16K PRL. a, The time course (0-24 h) of the stimulation by 16K hPRL of immunodetected PAI-1 in conditioned media (secreted) and cell lysates (cell associated). b, Quantitation by densitometric scanning.*

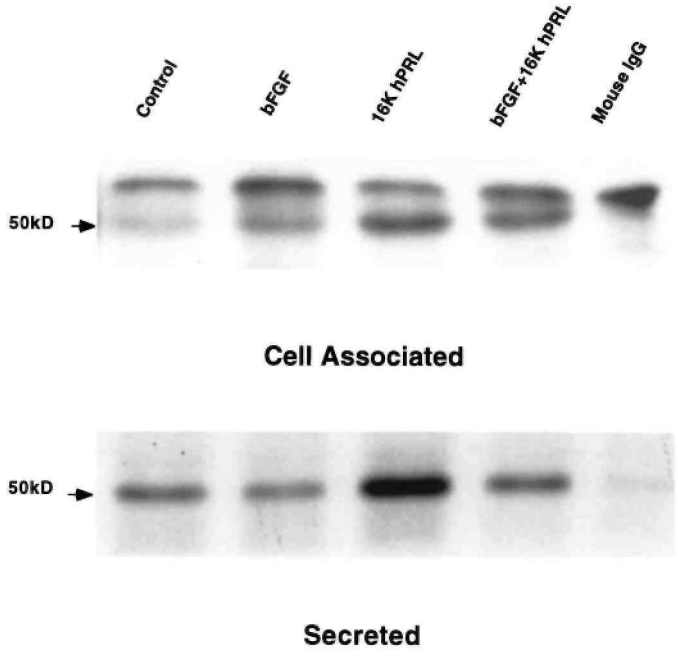


Stimulation of PAI-1 levels and synthesis by 16K PRL

Because urokinase activity can be negatively regulated by increased PAI-1 levels, we next determined whether treatment with 16K PRL altered PAI-1 levels. PAI-1 protein levels estimated by Western analysis were increased approximately 6-fold in cell lysates and 10-fold in the conditioned media by treatment for 16 h with 10 nM 16K hPRL (Fig. 3). This observation was confirmed in three independent experiments. The stimulation of cell associated and secreted PAI-1 by 16K PRL was first observed at 1 h and was maximal by 8 h in the cell associated fraction and by 24 h in the secreted fraction in time course experiments (Fig. 4). The same pattern was observed in two additional experiments. In the three experiments, the maximum stimulation in the secretory fractions at 24 h varied from 10- to 50-fold. The decrease in the cell associated PAI-1 at 24 h likely reflects the saturation of cell associated binding sites and release into the media. Treatment with 10 ng/ml of bFGF slightly stimulated cell associated PAI-1 protein levels (Fig. 3). The stimulation by bFGF was additive with the effect of 16K PRL

We next determined if 16K PRL stimulated the synthesis rate of PAI-1. Subconfluent cultures of BBEC were treated with 10 nM 16K hPRL for 16 h and then pulse-labeled with S^{35} methionine for 4 h. There was an approximate 2-fold stimulation of incorporation of radioactivity into immuno-precipitates of PAI-1 from the cell associated fraction and a 5-fold increase in the secreted fraction (Fig. 5, a and b). Treatment with bFGF at 5 ng/ml had only a minor effect on the metabolic labeling of PAI-1 in the cell associated fraction and no effect on PAI-1 in the secreted fraction. The cotreat-ment of cells with bFGF and 16K hPRL resulted in a decrease in the PAI-1 levels in the secreted fraction compared with treatment with 16K hPRL alone. As will be discussed this is likely due to the increased formation of PAI-1/uPA complexes resulting from the stimulation of uPA by bFGF. These findings were consistent with the increase in PAI-1 levels stimulated by 16K PRL being associated with an increase in protein synthesis. The finding that a larger proportion of the secreted PAI-1 was labeled suggests that the newly synthesized PAI-1 is preferentially released. We then asked if the increase in protein synthesis was associated with a change in the level of PAI-1 mRNA.

FIG. 5. *Effect of 16K hPRL on PAI-1 synthesis. BBEC treated with nothing, 16K hPRL (10 nM), bFGF (1 ng/ml) or both for 16 h were pulsed labeled with S-methionine for 4 h. PAI-1 was immunoprecipi-tated from cell associated (upper panel) and conditioned media (lower panel), separated by PAGE and autoradiographed. Treatment with 16KhPRL stimulated the intensity of a 50-kDa band in both fractions. Substitution of the PAI-1 antibody with normal mouse IgG demonstrated that, in the cell-associated fraction, the upper band observed was nonspecific, whereas the lower band was specific.*



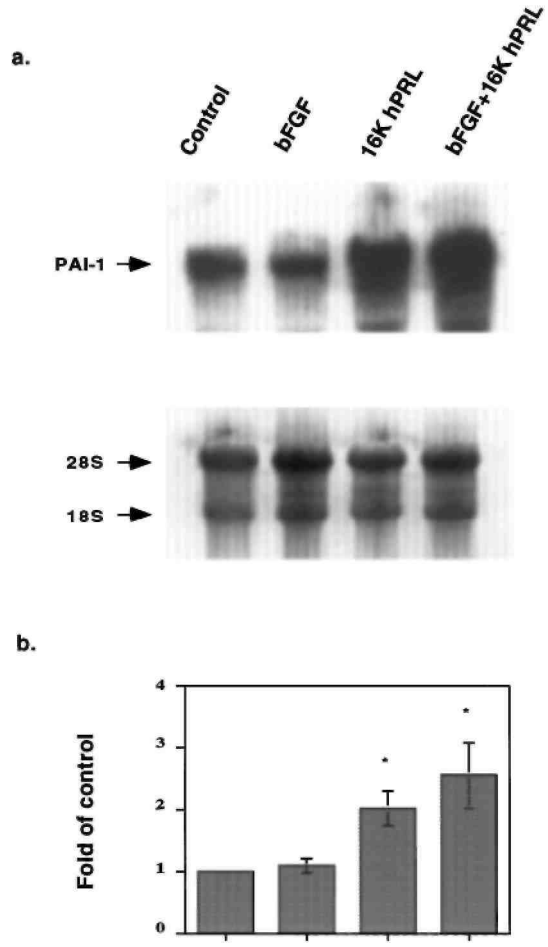
Stimulation of PAI-1 mRNA levels by 16K PRL

Treatment with 10 nM 16K hPRL stimulated PAI-1 RNA levels approximately 2-fold compared with the control (Fig. 6, a and b). Values were corrected for loading relative to the intensity of the 28S and 18S bands. These observations were confirmed in three independent experiments. Treatment with 10 ng/ml of bFGF had no effect

on PAI-1 mRNA levels, nor did it affect the 16K PRL-induced stimulation.

16K PRL could affect the accumulation of PAI-1 mRNA by increasing either PAI-1 mRNA stability or the transcription rate of the PAI-1 gene. To examine the effect of 16K PRL on PAI-1 mRNA stability, the rate of disappearance of mRNA levels, an estimate of mRNA stability, was performed using the mRNA synthesis inhibitor actinomycin D. For this purpose, BBEC were incubated with 10 nM 16K hPRL or vehicle alone for 6 h. Actinomycin D was then added to the culture medium, and PAI-1 mRNA levels were quantitated by Northern blot analysis at various times after addition of the inhibitor. As shown in Fig. 7, 16K hPRL treatment did not significantly affect the rate of disappearance of PAI-1 mRNA. This observation is consistent with 16K PRL increasing the rate of transcription of the PAI-1 gene rather than affecting mRNA stability.

FIG. 6. *Effect 16KhPRL on PAI-1 mRNA levels. a, Northern analysis for bovine PAI-1 mRNA of total RNA (10 μg) from BBEC treated for 16 h with nothing, bFGF (10ng/ ml), 16K hPRL (10 nM) or the two combined. Methylene blue staining of the 28S and 18S RNA showed even loading of the samples (lower panel). b, Quantitation of the PAI-1 bands by densitometric scanning showed that treatment with 16K hPRL doubled the level of PAI-1 mRNA while bFGF had no effect. Data are the mean ± SE of three experiments. Significant differentce from the control are indicated by * (P < 0.05).*



Formation of PAI-1 uPA complex

The major mechanism by which PAI-1 inhibits uPA activity is by forming stable complexes with the molecule. We therefore asked if uPA activity in BBEC could be inhibited by the addition of exogenous PAI-1, and secondly if there was evidence that the increased production of PAI-1 stimulated by 16K PRL resulted in the formation of complexes with uPA.

Addition of 1 or 2 μg of recombinant PAI-1 to lysates of BBEC resulted in the immunolocalization with an antibody to human PAI-1 of a new band migrating at 99 kDa (Fig. 8a). This is consistent with the expected size of the PAI-1/uPA complex. The uPA activity of these lysates in the PA substrate gel assay was partially inhibited by the addition of 1 μg of recombinant PAI-1, and totally inhibited by the addition of 2 μg (Fig. 8b).

The increased production of PAI-1 following treatment with 10 nM 16K hPRL for 20 h also resulted in the appearance of a band migrating at 90 kDa in a radioautograph of conditioned media from metabolically labeled BBEC, Fig. 9. This band was not present in the conditioned media from control or bFGF-treated cells. The conditioned media used were from cell cultures used in the experiment reported in Fig. 5. The more slowly migrating 90-kDa band was not seen in autoradiogram of the immunoprecipitates of PAI-1 in Fig. 5 because the antibody against PAI-1 used did not recognize the PAI-1/uPA complexes.

FIG. 7. *Stability of PAI-1 mRNA. a, The rate of disappearance of PAI-1 mRNA following treatment with actinomycin D (3 µg/ml) was by Northern blot analysis. Uniform loading was confirmed by methylene blue staining of the 28S and 16S RNA. b, Quantitation by densitometric scanning showed that the rate of disappearance of PAI-1 mRNA was similar in the 16K PRL treated (closed circles) and control (open circles) cultures.*

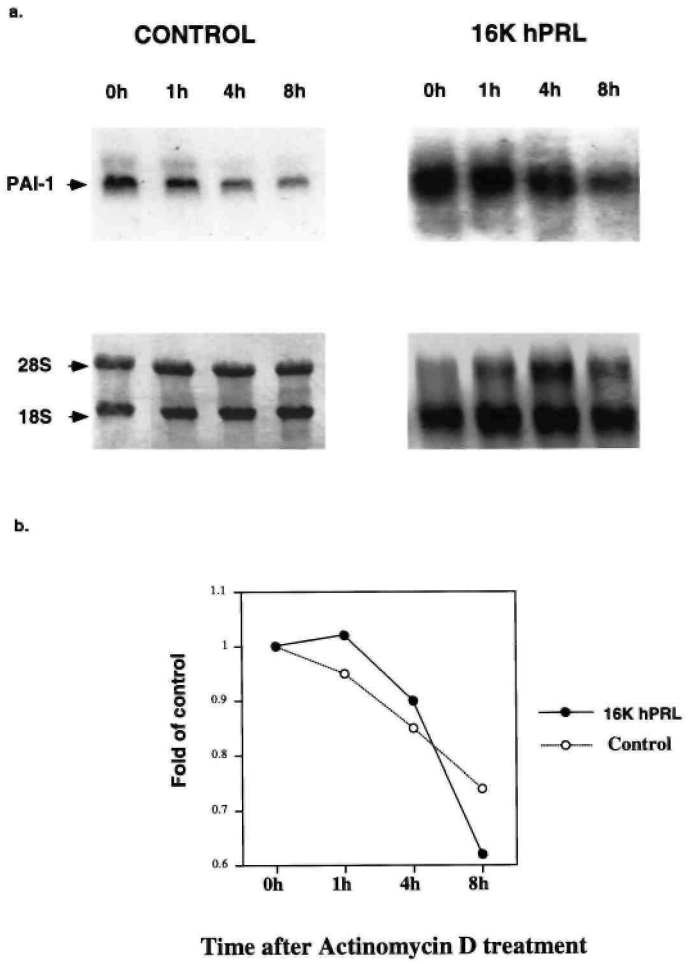
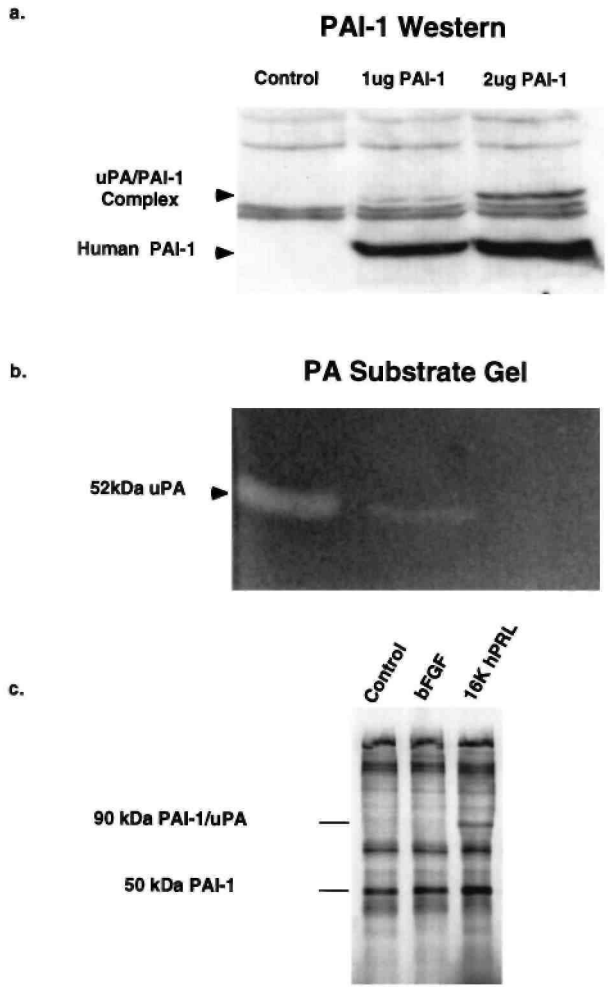


FIG. 8. *Formation of PAI-1/uPA complex. a, Western blot analysis was performed for human PAI-1 in BBEC lysates alone (control) or following the addition of 1 or 2 µg of recombinant human PAI-1. In the lysates in which recombinant human PAI-1 was added, a specific high molecular mass band of approximately 90 kDa can be observed at the expected size of the bovine uPA/human PAI-1 complex, as well as the 50-kDa PAI-1 band. b, Urokinase activity in the same samples was decreased by the addition of 1 µg of recombinant PAI-1 and completely inhibited by the addition of 2 µg. c, Conditioned media from the metabolically labeled BBEC used in Fig. 5 were separated by PAGE and autoradiographed. Treatment with 16KhPRL resulted in the appearance of a labeled band at 50 kDa, consistent with increased synthesis of PAI-1, and a 90-kDA band consistent with the formation of the PAI-1/uPA complex.*



Discussion

In addition to the action of the antiangiogenic factor, 16K PRL, on cell proliferation of capillary endothelial cells, it also inhibits their migration and organization into capillaries (10).

In this study, we tested the hypothesis that some of the antiangiogenic actions of 16K PRL are mediated via the regulation of protease activity, which plays an essential regulatory role in the formation of a new microvasculature. We have demonstrated that 16K PRL inhibits both basal or bFGF-stimulated uPA activity in BBEC. However, the inhibitory action of 16K PRL, rather than being mediated via a direct effect on uPA expression, appears to be mediated via a stimulation of the expression of the uPA inhibitor PAI-1.

Treatment of BBEC with 16K PRL decreased uPA activity measured in substrate gels. The decrease in uPA activity did not appear to be due to a decrease in uPA gene expression as assessed by measurement of uPA mRNA levels by Northern analysis or an RNase protection assay. The direct measurement of uPA protein levels was not possible because no antibody to bovine uPA was available for Western blot or immunoprecipitation studies. In several systems, increases in uPA protein levels have been shown to be associated with increases in uPA mRNA levels (29, 30). Therefore, although we cannot exclude the possibility that 16K PRL decreases the levels of uPA, the lack of change in uPA mRNA levels is consistent with no substantial change in uPA levels.

An important component of the regulation of uPA activity is the level of production of the plasminogen inhibitor PAI-1. Treatment with 16K PRL results in a substantial increase in the level of PAI-1 protein in BBEC. The stimulation of PAI-1 by 16K PRL is independent of the action of bFGF. The 16K PRL-induced increases in PAI-1 levels were rapid and were first detected at 1 h and reached a maximum by 8 h. The increase in PAI-1 synthesis appeared to be associated with an increase in the level of expression of the PAI-1 gene. PAI-1 mRNA levels were increased following treatment with 16K PRL, and the increase in mRNA levels was not associated

with any change in mRNA stability, findings consistent with the stimulation of the rate of transcription of the PAI-1 gene.

PAI-1 is known to form a complex with uPA that results in inactivation of the proteolytic activity of uPA, which can be measured in substrate gels. The formation of the complexes results in a band shift of uPA in Western blots to a higher molecular mass (90 kDa) (31). Addition of recombinant PAI-1 to BBEC lysates, caused a decrease in urokinase activity in substrate gels and formation of the 90 kDa uPA/ PAI-1 complex in Western blots. Long-term exposure of the PAI-1 Western blots of lysates from 16K PRL treated BBEC, in which the levels of PAI-1 were elevated, also showed the presence of the 90-kDa complex. These results are consistent with the explanation that the decrease in urokinase activity in the 16K PRL-treated BBEC is due to the increase of PAI-1 production.

Because LPS has been shown to stimulate PAI-1 production in endothelial cells (32), it was essential to show that the effect of recombinant 16K hPRL was not due to bacterial contamination. However, a 100-fold excess of the level of LPS found in the recombinant 16K hPRL preparations used only mildly stimulated PAI-1 production in BBEC precluding this possibility (data not shown). 23KhPRL contained a similar amount of LPS but had no effect on PAI-1 production in BBEC. Furthermore, this was not an issue with the 16K rPRL used because it was made by enzyme cleavage and contained low levels of LPS.

It appears that stimulation of PAI-1 expression may be the common action of several antiangiogenic factors in addition to 16K PRL including: thrombospondin (33); TGFβ (23); LIF (34); TNF-α (35, 36); and antiangiogenic steroids (26). In addition, the antiangiogenic steroid, medroxyprogesterone acetate, inhibited urokinase activity by stimulating PAI-1 expression (26).

Tissue remodeling associated with angiogenesis requires the delicate regulation of the proteolytic processes. Increased urokinase activity is necessary for degradation of the extracellular matrix and may also be involved in cell migration of capillary endothelial cells (15-17). Excess PAI-1 might result in endothelial cells being unable to penetrate through the basement membrane. Consistent with the hypothesis, addition of exogenous PAI-1 has been shown to inhibit endothelial cells migration (37). Antiangiogenic factors that have been shown to increase PAI-1 levels also inhibit capillary endothelial cell migration, *i.e.* 16K PRL, TGFβ (38), thrombospondin (6), and LIF (34). These findings strongly support the conclusion that an important component of the antiangiogenic action of 16K PRL is mediated via the stimulation of PAI-1 production.

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