Studies on the Antiangiogenic Action of 16K PRL: Regulation of Urokinase Type Plasminogen Activator System

by

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DISSERTATION

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DEDICATION

This thesis is dedicated to my loving parents and my wife, Ling-Fei Wu.

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I would like to thank all the people who have contribution in this work, including Drs. Gisela D'Angelo, Jean François Martini, Frauke Bentzien and Amy Choi in Dr. Richard Weiner's laboratory, Dr. Ingrid Struman in Dr. Joseph Martial's laboratory and Dr. Robert Woodward in Dr. David Dichek's laboratory. Without their help, I would not be able to finish this dissertation. I also thank all the members of the Reproductive Endocrinology Center for their supports during these years. I would like to thank Beth Vitalis, Janet Lee and Edward Robbie for helping me with my English writing.

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This thesis contains material in press in Endocrinology (Chapter 2). Chapter 4 and 5 has been submitted for publication at the time of presentation of this thesis.

At last, I would like to thank my parents and my wife, Ling-Fei. Without your unconditional support, I would not be able to obtain my degree.

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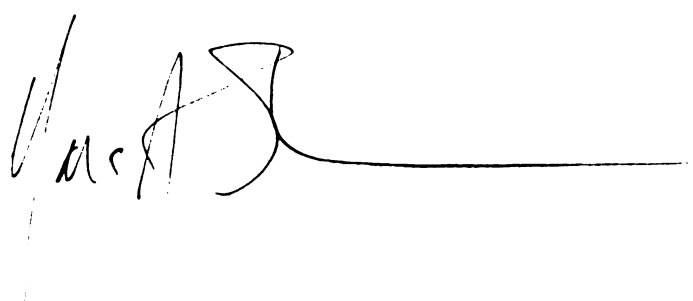
ABSTRACT

Angiogenesis is regulated by both stimulatory and inhibitory substances, namely angiogenic and antiangiogenic factors, respectively. An essential step in the regulation of angiogenesis is the regulation of uPA which in turn activates a cascade of proteases which play essential roles in endothelial cell migration and tissue remodeling. Work described in this thesis focuses on the effect of the antiangiogenic factor 16K PRL on urokinase activity in BBE cells. We show that 16K PRL inhibits bFGF-stimulated uPA activity and migration of BBE cells. 16K PRL has no effect on uPA mRNA levels; however, it stimulates the expression of the protein levels of PAI-1, a major inhibitor for uPA activity. The stimulatory effect of 16K PRL on PAI-1 appears to be at the transcriptional levels since 16K PRL increase PAI-1 mRNA levels while it has no effect on the stability of PAI-1 mRNA.

We further investigated the signaling mechanisms mediated by 16K PRL to stimulate PAI-1. By using kinase inhibitor, we show that 16K PRL PAI-1 is mediated through a serine/threonine kinase which is neither PKC nor PKA. Moreover, by luciferase reporter experiments, we show that the response element of 16K PRL is located between -6.4 kb and -1.5 kb region of the human PAI-1 promoter, which is different from the known TGF

β response element located within the first 800 bp of this promoter. These results suggest that 16K PRL and TGF β are mediated through a similar but distinct signaling mechanism.

Data presented also show that the members of 16K hPRL family members, including 16K hGH, 16K hGHV and 16K hPL, all stimulate PAI-1 production, while their full length counterparts have no effect. The signaling is not mediated through a intact GH or PRL receptor. Moreover, a serine/threonine kinase-dependent signaling mechanism is utilized by all these fragments to stimulate PAI-1. These findings suggest that the antiangiogenic effect of these factors are mediated through a same or similar family of receptors which is neither GH or PRL receptor.



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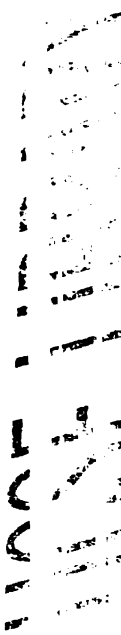

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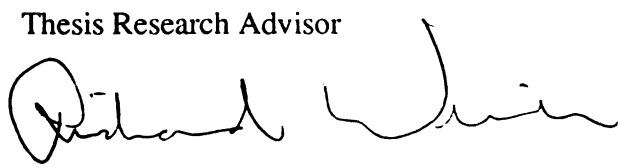
CO-AUTHORSHIP STATEMENT

In chapter 2, all the experiments and data described were performed and analyzed by H. Lee except for the preparation of 16K PRL. Recombinant h16K PRL was prepared by Ingrid Struman. Rat 16K PRL was prepared by Carmen Clapp. In chapter 4, H. Lee performed the experiments described in figures 3, 4 and 5. These experiments described the major difference between the 16K PRL and TGF β effect in endothelial cells and also provided information for the signaling pathway mediated by 16K PRL. The BBE cell proliferation experiments were performed by Frauke Bentzien. The experiments for 16K PRL effect on BBE cell apoptosis and MAPK activity were performed by Jean François Martini. The adenovirus vectors was prepared by Robert Woodward and he assited in the experiments. Ingrid Struman prepared the recombinant human 16K PRL. I served as primary advisor for both chapter 2 and 4. In chapter 5, H. Lee performed the experiments described in figure 5. These experiments provided important information defining the different receptor systems utilized by 16K PRL family and their full length counterparts. CAM assays and BBE cell proliferation assays were performed by Ingrid Struman, Véronique Mainfroid and Vincent Goffin. MAPK assays were performed by Frauke Bentzien. Joseph A. Martial and I served as advisors for this research.

Thesis Research Advisor



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Studies on the Antiangiogenic Action of 16K PRL: Regulation of Urokinase Type Plasminogen Activator System

by

Hsinyu Lee

Overview

Angiogenesis, or the formation of new blood vessels is a complicated process in which plasminogen activators are believed to play an important role. Regulation of these factors is essential for proper angiogenesis. The 16 kDa N-terminal fragment of PRL (16K PRL) is a potent antiangiogenic factor believed to regulate the activity of plasminogen activators. In this thesis, I investigated the effect of 16K PRL on the activity of plasminogen activators in capillary endothelial cells. In chapter 1, background information for the thesis is presented. In the first section of Chapter 1, a general review of angiogenesis is provided, including regulation of angiogenesis and the relationship between abnormal angiogenesis and pathological phenotypes. A review of models currently used to assess angiogenesis is also presented. In the second section, an overview of the proteolytic enzymes involved in angiogenic processes is provided, focusing on urokinase and its regulation. Urokinase activity is regulated by several factors including expression and synthesis of uPA, control of number of the specific receptors for urokinase (uPAR), and production of the specific uPA inhibitors, plasminogen activator inhibitors (PAI). This section will also include a review of another important component of angiogenesis-endothelial cell migration. In the final section, the antiangiogenic actions of 16 kDa N-terminal fragment of prolactin (16K PRL) and the signaling pathways mediating these actions are reviewed.

Chapter 2 includes the contents of a manuscript in press in Endocrinology. In chapter 2, we report that 16K PRL inhibits bFGF-stimulated urokinase activity. Futhermore, we show that 16K PRL stimulates the production of the major inhibitor of urokinase: plasminogen activator inhibitor 1 (PAI-1). 16K PRL stimulates both the protein and mRNA levels of PAI-1. The rate of turnover of PAI-1 mRNA is unaffected by 16K PRL treatment consistent with the stimulation of transcription of the PAI-1 gene.

In chapter 3, we showed that 16K PRL inhibits endothelial cell migration, another important component of angiogenesis.

Chapter 4 consists of a manuscript comparing the signaling mechanisms mediating the antiangiogenic actions of by 16K PRL and TGF β. The manuscript has been submitted for publication in Endocrinology. Using kinase inhibitors, we showed that 16K PRL and TGF β signal through a serine/threonine kinase which is neither PKA nor PKC. We went on to show that TGF β and 16K PRL signal do not signal through the same response element in the PAI-1 promoter.

Chapter 5 includes a manuscript submitted to PNAS and a appendix section. We report that the 16 kDa N-terminal fragments (16K) of three additional human PRL family members, human growth hormone (hGH), human placental lactogen (hPL) and human GH variant (hGHV), stimulate PAI-1 production, while the full-length molecules from which they were derived have no effect. The stimulation of PAI-1 production by 16K hGH was not mediated via the GH receptor.

In chapter 6, we ask whether 16K PRL family members signal through a same receptor system. By using kinase inhibitor, we show that 16K fragments of PRL family members may utilize the same signaling pathways and possibly share the same receptor.

The thesis is concluded with a general discussion section and a section on future research directions.

Chapter 1. INTRODUCTION

I. Angiogenesis

Capillaries are formed by two distinct mechanisms: vasculogenesis and angiogenesis. Vasculogenesis is defined as *de novo* formation of blood vessel from the lateral mesoderm of the embryo (Feinberg, 1983; Risau, 1988; Risau, 1995). On the other hand, angiogenesis is defined as the formation of new capillaries from existing vessels to produce "neovascularization" (Folkman, 1985; Pepper, 1996).

Angiogenesis is essential for many physiological functions such as reproduction, development, organ regeneration and wound repair (Folkman, 1992; Folkman, 1995; Klagsbrun, 1991). For example, ovulation, menstruation, implantation and pregnancy are angiogenic-dependent (Findlay, 1986; Gordon, 1995). Vascular changes specific to the menstrual cycle are a unique feature of the female reproductive tract. In the ovary, the differential development of the follicular capillary network may be involved in the selection of the dominant follicle. The rapid vascularization of the corpus luteum is crucial for the production of progesterone (Carr, 1982) which is important for the maintenance of pregnancy. These changes in the ovary are coincident with increased angiogenesis in the zona functionalis of the endometrium, a requirement for successful implantation and placentation. These changes require strict regulation of angiogenic processes (Edward 1980).

A series of morphogenic events have been described in the process of capillary formation (Auerbach, 1994; Findlay, 1986; Klagsbrun, 1991; Reynolds, 1992). They can be divided into distinct steps: the proliferation of endothelial cells, activation of proteolytic enzymes, migration of endothelial cells and finally, formation of tube-like structures (Fig. 1). Angiogenic stimulators, such as growth factors or cytokines, are produced from tumors or inflammatory cells (Peverali, 1994; Presta, 1986). These factors stimulate vascular cell proliferation and secretion of proteolytic enzymes, thereby promoting blood vessel growth and penetration of the basement membrane barrier. Activation of the

proteolytic enzymes can also release matrix bound growth factors and other angiogenic factors (Flaumenhaft, 1992; Vlodavsky, 1990), further promoting this process. Activated endothelial cells secrete both proteolytic enzymes and components of the extracellular matrix, thus modifying the extracellular microenvironment (Iruela-Arispe, 1991; Knudsen, 1988). The activated endothelial cells migrate and invade the remodeled extracellular matrix to form a capillary sprout. Endothelial cells also change their adhesion properties during these processes, allowing them to interact with the remodeled extracellular matrix environment. The proper interactions between endothelial cells and extracellular matrix provide signals to ensure the subsequent cell proliferation, migration, invasion and survival. Finally, the vascular sprouts begin to mature as the capillary lumen is formed. The newly formed sprouts secrete basement membrane components which maintain the endothelial cells in a differentiated and dormant state (Auerbach, 1994; Folkman, 1992; Stromblad, 1996). The complicated morphogenic events described above indicate that the angiogenic process is under strict regulation. A series of knockout experiments suggest that TIE receptors, a family of tyrosine kinase receptors expressed on endothelial cells, and their ligands play important roles in blood vessel formation. Angiopoietin-1, the ligand for the TIE-2 receptor, is suggested to regulate the proper recruitment of supporting cells to the vascular wall (Folkman, 1996; Suri, 1996; Vikkula, 1996). These observations provide more insight into how blood vessel formation is regulated. However, in order to fully understand the regulatory mechanisms of angiogenesis, further investigations are required.

Blood vessels are composed mainly of endothelial cells which interconnect to form the tubes that direct and maintain blood flow and tissue perfusion. Endothelial cells proliferate more slowly than most other cell types in the body with the exception of the central nervous system. It is estimated that in normal adult tissue only one out of every ten thousand endothelial cells has entered the cell cycle at any given time (Engerman, 1967; Hobson, 1984). This suggests that under normal conditions, endothelial cells are in a "dormant" state. However, the quiescent vasculature can become activated to grow new

capillaries under appropriate stimuli (Hanahan, 1996). In order to maintain the dormant state of angiogenesis, endothelial cells must maintain a delicate balance of positive and negative regulators (Pepper, 1996).

Many positive regulators of angiogenesis have been described both *in vivo* and *in vitro* (Folkman, 1987; Pepper, 1996), such as VEGF (Connolly, 1989; Ferrara, 1989), bFGF (Esch, 1985; Montesano, 1986), EGF, TGF α (Schreiber, 1986), TGF β *in vivo* (Pepper, 1993), angiogenin (Fett, 1985; Kurachi, 1985; Strydom, 1985), TNF α (Leibovich, 1987), PDGF (Conn, 1990; Ishikawa, 1989; Miyazono, 1987) and angiopoietin (Suri,1996). In addition, many antiangiogenic factors have also been described such as TGF β *in vitro* (Muller, 1987), platelet factor IV (PF-4) (Maione, 1990), thrombospondin (Good, 1990), angiostatin (O'Reilly, 1994), endostatin (O'Reilly, 1997), antiangiogenic steroids (Blei, 1993), interferons (Tsuruoka, 1988), integrin αvβ3 inhibitor (Brooks, 1994; Hammes, 1996) and the 16 kDa N terminal fragment of PRL (Ferrara, 1991). Maintenance of the dormant state of endothelial cells is likely due to the presence of negative regulators, since positive regulators are frequently detected in adult tissues in which angiogenesis is not apparent thereby keeping angiogenesis in check. The converse is also true, thus positive and negative regulators often coexist in tissues in which endothelial cell turnover is increased (Hanahan, 1996). It is currently believed that angiogenesis is regulated by a balance of positive and negative factors. In angiogenic endothelium, positive regulators predominate, whereas endothelial quiescence is achieved and maintained by the dominance of negative regulators (Bouck, 1990; Liotta, 1991; Hanahan, 1996; Pepper, 1996).

Abnormal angiogenesis is implicated in association with many different pathological phenotypes. These diseases include diabetic retinopathy, chronic inflammatory disorders such as rheumatoid arthritis, psoriasis, periondontitis, and solid tumors (Folkman, 1995; Leibovich, 1984). The importance of angiogenesis for the growth of solid tumors is now well recognized. A considerable body of research suggests that tumor growth and

metastasis require persistence of new blood vessel growth. This has been shown in experiments using the rabbit cornea as an angiogenic model (Gimbrone, 1972). Tumors or cultured tumor cell implants can grow in the cornea of a rabbit eye and attract in growth of new blood vessels. This results in the expansion of the tumor mass. If the capillaries were physically prevented from reaching the implant, tumor growth was dramatically impaired. These isolated tumor nodules were restricted to a maximum diameter of around 0.4 mm. Presumably this is due to the fact that unvasculized tumor tissues can only obtain nutrients by simple diffusion, thus limiting tumor growth. Subsequent experiments confirmed that in the absence of vasculature, tumors become necrotic (Brem, 1976) and/or apoptotic (Holmgren, 1995; Parangi, 1996). These results suggest that tumor progression is dependent on neovascularization, and implicate anti-angiogenic factors as possible targets for cancer treatment (Holmgren, 1996).

Both *in vivo and in vitro* assays are used to assess angiogenic activity. *In vitro* assays analyze individual steps in the angiogenic cascade while *in vivo* assays are focused on the entire angiogenic process. In the *in vitro* systems, assays including measuring endothelial cell proliferation, endothelial cell migration and tube formation of endothelial cells have been used to assess the angiogenic activity of a substance. *In vivo,* angiogenesis has been studied with either the chicken chorioallantoic membrane (CAM) assay (Klagsbrun, 1991) or the rabbit corneal assay (Gimbrone, 1974). In the CAM assay, a substance of interest is placed on the chorioallantoic membrane of the chicken embryo to evaluate its angiogenic ability. In the rabbit cornea assay, the angiogenic activity is similarly determined by the growth of new vessels toward a implant of factor of interest in the normally avascular cornea. Although each of these methods has its limitations, both are useful tools used to search for the biologic regulators of this important physiologic process.

II. Proteases and Angiogenesis

In order for angiogenesis to occur, endothelial cells need to break down the surrounding basement membrane (Menashi, 1993; Mignatti, 1996; Saksela, 1988). The basement membrane is composed of proteoglycans, glycoproteins, collagens, and elastins. Activation of various proteases is required for the degradation of these basement membrane components (Pintucci, 1996). As mentioned, protease activation is an important initiation step for angiogenesis.

Many studies have shown that among the proteases involved in the angiogenic pathway, components of the plasminogen activator-plasmin system play a central role in endothelial cell migration and activation (Dano, 1985; Fibbi, 1988; Mignatti, 1996; Odekon, 1992; Pepper, 1987; Pepper, 1992; Pepper, 1993; Pepper, 1996).

Plasmin, an ubiquitous protease, degrades many different types of extracellular matrix proteins (Chapman, 1984; Kleiner, 1993; Moscatelli, 1988; Matrisian, 1990; Mignatti, 1993; Saksela, 1985 Werb, 1977; Werb, 1980). Plasmin is activated from its inactive zymogen form (plasminogen) by specific plasminogen activators (Dano, 1985; Saksela, 1988). Because of high circulating plasminogen concentration (2 μM) in plasma (Collen, 1972; Lijnen, 1982), a small increase of plasminogen activator activity will induce high local concentrations of plasmin. Plasmin not only degrades many different types of extracelluar matrix proteins, it also activates several matrix degrading metalloproteases (MMPs) including interstitial collagenase (Werb, 1977), stromolyin type IV collagenase, and latent elastase (Chapman, 1984). Thus a small amount of plasminogen activator can result in the generation of high local concentration of active serine and metalloproteases with different substrate specificities (Mignatti, 1996).

The degradation pathway is safe-guarded by the presence of many protease inhibitors (Mignatti, 1996). The blockade of plasminogen activation by specific PA inhibitors will inhibit all subsequent events including the activation of matrix

metalloproteinase (MMPs) and elastase, but the specific MMPs inhibitors (TIMPs) will not affect plasmin activity. These results support the idea that the PA-plasmin system is a key regulator of proteolytic activity involved in angiogenic processes (Mignatti, 1996).

Two different types of plasminogen activators have been identified: urokinase type plasminogen activator (uPA) and tissue type plasminogen activator (tPA). They were originally found in urine and tissue extracts respectively (Dano, 1985), thus their names. uPA is a 50 kDa protein that is thought to be involved in tissue degradation and cell migration (Dano, 1985; Moscatelli, 1986). tPA has a molecular weight of 70 kDa and is believed to play a role in thrombolytic process. The human uPA gene is located on chromosome 10 (Rajput, 1985; Tripputi, 1985) while tPA is located on chromosome 8 (Rajput, 1985). The human 24 kb uPA gene contains 11 exons (Riccio, 1985) and encodes a 2.5 kb mRNA (Verde, 1984). The human uPA cDNA shows high homology with the uPA cDNA found in other species including bovine (Kratzschmar, 1993), porcine and murine (Belin, 1985). The human tPA gene is 29-32.7 kb in size and consists of 14 exons (Degen, 1986; Fisher, 1985; Ny, 1984). It encodes a 2.7 kb mRNA. The homology between the two proteins is around 40% (Degen, 1986), but the functional domains of the two enzymes are highly conserved, suggesting a close evolutionary relationship. Both enzymes have signal peptides which are removed after synthesis (Pennica, 1983; Riccio, 1985). The secreted polypeptide chains are cleaved and further processed to create two polypeptide chains connected via disulfide bonds (Dano, 1985). The carboxy-terminal of the proteins (B chain) contains the proteolytic active sites. The amino-terminal portion of the enzymes (A chain) contains domains which will interact with cellular proteins and receptors (Appella, 1987; Banyai, 1983; Gunzler, 1982). This portion of uPA contains one kringle domain- a structure contains three disulfide bonds, and one growth factor-like domain. In tPA, two kringle domains and one growth factor-like domain are present in this region (Saksela, 1988). The kringle domain is essential for binding to other proteins. The second kringle domain of tPA may be responsible for its

fibrin binding activity (van Zonneveld, 1986). The growth factor domain of PAs is structurally similar to the receptor binding region of EGF and TGF-α (Derynck, 1984; Gunzler, 1982). The function of this growth factor domain in tPA is unclear. In uPA, this growth factor-like region is responsible for the interaction with the uPA receptor (Appella, 1987). The proteolytic cleavage of pro-uPA at lysine 158 results in the exposure of the active site of the enzyme. (Verde, 1984). However, some studies suggest that the single chain form of the protein is also enzymatically active and regulated by plasminogen inhibitors (Manchanda, 1995). In contrast, tPA activity is greatly increased upon binding to fibrin (Dano, 1985; Hekman, 1987). Once bound to fibrin, both single-chain and two-chain forms of the molecules are equally active. The fibrin binding activity suggests that tPA is important in regulating the thrombolytic process.

The proteolytic enzyme responsible for cleavage of uPA is unclear. *In vitro*, plasmin can activate pro-uPA both in solution and on the cell surface (Cubellis, 1990; Nelson, 1995; Wun, 1982). The activation of uPA by plasmin, a down stream product of the pathway, suggests that a positive feedback might exist in the uPA-plasmin system (Saksela, 1988) (Fig. 2). This relationship also suggests that an increase in uPA activity might result in a rapid increase in local plasmin activity. Other known activators for pro-uPA include kalikrein, factor XIIa and cathepsin B.

In the uPA knockout experiments, mice appear to develop normally (Carmeliet, 1994). These observations contradict the idea that uPA is important in angiogenesis, a process necessary for normal development. However, the null effect of knocking out important factors in transgenic experiments has been reported by others (Li, 1993; Umanoff,1995; Eitzman, 1996). A reasonable explanation for these observations is that a compensate pathway is activated early in the development of these animals, providing them an alternative mechanism to bypass the requirement uPA. However, it is less likely to activate such a compensatory process in a fully developed animal since the differentiation program has already been initiated.

Transcription of uPA has been shown to be regulated by a variety of molecules such as phorbolester, growth factors, peptides and steroid hormones (Dano, 1985). The tumor promoter PMA stimulates the transcription of uPA suggesting that protein kinase C might be involved in the regulation of uPA transcription (Degen, 1985; Grimaldi, 1986). Dexamethasone inhibits uPA transcription in normal and malignant human mammary gland carcinoma cells, human fibrosarcoma cells, and human synorial fibroblast (Busso, 1986; Busso, 1987; Medcalf, 1988). In contrast, dexamethasone has no effect on tPA expression (Busso, 1986; Medcalf, 1988).

Several growth factors have also been shown to affect uPA transcription. These factors include EGF (Gross, 1983; Grimaldi, 1986; Lee, 1978), PDGF (Stoppelli, 1986), TGF β (Laiho, 1986; Saksela, 1987), γ INF (Collart, 1986), IL1 (Leizer, 1987; Mochan, 1986), CSF1 (Hume, 1984), TNF α (Frater-Schroder, 1987; Niedbala, 1992; Schleef, 1988), angiogenin (Jimi, 1995) and bFGF (Gualandris, 1995). cAMP and the tyrosine phosphatase inhibitor vanadate (Saksela, 1987) have also been shown to stimulate the transcription of uPA. These results support the notion that uPA regulation is complex.

The actual activity of uPA is regulated not only by the concentration of the enzymes but also their interaction with receptors, specific inhibitors and extracellular matrix proteins. The specific uPA receptor was first described by Vassalli et al (Vassalli, 1985). These investigators detected a high affinity binding receptor on monocyte U937 cells. A similar receptor has been found in a variety of cell types including endothelial cells (Fibbi, 1988), neutrophils (Gudewicz, 1987), smooth muscle cells (Reuning, 1994) and tumor cells (Ohtani, 1995; Stahl, 1994). uPA Receptor is anchored on the cell surface through a glycosylphosphatidylinositol (GPI) moiety. The receptor has been cloned and shown to encode a single-chain, highly glycosylated protein (Higazi, 1996). The uPAR plays a major role in the regulation of cell surface proteolysis by potentiating plasmin generation on the cell surface (Fazioli, 1994). Pro-uPA binds to the uPAR and is then converted to the active enzyme which remains bound to the receptor. Binding might increase the local

concentration of uPA and generate more specific and localized proteolytic activity to achieve more efficient proteolysis (Hebert, 1988; Pollanen, 1988; Saksela, 1988; van Hinsbergh, 1997).

The uPA receptor acts both as a site for focal pericellular proteolysis by uPA and also as a clearance receptor for the uPA-PAI-1 complex. Upon secretion, uPA binds to the receptor and is converted to the active two-chain form of uPA. Like free uPA, the receptor bound proteinase is also inhibited by PAI-1. PAI-1-uPA-uPAR is rapidly internalized followed by degradation of the uPA-PAI-1 complex (Andreasen, 1994; Cubellis, 1990; Olson, 1992). The empty uPAR is recycled to the cell surface for the next round of activity. GPI-linked uPAR is internalized via the interaction with 2 macroglobulin/low density lipoprotein receptor related protein (LRP) (Nykjaer, 1992). uPAR has also been shown to interact with the extracelluar protein vitronectin, suggesting that uPAR might be involved in cell adhesion (Wei, 1994). Moreover, the direct interaction between uPAR and integrins has been reported (Wei, 1996; Xue, 1997; Yerba, 1995). These interactions have been shown to modify the binding of integrins with extracellular matrix proteins thus affects cell adhesion and migration.

Blocking the interactions between uPA and uPAR by using synthetic inhibitors has been shown to inhibit angiogenesis in vitro and in vivo, suggesting that urokinase and uPAR interaction play an important role in this process (Min, 1996).

Urokinase activity is negatively regulated by specific inhibitors, including plasminogen activator inhibitor I, II, III (PAI-1, PAI-2, PAI-3) and protein nexin (PN) (Kruithof, 1988; Saksela, 1988). PAI-1, a major inhibitor of uPA activity, is secreted by different cell types. The 50 kDa PAI-1 is encoded by two mRNA fragments (3.0 kb and 2.2 kb) that differ at the 3' untranslated region (Ginsburg, 1986; Ny, 1986). PAI-1 inhibits PA activity by rapidly forming a 1:1 molar complex (Hekman, 1987). The reactive center of PAI-1 is located near the carboxyl terminal (Andreasen, 1986; Ny, 1986). PAI-1 binds only to two-chain, but not to single-chain, uPA (Andreasen, 1986). It is synthesized

in an active form and rapidly inactivated in serum, unless stabilized by forming a complex with the extracellular protein vitronectin (Wei, 1994). The inactive form of PAI-1 can be activated by incubation with a denaturant, heat and negatively charged phospholipid (Andreasen, 1986; Erickson, 1986; Hekman, 1985; Levin, 1986). The physiological relevance of these artificial modes of activation is unknown. PAI-1 is a major secretory protein of endothelial cells; this protein can comprise 10-15% of the cell's secreted proteins. This observation suggests that PAI-1 is the most important negative regulator of uPA in endothelial cells.

PAI-1 transcription is regulated by many different growth factors, steroids and other agents. Transcription can be stimulated by IL1 (Emeis, 1986), TNF (Schleef, 1988), TGF- β (Saksela, 1987), bFGF (Saksela, 1987), thrombospondin (Bagavandoss, 1993), thrombin (Gelehrter, 1986), LIF (Pepper, 1995), angiogenic steroid (Blei, 1993), glucocorticoid (van Zonneveld, 1988), bacterial LPS (Colucci, 1985), cAMP (Santell. 1988), and PMA (Saksela, 1987).

The transcriptional regulation of PAI-1 by TGF β has been carefully analyzed. The response element for TGF β is located within -800 bp of the human PAI-1 promoter (Dong, 1996; Keeton, 1991). A novel protein has been shown to bind to this region (Sandler, 1994). Recent experiments suggest that this protein might be a complex of smad family members (Yingling, 1997).

In addition to uPA, PAI-1 interacts with other proteins including tPA, vitronectin (Mimuro, 1989; Salonen, 1989; Wei, 1994), and LRP (Nykjaer, 1992). Complexes of PAI-1 with these proteins are involved in degradation of the proteolytic enzymes, recycling of the receptors, and interaction with extracellular matrix proteins which might be involved in apoptosis of certain cell types (Brooks, 1994). Thus PAI-1 may have multiple biological functions distinct from that as a proteolytic inhibitor.

III. Endothelial Cell Migration

Endothelial cell migration is an important component of many biological processes including angiogenesis. Following a cascade of highly coordinated steps which restore the integrity of tissue function, endothelial cells, guided by a gradient of chemoattractant factors migrate from pre-existing microvessels toward the involved tissue (Folkman, 1992; Folkman, 1997; Hanahan, 1996; Presta, 1986; Peverali, 1994). As leading capillary tip migrates toward the angiogenic stimulus, the endothelial cells undergo mitosis to ensure an adequate supply of new endothelial cells. Capillary sprouts then begin to branch at their tips, joining with one another to restore blood flow and supply in the affected tissues.

Endothelial cell migration can be regulated by multiple mechanisms including the composition of the extracellular matrix (ECM) proteins, the pattern of integrins expression on the endothelial cell surface, changes in the cytoskeletal protein structure (Abedi, 1995), and interactions with urokinase (Fibbi, 1988; Odekon, 1992). Dynamic interactions between cell surface integrins and ECM components play a central role in cell migration by providing cell-substrate traction for the generation of intracellular mechanical stress (Devreotes, 1988; Singer, 1986). Organization of the cytoskeletal proteins and their interaction with the associated components generate forces required for endothelial cell migration. Endothelial cell migration is also thought to be regulated by the interaction between uPA and its receptor (Blasi, 1996; Fibbi, 1988; Odekon, 1992). Any changes in these factors might affect endothelial cell migration.

IV. 16K PRL as an Antiangiogenic Factor

Among all the pituitary hormones, prolactin is most versatile in terms of its biological actions (DeVlaming, 1979; Sinha, 1995). More than 100 distinct biological effects for prolactin have been described, ranging from the initiation of lactation in mammals to nesting behavior of birds, and osmoregulation of fish. The human prolactin is a 23 kDa protein hormone. It was first found in the extracts of the pituitary gland (Riddel,

1932; Stricker, 1928). PRL is secreted by acidophilic staining cells known as mammotrophs (Frawley, 1991). Recent reports suggested that prolactin can be produced in a variety of tissues including the placenta (Fuxe, 1977; Handwerger, 1991; Rosenberg, 1980), uterus (Chapitis, 1988; Gellersen, 1989; Maslar, 1979; Nowak, 1993; Walters, 1983), brain (Golander, 1978; Schachter, 1984; Valatx, 1992; Wilson, 1992) and immune system (DiMattia, 1988; Hiestand, 1986; Montgomery, 1992; Pellegrini, 1992; Sabharwal, 1992). Human prolactin is structurally similar to growth hormone and placenta lactogen (Nicoll, 1988), suggesting that these three hormones are derived from a common ancestral gene. A three-dimensional structure of human PRL has been derived from the structure of human GH (Goffin, 1996). This model predicts that PRL is composed of four alpha helixes arranged in an anti-parallel fashion. These helical structures mediate PRL binding to its receptor.

Encoded by a single gene, human PRL consist of 197-199 amino acids with three intra-molecular disulfide bridges (Goffin, 1996; Sinha, 1995). The amino-terminal disulfide bridges between amino acids 4 and 11 are absent in hGH and hPL giving hPRL a unique structure. In contrast, hGH is encoding by two genes: hGH-n and hGHV (Frankenne, 1987; Frankenne, 1990). The PRL gene consist of five exons and four large introns (Miller, 1983). All intron and exon boundaries of the rat gene consist of potential multiple splicing sites suggesting that multiple PRL mRNA isoforms might exist (Maurer, 1981). In fact, different sizes of PRL mRNAs have been found in both the pituitary and the brain of the rat (Emanuele, 1992). In addition, PRL of some species contains two sets of basic amino acid pairs that are characteristic of protease processing sites. This will generate multiple forms of PRL. In accordance with these observationss, a number of studies has shown that PRL is processed extensively after synthesis (Baldocchi, 1993; Clapp, 1987; Clapp, 1988; Clapp, 1989; Mittra, 1980; Mittra, 1980). A major post-translational modification is proteolytic cleavage. These modifications produce biologically active fragments which, in some cases, have activity different from the parental molecule.

Activity of differentially cleaved PRL has been found in the anterior pituitary (Mittra, 1980), brain (DeVito, 1988), prostate gland (Compton, 1984) and mammary gland (Clapp, 1987). The cleavage activity changes according to the physiological state of the tissue (Clapp, 1987). For instance, a pregnant rat mammary gland has the highest cleavage activity. A 16 kDa PRL fragment can also be detected when PRL is produced recombinantly in bacteria (Yamamoto, 1992). The enzyme responsible for the cleavage of rat PRL has been shown to be cathepsin D (Baldocchi, 1993). The cleavage produces 16.4 kDa and 5.8 kDa fragments. 16 kDa PRL has been immunologically detected in the circulation of rats, mice (Torner, 1991) and human (Sinha, 1985). Initial reports suggest that the 16K PRL has higher activity than its parent molecule in stimulating pigeon crop-sac, Nb2 lymphoma cell, and mammary cell proliferation (Clapp, 1988). Later reports have shown that its mammary mitogenic and lactogenic potency are actually lower than 23K PRL (Sinha, 1995).

During the formation of PRL-secreting tumors in rats, a direct arterial blood supply is formed (Elias, 1984). Normally, the vascular supply of the anterior pituitary is exclusively through the hypophyseal portal vessels, thus the gland receives no arterial blood supply directly. In attempting to identify the factors that are involved in the regulation of the vasculature of the anterior pituitary, PRL was tested for its angiogenic activity *in vitro* . Intact rat 23 kDa PRL had no effect on basal or bFGF-stimulated growth of BBE cells (Ferrara, 1991). Since the 16 kDa N-terminal fragment of rat PRL (16K rPRL) is a mitogen for the mammary gland (Clapp, 1988), experiments were performed to see if 16K rPRL is an angiogenic factor. Surprisingly, 16K rPRL inhibited endothelial cell proliferation with high potency and in a dose dependent fashion (Ferrara, 1991). Ligand binding and cross linking experiments showed that 16K rPRL acted through a unique, high affinity saturable receptor that is neither the FGF nor the PRL receptor (Clapp, 1992). 16K PRL can be generated by two different methods: by proteolytic cleavage of NIH rat PRL and recombinant human 16K PRL (16K hPRL) was generated by inserting a stop codon in

place of amino acid 139 (Clapp, 1993). *In vitro*, 16K PRL from either source inhibits angiogenesis in endothelial cell proliferation and tube formation assay. *In vivo*, 16K PRL from both origins inhibited angiogenic activity in chicken chorioallantoic membrane assay (Clapp, 1993). Thus, *in vitro* and *in vivo* studies point to 16K PRL as a possible inhibitor of tumor growth

Clapp et al., reported that the PRL gene is expressed in the hypothalamic-neurohypophyseal system. Here the protein is processed into a 14 kDa fragment with antiangiogenic activity similar to that of 16K PRL (Clapp, 1994). A subsequent report showed that cultured neurohypophyseal explants release 14K PRL molecule into the incubation media. Secretion of the 14K rPRL increased 3-fold after incubation with calcium or depolarization with high concentration of potassium. 14K PRL is also detected in the rat circulation. These results suggest that the 14K PRL is synthesized locally and released in a calcium dependent fashion from the neurohypophyseal lobe of the pituitary where it could have physiological antiangiogenic activity.

The angiogenic factors, bFGF and VEGF, bind to receptors in the tyrosine kinase family (RTK) (de Vries, 1992; Lee, 1989). Growth factor binding results in receptor dimerization, activation of the intracellular tyrosine kinases, and receptor autophosphorylation (Ullrich, 1990). Phosphorylated tyrosine residues are recognized by adapter molecules via Src homology (SH2) domains, leading to activation of Ras which triggers a phosphorylation cascade. Ras activation leads to activation of the serine/threonine kinase Raf-1 which phosphorylates and activates the threonine/tyrosine kinase MEK. Activated MEK phosphorylates mitogen activated protein kinases (MAPK, p44 and p42) resulting in increased expression of early response genes and stimulation of cell proliferation (Dent, 1992; Howe, 1992; Kyriakis, 1992). Both bFGF and VEGF have been shown to activate MAPK in capillary endothelial cells (D'Angelo, 1995).

Increasing the level of intracellular cAMP has been reported to inhibit basal proliferation of endothelial cells (Leitman, 1986). The inhibitory effect of cAMP on cell

proliferation is mediated through the activation of the cyclic AMP-dependent protein kinase (PKA) (Burgering, 1993; Cook, 1993; Graves, 1993; Sevetson, 1993; Wu, 1993). Activation of PKA inhibits activation of MAPK by PGDF, EGF and bFGF in a variety of cell types (Assender, 1992; Cook, 1993; Chabre, 1995; Hordijk, 1994; Loesberg, 1985; Nilsson, 1984; van Corven, 1993). The target for inhibition by PKA in the MAPK pathway was shown to be Raf-1. Phosphorylation of serine 43 by PKA inhibits Raf-1 kinase activity thus blocks the MAPK signaling cascade (Hafner, 1994; Wu, 1993).

16K PRL inhibits both bFGF and VEGF-stimulated tyrosine phosphorylation and activation of MAPK. The inhibition of MAPK activity is associated with inhibition of Raf activity (D'Angelo, 1995). Inhibition of Raf is in turn a result of inhibition of Ras, or factors associated wuth Ras. 16K hPRL does not prevent the association of GRB2 or Sos with the phosphorylated FLK receptor. We recently showed that 16K hPRL inhibits Ras activation by stimulating the hydolysis of GTP bound to Ras to GDP (D'angelo, unpublished results).

A rapid increase of intracellular cAMP was observed following the addition of 16K hPRL. As described earlier, 16K hPRL might inhibit endothelial MAPK activity by increasing cAMP levels. However, no increase of adenylate cyclase activity was observed in membrane preparations of bovine brain capillary endothelial cells (BBEC) after 16K hPRL treatment. A PKA inhibitor H89, had no effect on 16K PRL's ability to inhibit RAF/MAPK but did block the inhibition induced by forskolin, the direct stimulator of adenylate cyclase activity. These results suggest that the 16K hPRL receptor is not directly coupled to adenylate cyclase, and that activation of PKA is not necessary for the inhibitory effect of 16K hPRL. However, the indirect increase in cAMP following treatment with 16K PRL is consistent with the presence of an additional mechanism by which 16K PRL inhibits endothelial cell proliferation in response to angiogenic factors.

V. Hypotheses

1) We hypothesize that the antiangiogenic factor, 16K PRL. inhibits angiogenesis by inhibiting the activity of proteases.

2) We hypothesized that 16K hPRL inhibits angiogenesis by interfering with endothelial cell migration.

3) Since 16K hPRL and TGF-β have similiar activities, we hypothesize that similar mechanisms mediated the signals these two hormones generate.

4) 16K hPRL family members, including 16K hGH, 16K hGHv and 16K hPL have been shown to be antiangiogenic, while the full length molecules from which they are formed are angiogenic. We hypothesize that the antiangiogneic actions of the 16K fragments are mediated at receptors which are neither the PRL or GH receptors.

VI. Model System

We used bovine brain capillary endothelial (BBE) cells to investigate the effect of 16K PRL since they have been used by many different research groups as a capillary endothelial cell model system and thus have been carefully characterized.

VII. Specific Aims

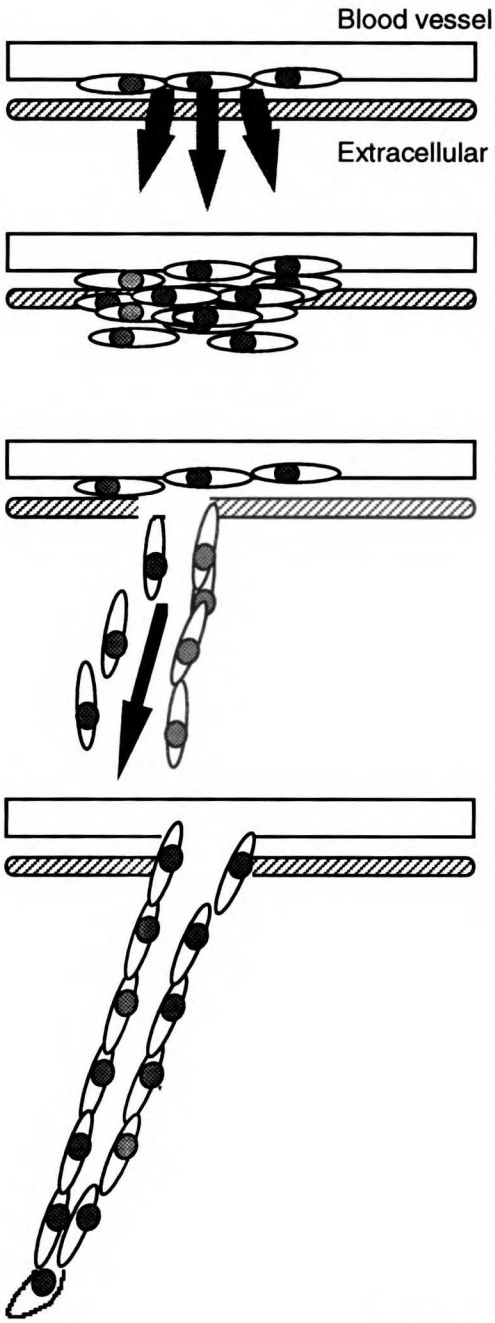
Our first specific aim was to determine if the antiangiogenic factor 16K PRL affects the activity of plasminogen activators in the BBE cell model, and if so, to identify the mechanisms mediating the inhibitory effect.

Our second specific aim was to determine if 16K PRL affects bFGF-stimulated BBE cell migration by using a modified Boyden chambers assay.

The third specific aim was to compare the signaling mechanisms triggered by 16K PRL and TGF-β in BBE cells. This was accomplished by using kinase inhibitors. We also compare the response elements used by these two factors in a reporter gene system.

The final specific aim was to investigate whether all of the 16K hPRL family members stimulate PAI-1 expression, and to show that they do not signal via the receptors for the intact molecules. By using pharmacological agents that block serine/threonine kinase activity, we investigated whether a similar signaling mechanism was used by all of the 16K family members.

Anatomy of angiogenesis



Activation of proteolytic enzymes

Proliferation and invasion by endothelial cells

Migration of endothelial cells

Formation of capillary tube-like structure

FIGURE 1-1

Urokinase activation pathway

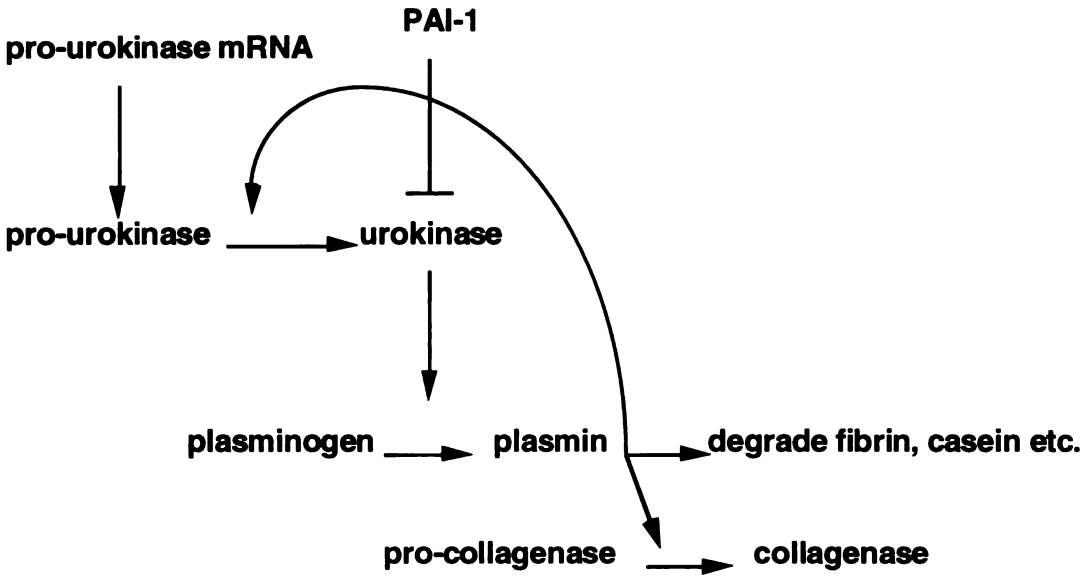


FIGURE 1-2

Chapter 2. 16K PRL Inhibits Urokinase Activity in BBE Cells

Introduction:

16K PRL has been shown to be a potent antiangiogenic factor in both *in vitro* and *in vivo* systems. Since the elaboration of proteases is believed to be an important component of angiogenesis, we investigated whether 16K PRL inhibited the plasminogen activator activity of endothelial cells stimulated by the angiogenic factor, basic fibroblast growth factor (bFGF). Chapter 2 includes a publication showing that 16K rPRL and 16K hPRL inhibited urokinase activity in BBE cells. However, 16K PRL had no effect on uPA mRNA levels. PAI-1, a major inhibitor of urokinase activity, was increased by 16K PRL at the transcriptional level. This study suggests that the antiangiogenic action of 16K PRL is mediated by indirectly inhibiting uPA activity by stimulating PAI-1 production.

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

Hsinyu Lee, Ingrid Struman, Carman Clapp, Joseph Martial and Richard I. Weiner

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ABSTRACT

The N-terminal fragment of prolactin (16K PRL) is an antiangiogenic factor which *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 which 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 BBE cells as detected by plasminogen substrate gel assay. 16K PRL did not appear to be acting via an effect on uPA expression, since no change in mRNA 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.

INTRODUCTION

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 e.g. wound healing; tissue remodeling e.g. formation of the corpus luteum; or various disease processes e.g. 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], while 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 prolactin (16K rPRL) [7] and human prolactin (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 inhibit bFGF and VEGF-induced cell proliferation of cultured capillary endothelial cells from bovine and human [7,10]. Furthermore, 16K PRL inhibits the organization of BBE cells cultured in type 1 collagen gels into capillary-like structures [10], and the migration of BBE cells 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 [12]. 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 BBE cells 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 RESULTS

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 cDNA was site-directed mutagenized as reported. Briefly, Cys-58 (TGG) of the constructed 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/160ng.

16K rPRL:

Rat 16K PRL (16K rPRL)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 dithiothreitol (20-fold molar excess over the disulfide bond molecular content) in the presence of 4 M guanidine HCl in 0.1 M NH_4HCO_3, pH 8.5, and subsequently alkylated with a 40 M excess of iodoacetamide over the dithiothreitol concentration. The sample was dialyzed, and its purity and concentration were assessed by SDS-PAGE and densitometry.

Cell Culture

Bovine brain capillary endothelial (BBE) cells were isolated as previously described (25). The cells were grown and serially passaged in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 2 mM L-glutamine, and antibiotics (100 units of 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 two days. Experiments were initiated with confluent cells between passages 5-13.

Cell Stimulation and Preparation of Cell Extracts

Confluent culture of BBE cells were enzymatically dispersed and plated at a density of 5×10^5 cells per well in 6 well plates in 1 ml of BBE media. Twenty four hours after plating, cells were transferred to MEM containing low serum for 24 hours (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 X100) shake at 4 C for 20 min. The insoluble fraction was removed by centrifugation at 4 C for 10 min at 14,000 x g and the protein concentrations of the soluble fraction were determined by BCA assay kit (Pierce).

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 non-reducing conditions by sodium dodecyl sulphate polyacrylamide gel electrophoresis (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 which 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 BBE cells 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), 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 provides by Dr. Schuring), 600 bp of PstI fragment of PAI-1 cDNA (kindly provided by Dr. M.J. Pepper) and 110 bp of PstI fragment of cyclophillin cDNA (kindly provided by Dr. M. Skinner) were labeled by [^{32}-P]dCTP with Oligolabeling kit (Pharmacia) and purified by Quick Spin columns (Boehringer Mannheim). The hybridization reactions were performed at 68 C for 1h in Quickhyb solution(Stratagene). The hybridized blots were washed 2 times with 2X SSC/0.1% SDS at RT for 15 min and once with 0.1X SSC/0.1% SDS at 65 C for 30 min. The resulting blots were subjected to autoradiography. Quantification of the mRNA levels was performed by densitometric scanning of the autoradiograms of cyclophillin or methylene blue staining of 28s and 18s rRNA 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 EcoRI-BglII fragment of the bovine urokinase cDNA was transcribed with ^{32}P UTP using T7 promoter to generate a radiolabelled riboprobe. Ten ug of the RNA isolated from BBE cells were hybridized with 2.5 x 10^5cpm of the riboprobes at 42 C for 12 h, digested with RNase A and T1 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 BBE cells were resolved by SDS/PAGE (4-10%) and transferred to nitrocellulose membrane using a semi-dry 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 anti-bovine PAI-1 mouse monoclonal antibody at a 1:2,000 dilution for 2 h (Gibco, BRL, Gaithersburg, MD). The antigen-antibody complexes were detected with horse-radish peroxidase conjugated secondary antibody and the enhanced chemiluminescence system (ECL, Amersham). The blots were exposed on reflection NEF films (NEN) to visualize the bands.

Immunoprecipitation of Bovine PAI-1

Subconfluent cultures of BBE cells 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, Boston, MA) 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 X100, 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 (PMSF), 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 X 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 5ug of the antibovine PAI-1(Gibco) or 5 ug normal mouse IgG at 4 C for 1 h. Fifty µl 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 X100 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

BBE cells were initially treated with 10 nM 16K hPRL for 6 h to stimulate PAI-1 mRNA expression. The treated BBE cells were then washed and fresh medium containing actinomycin D (3 ug/ml) added to arrest transcription. Cells were harvested 0,1,4,8 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

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 BBE cells for 16 h with both 3ng/ml and 30 ng/ml of bFGF resulted in a large

increase in the size of the lysis zone formed by proteins in the cell associated fraction migrating at 52 kDa, the expected size for uPA, Fig 1a. 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-stimulated uPA activity, Fig 1b. These observations were repeated in 3 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).

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. Since, no reagents were available to perform Western blotting or immunoprecipitation studies for bovine uPA, and since 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 BBE cells with bFGF (3ng/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 2a-d. Treatment with 40 nM 16K rPRL had no effect on the stimulation of uPA mRNA levels by bFGF nor on basal levels, Fig 2a-d. Similar results were obtained in 3 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.

Stimulation of PAI-1 Levels and Synthesis by 16K PRL

Since, 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 3 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 2 additional experiments. In the three experiments, the maximum stimulation in the secretory fractions at 24 h varied from 10-50 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 BBE cells were treated with 10 nM 16K hPRL for 16h and then pulse labeled with S^{35} methionine for 4 h. There was an approximate 2 fold stimulation of incorporation of radioactivity into immunoprecipitates of PAI-1 from the cell associated fraction and a 5 fold increase in the secreted fraction, Fig 5a,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 cotreatment 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.

Stimulation of PAI-1 mRNA levels by 16K PRL

Treatment with 10 nM 16K hPRL stimulated PAI-1 RNA levels approximately two fold compared to the control, Fig 6a,b. Values were corrected for loading relative to the intensity of the 28s and 18s bands. These observations were confirmed in 3 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, BBE cells were incubated with 10 nM 16K hPRL or vehicle alone for 6h. 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.

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 ug 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 ug of recombinant PAI-1, and totally inhibited by the addition of 2 ug, 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 utilized 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, since the antibody against PAI-1 used did not recognize the PAI-1/uPA complexes.

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,

since 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 can not 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 was 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 which 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 weight (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.

Since, 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 similar amount of LPS had no effect on PAI-1 production in BBEC. Furthermore, this was not an issue with the 16K rPRL used, since it was made by enzyme cleavage and contained low levels of LPS.

It appears that stimulation of PAI-1 expression may be common action of several antiangiogenic factors in addition to 16K PRL including: thrombospondin [32]; TGFβ [23]; LIF [33]; TNF α [34, 35] 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 [36]. Antiangiogenic factors which have been shown to increase PAI-1 levels also inhibit capillary endothelial cell migration i.e. 16K PRL, TGFβ [37], thrombospondin [6], and LIF [33]. 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.

FIGURE LEGENDS

Figure 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.

Figure 2. Effect of 16K PRL on Urokinase mRNA Levels

a. Northern blot analysis for bovine uPA mRNA of total RNA harvested from BBE cells treated for 16h with bFGF (3ng/ml), 16K rPRL (40nM), or the two combined. b. Blots were stripped and reprobed with a bovine cyclophillin 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 (3ng/ml), 16K rPRL (40nM) or the two combined. The tRNA lane was added to control for nonspecific background and the antisense lane to identify the size of the protected fragment.

Figure 3. Effect of 16K hPRL on PAI-1 Levels

Conditioned media (secretory) or cell lysates (cell associated) from BBEC treated with bFGF (10ng/ml), 16K hPRL (10 nM), or the two combined were immunoblotted using an anti-bovine 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.

Figure 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.

Figure 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 4h. PAI-1 was immunoprecipitated from cell associated (upper panel) and conditioned media (lower panel), separated by PAGE and autoradiographed. Treatment with 16K hPRL 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 while the lower band was specific.

Figure 6. Effect 16K hPRL on PAI-1 mRNA Levels

a. Northern analysis for bovine PAI-1 mRNA of total RNA (10 ug) from BBEC treated for 16h with nothing, bFGF (10 ng/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 * ($P<0.05$).

Figure 7. Stability of PAI-1 mRNA

a. The rate of disappearance of PAI-1 mRNA following treatment with actinomycin D (3ug/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.

Figure 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 ug of recombinant human PAI-1. In the lysates in which recombinant human PAI-1 was added a specific, high molecular weight 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 ug of recombinant PAI-1 and completely inhibited by the addition of 2 ug. c. Conditioned media from the metabolically labeled BBEC used in Fig. 5 were separated by PAGE and autoradiographed. Treatment with 16K hPRL 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.

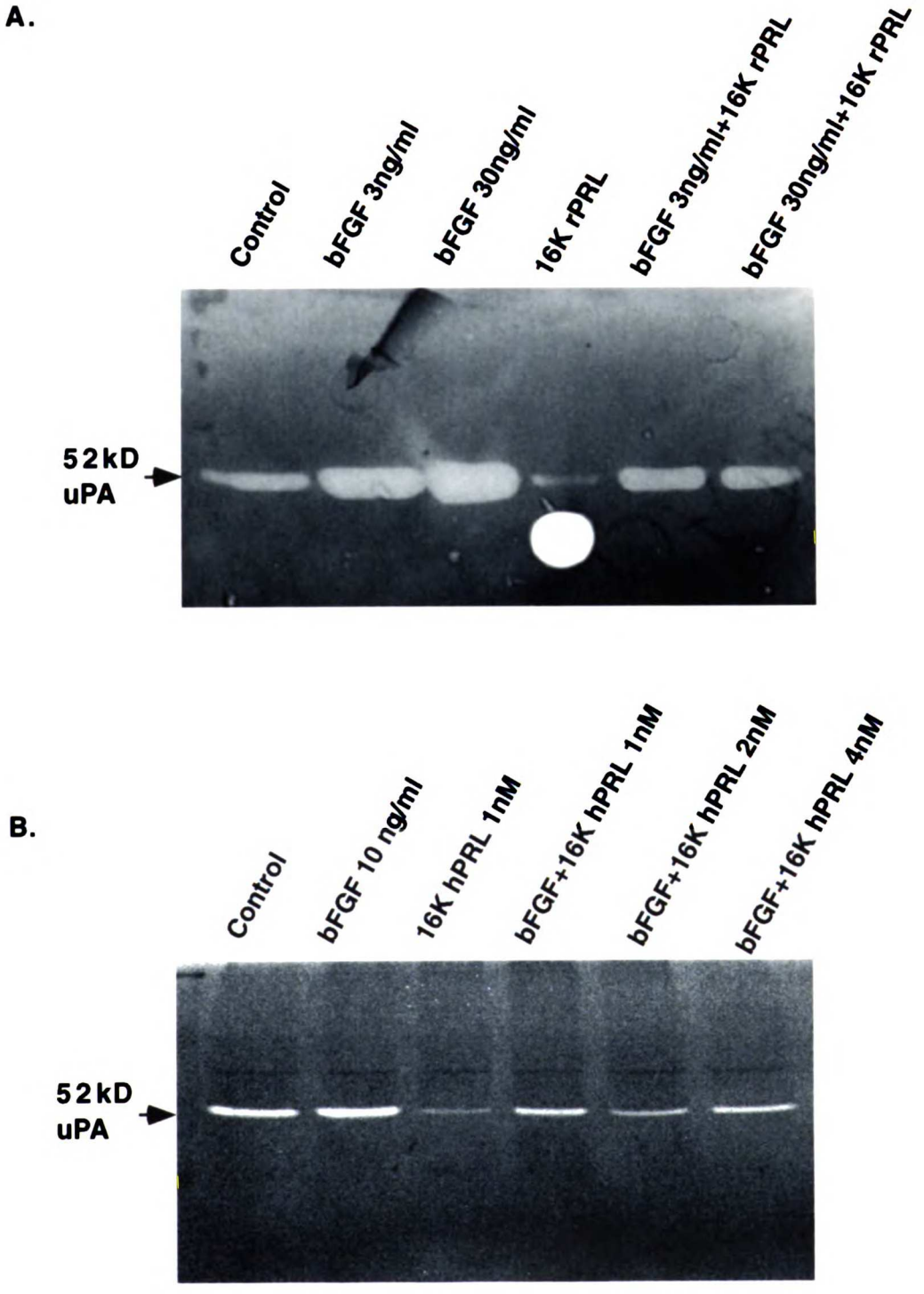

A.
Control
bFGF 3ng/ml
bFGF 30ng/ml
16K rPRL
bFGF 3ng/ml+16K rPRL
bFGF 30ng/ml+16K rPRL
52kD
uPA
B.
Control
bFGF 10 ng/ml
16K hPRL 1nM
bFGF+16K hPRL 1nM
bFGF+16K hPRL 2nM
bFGF+16K hPRL 4nM
52kD
uPA

FIGURE 2-1

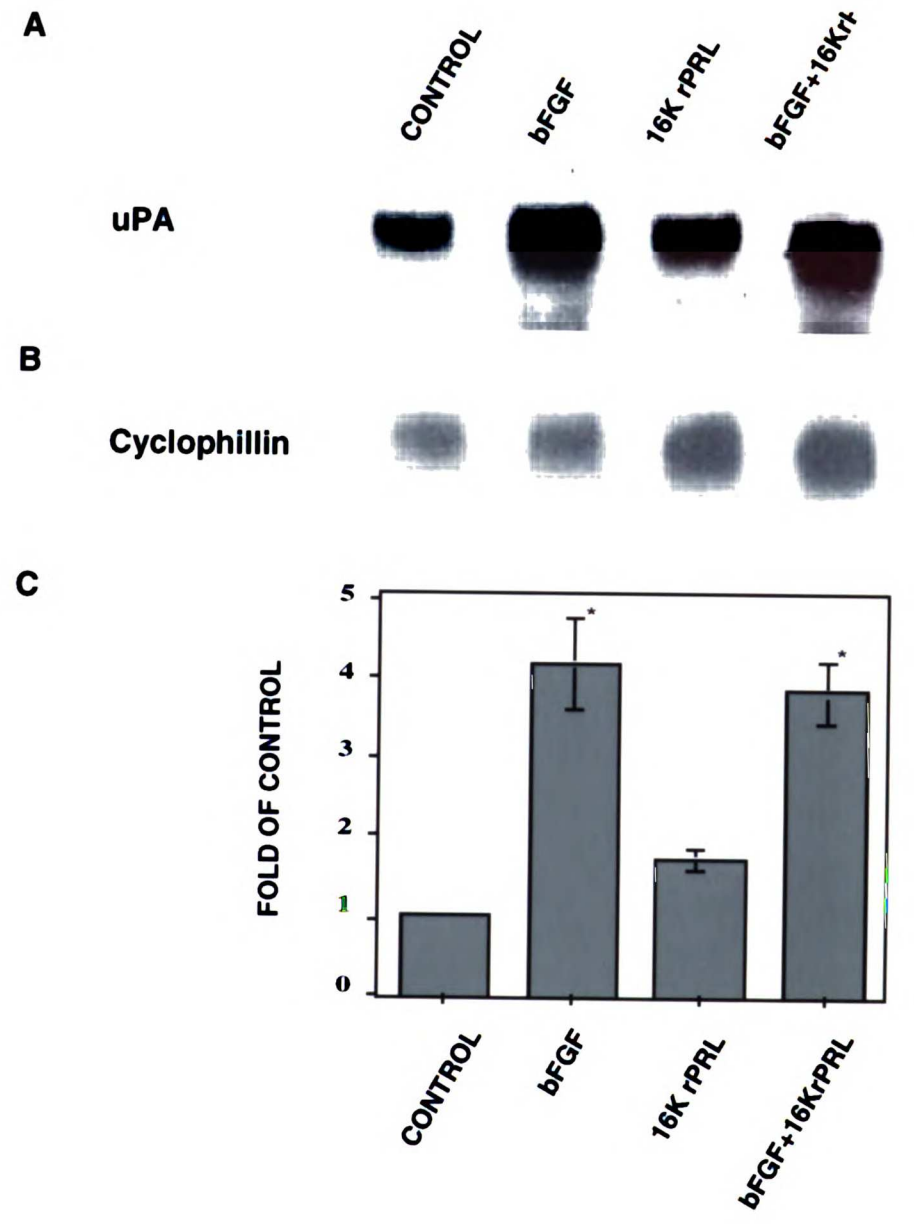

A
CONTROL
bFGF
16K rPRL
bFGF+16Krt
uPA
B
Cyclophillin
C
FOLD OF CONTROL
5
4
3
2
1
0
CONTROL
bFGF
16K rPRL
bFGF+16KrPRL
D
PROBE
tRNA
CONTROL
bFGF
16K rPRL
bFGF+16KrPRL
ANTISENSE

FIGURE 2-2

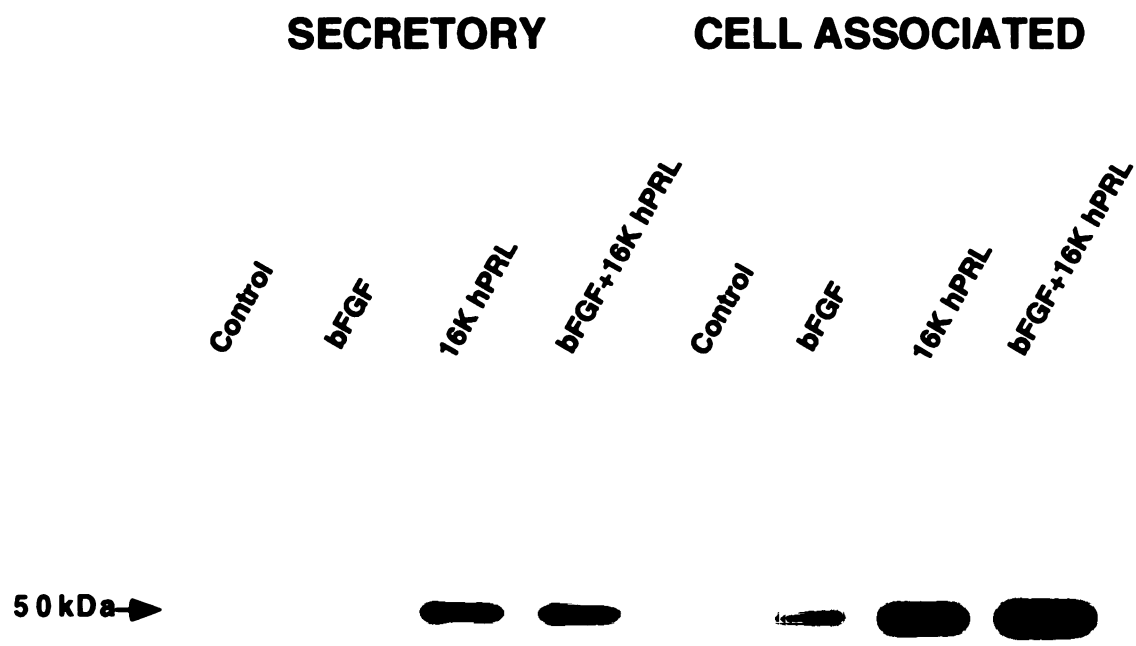
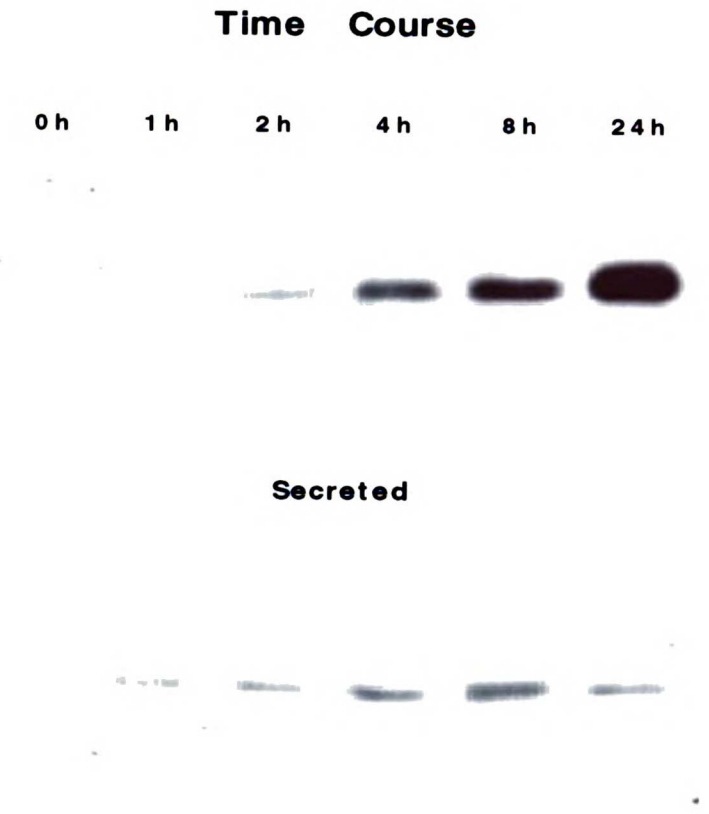

SECRETORY
CELL ASSOCIATED
Control
bFGF
16K hPRL
bFGF+16K hPRL
Control
bFGF
16K hPRL
bFGF+16K hPRL
50kDa

FIGURE 2-3

A.


Time Course
0 h
1 h
2 h
4 h
8 h
24 h
Secreted
Cell associated

B.

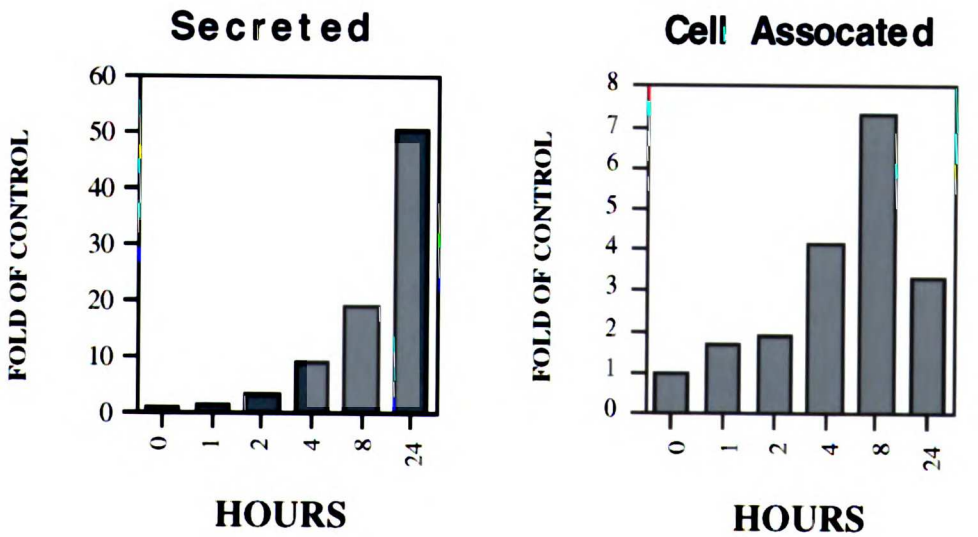
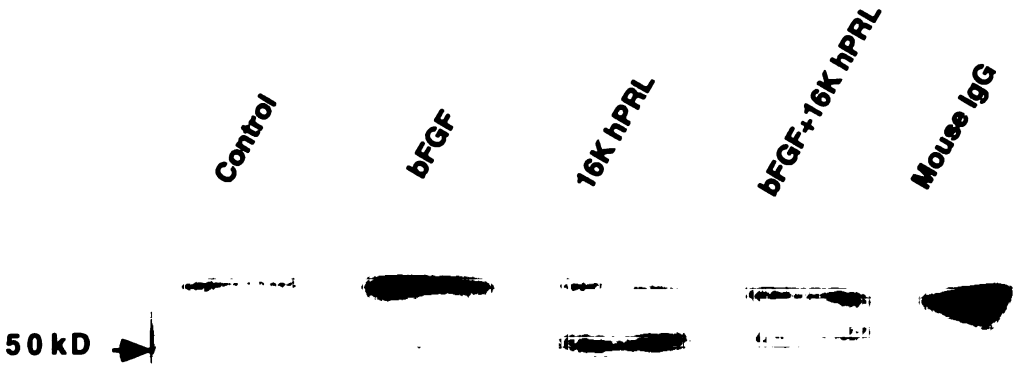

Secreted
FOLD OF CONTROL
60
50
40
30
20
10
0
0
1
2
4
8
24
HOURS
Cell Assocated
FOLD OF CONTROL
8
7
6
5
4
3
2
1
0
0
1
2
4
8
24
HOURS

FIGURE 2-4

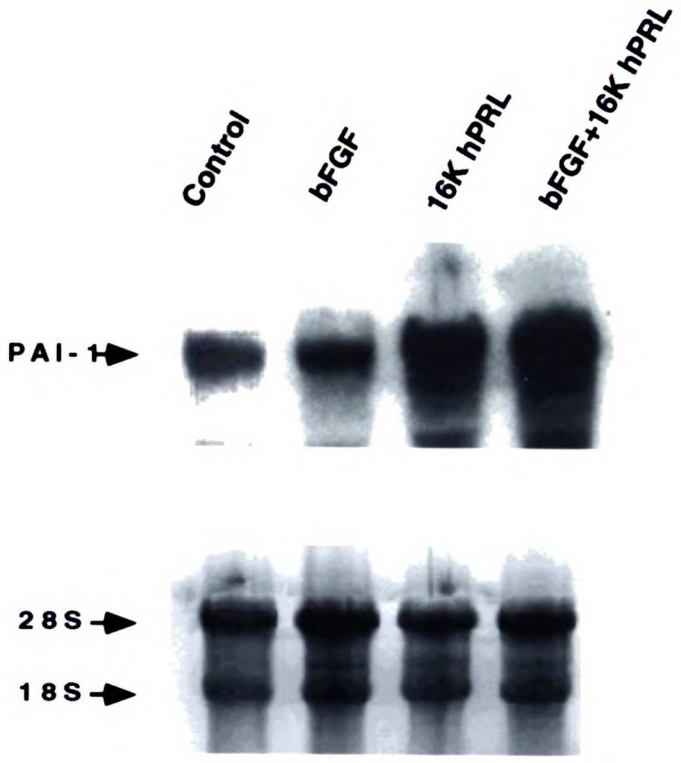


Cell Associated

Secreted

FIGURE 2-5

A.


Control
bFGF
16K hPRL
bFGF+16K hPRL
PAI-1
28S
18S

B.

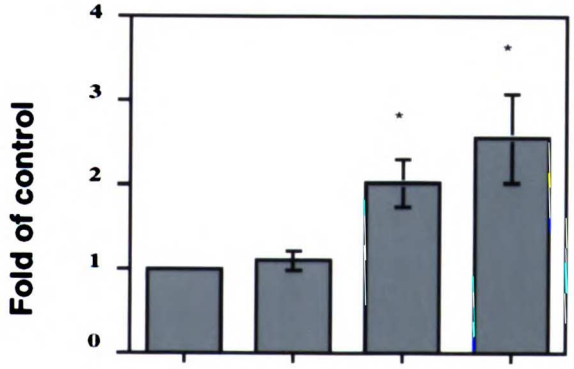
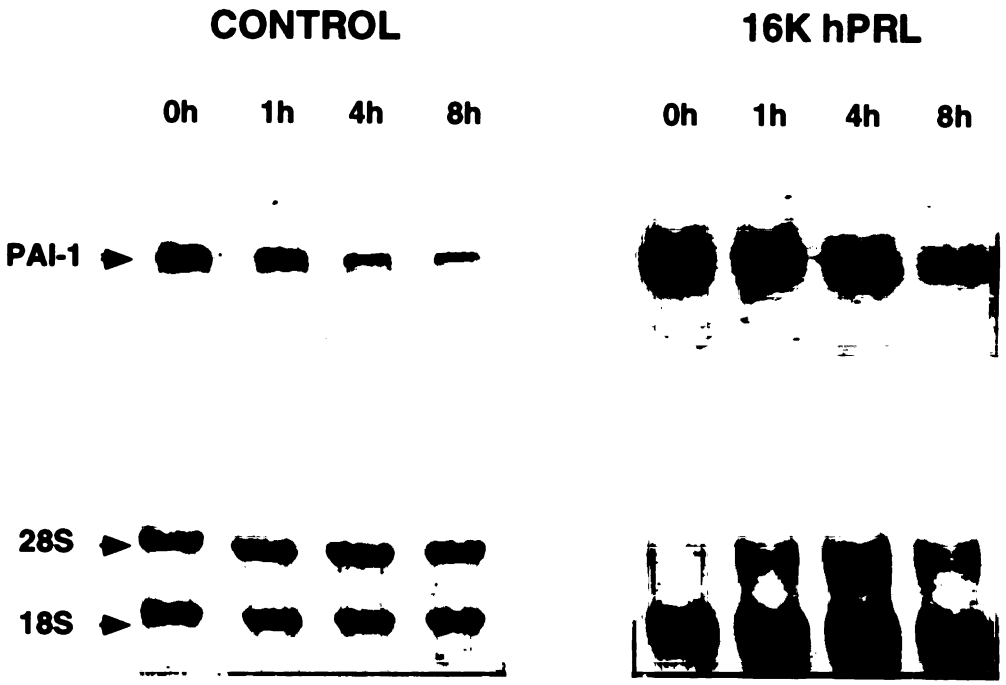
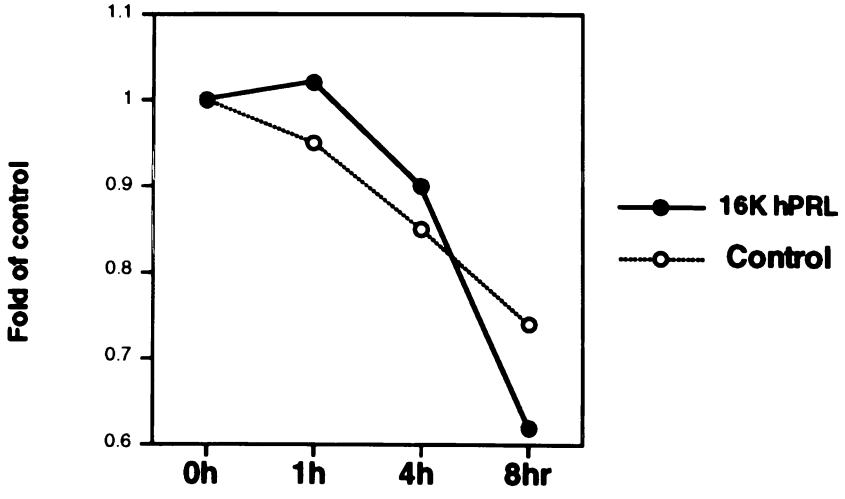

Fold of control
4
3
2
1
0
*
*

FIGURE 2-6

A.



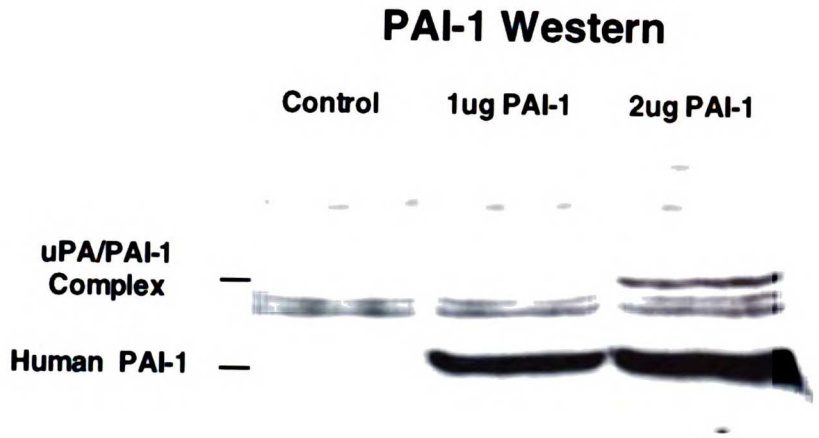
B.



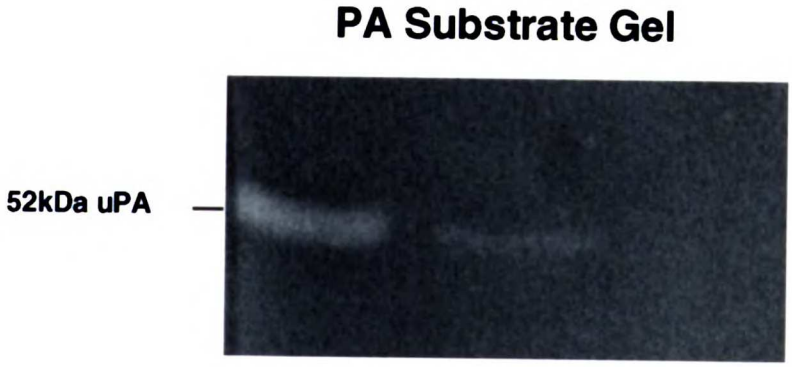
Time after Actinomycin D treatment

FIGURE 2-7

A.



B.



C.

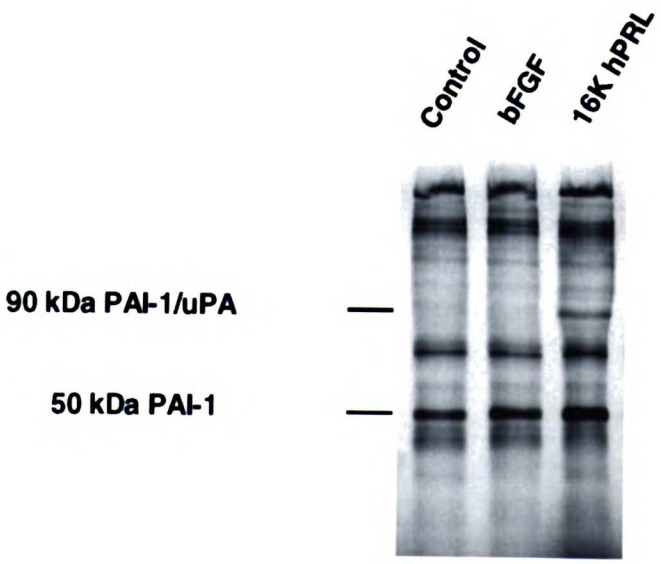


FIGURE 2-8

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Chapter 3. 16K hPRL Inhibits bFGF-Stimulated Endothelial Cell Migration

INTRODUCTION

Endothelial cell migration is another essential component of angiogenesis (Fibbi, 1988; Folkman, 1992; Lindner, 1989; Madri, 1982; Mignatti, 1996; Odekon, 1992). It has been shown that endothelial cell migration is regulated by many different factors at distinct stages. For example, the release of endogenous basic fibroblast growth factor (bFGF), and coating the culture surface with collagen of types I and IV have been identified as requirements for endothelial cell migration *in vitro* (Madri, 1982; Madri, 1991; Rosen, 1989; Sato, 1988; Tsuboi, 1990). Recent reports also suggest that modulation of endothelial cell surface protein expression and organization often occurs in response to changes in adhesive substratum, soluble factors, and the migration of endothelial cells (Basson, 1990; Ingber, 1990; Lampugnani, 1991). We tested the hypothesis that 16K hPRL might interfere in endothelial cell migration stimulated by angiogenic factor bFGF. Data presented here showed that 16K hPRL inhibits migration of bovine capillary endothelial cells.

MATERIALS AND METHODS

Migration of endothelial cell was determined by a previously published method using a modified version of Boyden chambers (Good, 1990). Briefly, 10 μg of type I collagen was added to the upper chamber which consisted of a membrane with 8 μM pores (Collaborative Biomedical Products). Ten thousand endothelial cells were added in the top chamber. Ten ug/ml of the bFGF was added to the bottom wells to induce endothelial cell migration. 16K hPRL (10 uM) was added to both the top and bottom wells, and the chambers were incubated at 37° C with 5% CO_2 for 16 hrs. The non-migrating cells remaining in the top wells were removed by scraping the membrane with a cotton swab. The membranes were fixed, stained with 1% crysyl violet and mounted on microscopic

slides. The migrating cells were quantified by light microscopy at a magnification of 400x by counting the stained cells from 10 randomly selected fields.

RESULTS

16K PRL Inhibits bFGF Stimulated Endothelial Cell Migration

In the absence of bFGF, few endothelial cells migrated across the collagen-coated membrane. As previously reported, bFGF at 10 ng/ml concentration stimulated endothelial cell migration when compared to control BBE cells (Fig. 1). 16K hPRL (10nM) significantly ($P<0.05$) inhibited bFGF-stimulated BBE cell migration by 50% (Fig. 2).

DISCUSSION

Endothelial cell migration is regulated by many different factors. It is negatively regulated by anti-angiogenic factors including thrombospondin (Good, 1990), angiostatin (O'Reilly, 1994), endostatin (O'Reilly, 1997), SPARC (Hasselaar, 1992) and proliferin (Jackson, 1994). By using the collagen-coated membrane assay system, we show that 16K PRL also inhibits endothelial cell migration.

Migration of endothelial cells can be affected by many different factors (Abedi, 1995). The integrin family of cell adhesion receptors mediate cell attachment to extracellular matrix proteins and is known to play a critical role in cell motility (Lauffenburger, 1996; Lawson, 1995; Klemke, 1994; Yebra, 1995). Integrins are involved in a variety of biological processes including angiogenesis, wound healing and tumor cell invasion and metastasis (Brooks, 1996; Lindner, 1989; Folkman, 1985; Friedlander, 1995; Madri, 1991; Nip, 1995). The inhibitory effect of 16K hPRL on endothelial cell migration could be due to the change of cell surface integrin composition. Moreover, the extracellular matrix protein composition might affect the cell-matrix interaction and thus change the endothelial cell migration pattern. Thus 16K hPRL may inhibit cell migration via more than a single mechanism.

The inhibition of endothelial cell migration might also be due to inhibition of uPA (Blasi, 1996). uPA receptor binding has been reported to stimulate endothelial cell migration by different groups (Fibbi, 1988; Odekon, 1992). Endothelial cell migration seems to be stimulated by receptor binding, but not uPA enzymatic activity. Enzymatically inactive uPA amino-terminal fragments also stimulate endothelial cell migration. In fibroblasts, uPA regulates cell migration by activation of a threonine/serine protein kinase (Busso, 1994; Dumler, 1993). Two intermediate filament proteins, cytokeratin 8 (CK8) and 18 (CK18), are phosphorylated by the treatment of fibroblasts with uPA. The phosphorylation of CK proteins in endothelial cells by uPA has not been reported. It would be interesting to see if 16K PRL affects the phosphorylation status of these proteins in endothelial cells.

In conclusion, we showed that 16K hPRL inhibited bFGF-induced migration of bovine capillary endothelial cells. A finding consistent with the ability of 16K hPRL to inhibit *in vivo* angiogenesis.

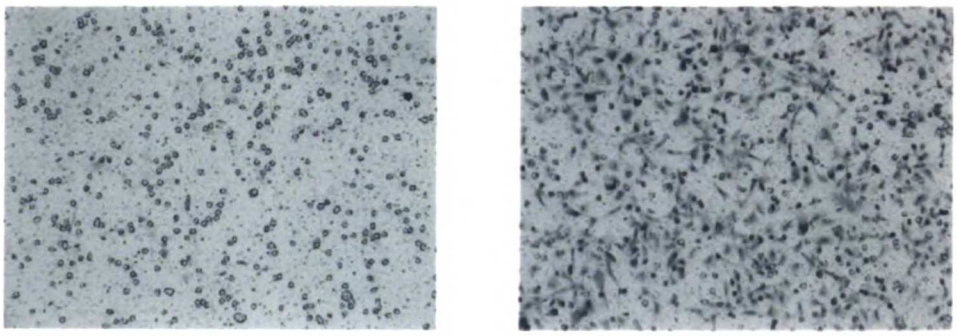
FIGURE LEGENDS

Figure 1. 16K PRL effect of BBE cell migration

16K hPRL inhibits bFGF-stimulated endothelial cell migration. Endothelial cell migration was assayed by a modified Boyden chamber assay. Endothelial cell migration was stimulated by 10 ng/ml of bFGF treatment (bFGF) . bFGF-stimulated endothelial cell migration was inhibited by 10 nM 16K hPRL treatment (bFGF+16K PRL).

Figure 2. Quantification of endothelial cell migration.

The migrating cells in Fig. 8 were quantitated by counting the stained cells from 10 randomly selected fields at 400x magnification. Values represent the means ± SD (n=10).



CONTROL

bFGF

16K PRL

bFGF + 16K PRL

FIGURE 3-1

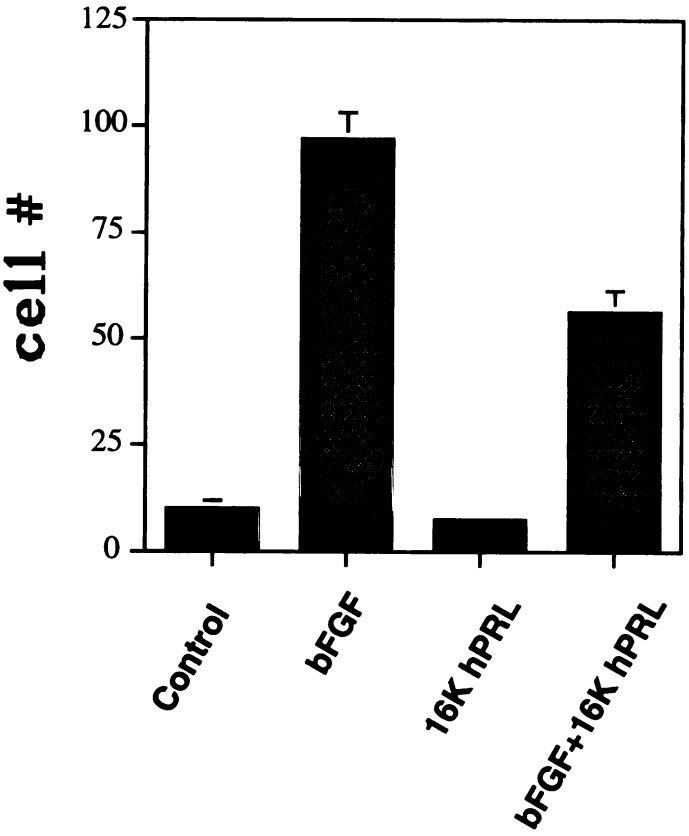


FIGURE 3-2

Chapter 4. Signaling Mechanisms Mediated by 16K PRL

Introduction

In chapter 2, we showed that 16K PRL stimulates PAI-1 produciton in BBE cells. Chapter 4 includes a submitted publication which represents collaborative studies between Drs. Richard Weiner and David Dichek's laboratories. In this publication, we compare the signaling mechanisms mediated by 16K PRL and TGF β, since these two hormone inhibit *in vitro* angiogenesis in a similar fashion. We show that both hormones inhibit gowth factor-stimulated endothelial cell proliferation and MAPK activity. However, TGF-β1 inhibits [^{3}H]-thymidine uptake in serum starved culture while 16K PRL has no effect. Furthermore, we show that 16K hPRL stimulates apoptosis in BBE cells, while TGF-β1 has no effect in this cell type. Using PAI-1 as a marker, we further investigate the signaling mechanisms mediated by these two hormones. By using kinase inhibitors, we show that 16K PRL and TGF β-stimulated PAI-1 is mediated through a serine/threonine kinase dependent pathway. We exclude PKA and PKC as mediator in these pathways since the specific inhibitors for these kinases have no effect on 16K PRL-stimulated PAI-1 production. By reporter gene experiments, we show that different response elements were used by 16K PRL and TGF-β1 to stimulate PAI-1. All these results provide evidences that 16K PRL and TGF β, a factor that share many biological effects with 16K PRL, utlize a distinct siganling mechanism in BBE cells.

MANUSCRIPT 2

H Lee, J-F Martini, F Bentzien, RN Woodward, I Struman, J Martial, RI Weiner (1998). Signaling pathways for the antiangiogenic actions of 16K PRL and TGF β. Endocrinolgy (submitted).

ABSTRACT

In vitro the 16 kDa N-terminal fragment of human prolactin (16K hPRL) has multiple antiangiogenic actions on capillary endothelial cells including: inhibition of bFGF-induced cell proliferation, activation of apoptosis and stimulation of type 1 plasminogen activator inhibitor (PAI-1) expression. TGF-β1 shares many of the *in vitro* antiangiogenic actions of 16K hPRL, and we asked whether the factors utilized convergent or parallel signaling pathways to mediate their actions. Both factors inhibited bFGF-induced bovine brain capillary endothelial (BBE) cell proliferation in a dose dependent manner. They also inhibited VEGF-induced stimulation of MAPK, indicative of the convergence of their anti-proliferative signaling pathways. Although 16K hPRL stimulated apoptosis in BBE cells, TGF-β1 had no effect as determined by measurement of DNA fragmentation and caspase-1 cleavage/activation. Both 16K hPRL and TGF-β1 stimulated PAI-1 expression in BBE cells. The signaling pathway for the stimulation of PAI-1 expression by both factors appeared to involve a serine/threonine kinase, since the stimulation of PAI-1 was blocked by treatment with the non-specific inhibitor, H-7, but not by specific inhibitors of protein kinase A (PKA) and protein kinase C (PKC). The response element for the stimulation of PAI-1 expression in BBE cells by TGF-β1 was within the -800 bp fragment of the human PAI-1 reporter gene, while that for 16K hPRL was located upstream within -6.4 and -1.5 kb fragment. These results show that the antiangiogenic actions of 16K hPRL and TGF-β1 on BBE cells only partially overlap with only 16K hPRL activating apoptosis. While both factors appear to signal via parallel pathways to inhibit cell proliferation, they stimulate PAI-1 gene expression via different response elements. Activation of the 16K hPRL receptor in BBE cells was capable of activating caspase 1 and DNA fragmentation, while TGF-β1 had no effect.

INTRODUCTION

Angiogenesis is controlled by a balance of both positive and negative regulators, namely angiogenic and antiangiogenic factors [1-4]. The 16 kDa N-terminal fragment of

human prolactin (16K hPRL), a proteolytic cleavage product of PRL, inhibits angiogenesis both *in vivo* and *in vitro*. *In vitro* 16K PRL has multiple antiangiogenic effects on capillary endothelial cells including: inhibition of VEGF and bFGF-induced proliferation [5] (Ferrara, 1991); activation of apoptosis [6]; inhibition of the organization cells into capillary-like structures [7], and inhibition of cell migration (Lee, unpublished results).

An essential component of endothelial cell migration and organization is the activation of urokinase (uPA). Endothelial cells also express and secrete PAI-1, which is a major inhibitor of uPA activity [8, 9]. The balance of proteolytic activity between uPA and PAI-1 has been suggested to be an important factor regulating angiogenesis [10]. Reduction of urokinase activity by PAI-1 may decrease breakdown of extracellular matrix components, subsequently retarding the migration of endothelial cells. 16K PRL stimulates PAI-1 expression which appears to indirectly inhibit uPA activity [11].

The actions of 16K hPRL appear to be mediated via a novel receptor which is not the PRL receptor. High affinity, saturable, selective binding sites for rat 16K PRL have been characterized in bovine brain capillary endothelial (BBE) cells [12]. The receptor appears to be unique from several angiogenic and antiangiogenic factor receptors including the TGF-β1 receptor, since addition of the factors do not compete for 16K PRL binding. Although the identity of the receptor is currently unknown, the downstream signaling pathways mediating the actions of 16K hPRL have been partially elucidated. 16K hPRL inhibits both bFGF and VEGF-induced activation of the MAPK signaling cascade, a well characterized pathway mediating the mitogenic action of these angiogenic factors [13]. Inhibition of VEGF-induced activation of MAPK appears to be mediated via the blockade of the activation of Ras [14], an observation consistent with the findings that 16K hPRL blocks Raf-1 and MAPK activation. The stimulation of apoptosis by 16K hPRL is mediated by activation of caspases 1 and 3, but the upstream signaling events are unknown [6]. The signaling pathway mediating the increased expression of PAI-1 by 16 K hPRL have not been identified.

TGF-β1 was reported to have similar antiangiogenic actions as 16K hPRL *in vitro* [15]. TGF-β1 inhibits proliferation, migration and organization of capillary endothelial cells and increases the expression of PAI-1 [10, 16, 17]. TGF-β1 signals through a heteromeric complex of TGF β type I and type II receptors (TβR-I and TβR-II) which belong to the serine/threonine kinase receptor family [18]. Activation of the TGF-β1 receptor complex results in phosphorylation and activation of smad proteins, which are subsequently translocated into the nucleus and bind to a response element on the PAI-1 gene [19, 20].

Because of the similarities in the *in vitro* antiangiogenic actions of 16K hPRL and TGF-β1, we determined whether the two factors shared signaling pathways in mediating their antiangiogenic actions. We asked whether in addition to known effects of 16K hPRL and TGF-β1 on capillary endothelial cell proliferation and PAI-1 expression, if TGF-β1 could also activate apoptosis in BBE cells. Furthermore, we determined if the actions of 16K hPRL and TGF-β1 utilized: the MAPK signaling cascade for inhibiting cell proliferation; activation of caspase 1 for inducing apoptosis; and the same response elements for increasing PAI-1 gene expression.

MATERIALS AND METHODS

Preparation of Recombinant Human 16K PRL

Recombinant human 16K PRL (16K hPRL) was prepared as described [7]. A Pt7L plasmid containing human 23K PRL 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 TGA 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 level was < 0.01 EU/160 ng.

BBE Cell Culture

Bovine brain capillary endothelial (BBE) cells were isolated as previously described [21]. The cells were grown and serially passaged in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (CS; Hyclone), 2 mM L-glutamine, and antibiotics (100 units of 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 other day. Experiments were initiated with confluent cells between passages 5-13.

Cell Proliferation Assay

BBE cells were plated at a density of 10,000 cells per well (6-well plates, Falcon) in 1 ml of 10% CS supplemented DMEM medium. After 24 hrs, cells were serum starved for 24 hrs in 0.5% CS containing culture medium, and then incubated for an additional 24 hrs in the presence of bFGF (50 pM) alone or in combination with 16K hPRL or TGF-β1 in concentrations as indicated. For the last 4 hrs of the stimulation period 0.6 mCi [^{3}H]-thymidine (Dupont) was added to the culture wells. The reactions were stopped by addition of 0.5 ml of cold 5% trichloroacetic acid (TCA; Fisher), nonincorporated [^{3}H]-thymidine was removed with three washes of cold 5% TCA, and cells were solubilized at room temperature for 60 min with 0.25 N NaOH. [^{3}H]-thymidine incorporation was measured in a liquid scintillation counter.

MAPK Activity

For phosphorylation status of MAPK, BBE cells were plated at a density of 500,000 cells per 60 mm plate in 3 ml of DMEM containing 10 % CS (Gibco BRL). After 48 h cells were serum starved for additional 48 h and then stimulated for 5 min with VEGF (2 nM) alone or in combination with either 2 nM 16K hPRL or 80 pM TGF-β1. Cell lysates were harvested at 4°C in lysis buffer containing 1% Triton X-100; 20 mM Tris-HCl (pH 8); 137 mM NaCl; 10% glycerol; 2 mM EDTA; 1 mM Pefablock; 0.14 U/ml aprotinin;

20 mM leupeptin; and 1 mM sodium orthovanadate. Insoluble material was removed by centrifugation at 4°C for 10 min at 14,000 x g and the protein concentration of the soluble fraction was determined by BCA assay kit (Pierce).

Equal quantities of cell lysates (200 μg protein) from control or stimulated BBE cells were immunoprecipitated overnight at 4°C with 0.5 μg of MAPK polyclonal antiserum (Erk1-CT, Upstate Biotechnology). Immune complexes were purified with protein A-Sepharose and washed three times in RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS) and once in kinase buffer (30 mM Tris-HCl, pH 8.0; 20 mM $MgCl_2$; 1 mM DTT). The precipitate was resuspended in 30 μl of kinase assay buffer containing 20 μg of myelin basic protein (MBP, Sigma), 2 μM ATP and 5 μCi of [γ-^{32}P]ATP. Samples were incubated for 20 min at 30°C and the assay was terminated by addition of hot 4x SDS/PAGE sample buffer followed by boiling for 5 min. Reaction products were resolved on SDS/PAGE (15%). Gels were dried and subjected to autoradiography. The radioactivity incorporated into the MBP band was quantitated by PhosphorImager (Molecular Dynamic).

PAI-1 Western Blot Analysis

To prepare cell lysates for PAI-1 Western blot analysis, confluent cultures of BBE cells were enzymatically dispersed and plated at a density of 5 x 10^5 cells per well in 6 well plates in 1 ml of BBE media. Twenty four hours after plating, cells were transferred to MEM containing low serum for 24 hours (0.5% calf serum). Cells were treated with 16K hPRL or TGF-β1 for 16 h. The incubation was terminated by aspiration of the media, the addition of 200 μl of lysis buffer (0.1 M Tris, pH 8.1 and 0.5% Triton-X100), and shaken at 4°C for 20 minutes. The insoluble fraction was removed by centrifugation at 4°C for 10 minutes at 14,000 x g and the protein concentration of soluble fractions was determined by BCA assay kit (Pierce). Cell homogenates or conditioned media from BBE cells were resolved by SDS/PAGE (10%) and transferred to nitrocellulose membrane. The

membranes were stained with Ponceau Red for 1 min to visualize the protein to confirm the even transfer. The blots were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h and incubated with anti-bovine PAI-1 mouse monoclonal antibody at a 1:2,000 dilution for 2 hrs (Gibco, BRL, Gaithersburg, MD). The antigen-antibody complexes were detected with horse-radish peroxidase conjugated secondary antibody and the enhanced chemiluminescence system (Renaissance, NEN). The blots were exposed to reflection NEF films (NEN) to visualize the bands.

Inhibitor Studies

To nonspecifically inhibit serine/threonine kinases, cells were treated with 50 μM H7 (Sigma). To inhibit PKA activity, BBE cells were incubated with 10 μM of the specific inhibitor, H89 (Sigma). PKC activity was inhibited by treatment with 100 nM of the specific inhibitor Calphostin C (Calbiochem). BBE cells were treated with 16K hPRL alone or together with the inhibitors for 16 h. Treated cell lysates were subjected to PAI-1 Western blot analysis.

Transfection of BBE cells

Three different luciferase expression vectors containing 800 bp, 1500 bp and 6400 bp of human PAI-1 promoter regions were kindly provided by Dr. David Loskutoff from Scripp Clinic and Research Foundation. Briefly, human PAI-1 promoter fragments were isolated from a cosmid library. All fragments were subcloned into a p19LUC reporter construct [22] with an identical 3' end (EcoRI site at position +71) and different 5' ends. For P800LUC, the HindIII site at position -800 was used to subclone into the multiple cloning sites. For P1500LUC, the KpnI site at -1.5 kb was used for subcloning. For P6400LUC, the SmaI site at -6.4 kb was used. These constructs have been used to identify the response elements for glucocorticoid in human PAI-1 promoter in rat heptoma

cell line FTO2B and the response elements for TGF-β1 in human hepatoma cell line Hep3B.

$5X10^5$ BBE cells were plated in 12-well plates overnight in complete medium to allow cells to attach. The plated cells were then transfected by calcium phosphate methods with 1 μg of each of the three different vectors described above together with 0.1 μg of prLucCMV vector (Promega). BBE cells were transfected in DMEM H16 containing 0.5% CS overnight. Cells were washed three times with serum free media to remove transfection reagents and incubated in DMEM H16 containing 0.5% CS for 48 hrs. The transfected cells were then treated with 10 nM 16K hPRL or 80 pM of TGF-β1 for 4 hrs. The treated cells were then harvested and assayed for luciferase activity by Dual luciferase assay system (Promega) as described by the provider.

Adenovirus Infection Studies

The preparation of the virus vector (shown in Fig. 4A) was as described [23]. The titer of the virus used in these experiments ($5x10^{10}$ plaque forming unit (pfu)/ml) was determined by plaque titration on 293 cells. 1.5×10^5 BBE cells were plated in each well of a 24 well plate. The cells were infected for 90 min at 37°C with virus stock diluted in OptiMEM (Life Technologies, Inc.) to an moi (multiplicity of infection) of 500. The infection media was replaced with growth medium containing either no hormone, 10 nM 16K hPRL or 1 ng/ml TGF-β1 and incubated for 24 h. The treated cells were lysed in 100 μl of lysis buffer containing 100 mM potassium phosphate, pH 7.2, and 0.2% Triton X100. Extracts were assayed for β galactosidase activity by a chemiluminescence assay (Tropix, Inc., Bedford, MA). The β galatosidase activity was normalized the protein concentration of the cell lysate determine by the BCA assay (Pierce).

Apoptosis Assay

(a) DNA fragmentation assessment by ELISA: Endothelial cells were cultured as described above and, after stimulation, lysed for analysis with the Cellular DNA Fragmentation ELISA Kit (Boehringer Mannheim) according to the manufacturer's instructions. Three separate spectrophotometric measurements (A405/A490) were averaged, and the background value of the assay was subtracted from each of these averages. (b) Western Blotting: To detect processing of the apoptotic protease, caspase 1 which is also known as interleukin-1-β-converting enzyme (ICE), and to evaluate expression of Bax, Bak and Bcl-2 proteins, equal quantities of lysate were resolved by SDS/PAGE (15%) and transferred to Immobilon-P membranes (Millipore). Separate Western blots were performed using the following antibodies from Upstate Biotechnology: (1) an anti-human caspase 1 rabbit polyclonal antibody (1:1000 dilution) that recognizes the 45 kDa proenzyme and the 20 kDa subunit of active caspase 1; (2) an anti-human Bcl-2 mouse monoclonal antibody (1:500) that recognizes a 26 kDa protein ; (3) an anti-human Bax rabbit polyclonal antibody (1:500) that recognizes a 23 kDa protein ; and (4) an anti-human Bak rabbit polyclonal antibody (1:1000) that recognizes a 29 kDa protein . Western blots were incubated with the appropriate antibody and then washed in Tris-buffered saline containing 0.1% Tween 20. Antigen-antibody complexes were detected with horseradish peroxidase-coupled secondary antibodies and the enhanced chemiluminescence system (Renaissance, NEN). Finally, the blots were exposed to reflection NEF films (NEN). Western blots were "stripped" for reprobing with a buffer containing 0.2 M glycine (pH 2.5) followed by three washes in PBS. Autoradiographs of anti-Bcl-2 and Bax western blots were analyzed by densitometry and quantified using Intelligent Quantifier (Bio Image) software.

Statistical Analysis

All data are presented as the mean±SD. 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

Inhibition of bFGF-induced BBE Cell Proliferation by 16K hPRL and TGF-β1

To compare the effect of 16K hPRL and TGF-β1 on BBE cell proliferation, subconfluent, serum-starved cultures were incubated for 24 hrs with or without 50 pM bFGF and varying concentrations of either TGF-β1 or 16K hPRL. The rate of cell division was estimated by measuring the level of ^{3}H-thymidine incorporation into DNA during the final 4 hrs of the 24 hr treatment. Basic FGF stimulated the incorporation of ^{3}H-thymidine two fold over that observed in the untreated, basal wells. Both TGF-β1 and 16K PRL inhibited bFGF stimulated ^{3}H-thymidine incorporation in a dose dependent manner (Fig. 1A and 1B). Treatment with 10 nM 16K hPRL blocked 61% of the bFGF-induced stimulation, while 25 pM TGF-β1 inhibited the response by 82%. We also asked if treatment with 16K hPRL or TGF-β1 would inhibit the rate of ^{3}H-thymidine incorporation into BBE cells grown under basal conditions, 0.5% CS. Treatment with TGF-β1 inhibited the basal rate of ^{3}H-thymidine incorporation in a dose dependent fashion (Fig. 1D), while 16K hPRL treatment had no effect (Fig 1C). Treatment with 25 pM TGF-β1 suppressed basal growth by 64%.

Inhibition of VEGF-stimulated MAPK Activity by 16K hPRL and TGF-β1

The MAPK signaling cascade was shown to be activated in BBE cells by the angiogenic factors, VEGF and bFGF [13]. Treatment with 16K hPRL was shown to block the activation of MAPK by both factors. We compared the effect of 16K hPRL and TGF-β1 on activation of MAPK by VEGF by evaluating the ability of immunoprecipitated

MAPK (Erk-1) to phosphorylate myelin basic protein (MBP). Five min after addition of 2 nM VEGF the activity of MAPK was stimulated 3-fold (Fig. 2A and B). Incubation of cells with either 2 nM 16K hPRL or 80 pM TGF-β1 greatly reduced the stimulatory effect of VEGF on MAPK activity by 73% and 82%, respectively. In the absence of treatment with VEGF, the addition of 2 nM 16K hPRL or 80 pM TGF-β1 had no significant effect on MAPK activity (Fig. 2A and B).

Role of Serine/Threonine Kinases in the Stimulation of PAI-1 by 16K hPRL and TGF-β1

Both 16K hPRL and TGF-β1 are potent stimulators of PAI-1 production in endothelial cells. To compare the effect of 16K hPRL and TGF-β1 on PAI-1 production, cell associated and secretory fractions of treated BBE cells were analyzed by Western blot using a bovine PAI-1 monoclonal antibody. Both 16K hPRL and TGF-β1 stimulated the production of PAI-1 in the secreted and cell associated fractions (Fig. 3A).

H7, a non-specific serine/threonine kinase inhibitor, was shown to inhibit the TGF-β1-induced stimulation of PAI-1 in endothelial cells [24]. We asked if H7 also blocked the stimulatory effect of 16K hPRL on PAI-1 production. Treatment with 50 μM H7 inhibited both the TGF-β1 and 16K hPRL-induced increase in PAI-1 protein levels (Fig. 3B). H7 alone had no effect on basal PAI-1 expression. These results suggest that, like TGF-β1, 16K hPRL-induced stimulation of PAI-1 is mediated through a serine/threonine kinase dependent signaling pathway.

Since PKA and PKC are the two major serine/threonine protein kinases involved in hormone signaling, we asked if 16K hPRL or TGF-β1 stimulated PAI-1 production via activation of either kinase. H89, a specific inhibitor of PKA, had no effect on stimulation of PAI-1 by 10 nM 16K hPRL (Fig 3B). Similarly, treatment with the specific PKC inhibitor, Calphostin C (100 nM) had no effect on PAI-1 levels (Fig 3B). 16K hPRL-induced PAI-1 production was also unaffected by the down regulation of PKC by long

term treatment with TPA (data not shown). Therefore, neither PKA nor PKC appeared to mediate the blockade by H-7 of the action of 16K hPRL and TGF-β1 on PAI-1 production.

Promoter Elements Mediating the Stimulation of PAI-1 Expression by 16K hPRL and TGF-β1

Because of the similarities in the signaling pathways for the actions of 16K hPRL and TGF-β1, we asked whether the stimulation of the expression of the PAI-1 gene by the factors was mediated via the same response elements. The major TGF-β1 response elements have been identified within the first 800 bp of the 5' flanking region of the human PAI-1 gene in several different cell types [25]. BBE cells were transfected with a luciferase reporter gene driven by -800 bp, -1500 bp and -6400 bp region of the human PAI-1 promoter (Fig.4A). A prLucCMV vector, which contains renilla (sea pansy) luciferase gene driven by CMV promoter, was cotransfected to serve as an internal transfection control. In all experiments, TGF-β1 stimulated reporter activity approximately 2-fold. Although the level of stimulation of the reporter gene was low, the values, corrected relative to the transfection control, were statistically different than controls. Treatment with 16K hPRL had no effect with either the -800 bp or -1500 bp reporter genes, however, with the -6400 bp reporter gene 16K hPRL significantly stimulated luciferase activity (Fig. 4B). Similar results were obtained in another experiment. These results were consistent with the TGF-β1 response element being located in the first 800 bp of the 5' flanking region, confirming earlier reports [25]. The 16K hPRL response element appeared to be located within -1500 and -6400 bp of the human PAI-1 gene.

Because of the poor level of expression in BBE cells using a classical transfection approach, Adenovirus (Ad) vectors were used to obtain high transfection efficiencies. Ad mediated gene transfer has been used to study the TGF-β1 response of the human PAI-1 promoter in intact arterial endothelium [23]. We used an Ad vector containing the -800 bp human PAI-1 promoter driving β galactosidase reporter gene (Fig.5A) to compare the

effects of 16K hPRL and TGF-β1 on this region of the promoter. BBE cells infected with AdIAPβgal were treated with 5 or 10 nM 16K hPRL or 80 pM of TGF-β1. TGF-β1 significantly stimulated reporter gene activity up to 4-fold. However, treatment with either 5 or 10 nM 16K hPRL had no effect on reporter gene expression (Fig. 5B). Similar data were obtained in another experiment. These data clearly confirmed the findings that the response element for TGF-β1, but not 16K hPRL, was located within the -800 bp fragment.

Stimulation of Apoptosis by 16K hPRL but not TGF-β1

Since, we had previously shown that 16K hPRL stimulated apoptosis, we compared the effects of 16K hPRL and TGF-β1 on programmed cell death of BBE cells. Fragmentation of DNA into mononucleosomes and oligonucleosomes was quantitated with an ELISA specific for cytosolic histone-bound DNA. As shown Fig. 6A, DNA fragmentation was increased 5-fold in BBE cells after serum deprivation. Treatment with 16K hPRL increased DNA fragmentation in a dose dependent manner compared to 0.5% CS. 10 nM 16K hPRL stimulated the cytosolic nucleosome level 25-fold compared to the control. However, treatment with 80 pM TGF-β1, a concentration which supramaximally inhibited the mitogenic effect of bFGF, had no significant effect on cytosolic nucleosome levels compared to 0.5% CS. As a control for the small amounts of endotoxin present in the recombinant 16K hPRL preparations, addition of 5 times the amount of endotoxin present in a 10 nM 16K hPRL solution had no significant effect on DNA fragmentation compared to 0.5% CS (Fig. 6A). To eliminate the possibility that the stimulation of apoptosis by 16K hPRL was indirect via stimulation of PAI-1 production, we tested the effect of PAI-1 alone. Human PAI-1 (50 μM) had no effect on DNA fragmentation (Fig. 6A).

To characterize the effect of both 16K hPRL and TGF-β1 on the cell death pathway, we performed Western blot analysis for activation of caspase 1. As illustrated in Fig. 6B,

an immunoreactive band of 45 kDa corresponding to the proform of caspase 1 was observed with all treatments. However, the cleaved and active 20 kDa subunit of caspase 1 was markedly increased in the presence of 10 nM 16K hPRL. Treatment with 80 pM TGF-β1, endotoxin and human PAI-1 had no effect on caspase 1 cleavage and activation (Fig. 6B).

Another control point for the cell death pathway is the level of expression of anti- and pro-apoptotic proteins, Bcl-2 and Bax, respectively [26]. The level of Bcl-2 was stimulated by a decrease in the level of serum from 10% to 0.5%, however, neither 16K hPRL nor TGF-β1 had any effect relative to the 0.5% control (Fig. 6C). The expression of Bax remained unchanged under all conditions. The ratio of the Bcl-2/Bax levels was increased 8-fold by serum deprivation, but neither 16K hPRL nor TGF-β1 had a significant effect on the ratio (Fig. 6D). The expression of Bak, another Bax family member, was increased by both 16K hPRL and TGF-β1 compared to 10% and 0.5% CS conditions (Fig. 6C).

DISCUSSION

As with many biological responses, the regulation of angiogenesis involves considerable redundancy in factors which stimulate or inhibit the process. An important question concerning the regulation of capillary endothelial cell function is whether the actions of multiple angiogenic and antiangiogenic factors are mediated via convergent or parallel signaling pathways. The main purpose of this study was to compare the actions and signaling pathways of the antiangiogenic factors, 16K hPRL and TGF-β1 in BBE cells. We studied the effects of the factors on BBE cell proliferation, programmed cell death and regulation of PAI-1 production. The participation of the MAPK signaling pathway in BBE cell proliferation was studied, as was the regulation of the caspase proteolysis pathway in apoptosis. Finally, we determined if activation of a serine/threonine kinase was necessary for stimulation of PAI-1 production, and if the

response elements regulating PAI-1 gene expression were the same for 16K hPRL and TGF-β1.

Both 16K hPRL and TGF-β1 inhibited bFGF-stimulated BBE cell proliferation in a dose dependent fashion. In a parallel fashion, both 16K hPRL and TGF-β1 inhibited the stimulation of MAPK activity by VEGF. We previously showed that 16K hPRL also inhibited the bFGF-induced activation of MAPK [13]. The MAPK cascade is activated by the binding of numerous growth factors and cytokines to their receptors [27-29]. Furthermore, the MAPK cascade has been shown to be important for growth factor stimulated cell proliferation in many different cell types including endothelial cells [13]. The blockade of the MAPK cascade blocks the proliferative action of VEGF [30]. These findings do not eliminate the participation of other signaling pathways in mediating the anti-proliferative actions of 16K hPRL and TGF-β1, but they do support the idea that the factors inhibit mitogen-driven cell proliferation by blocking activation of MAPK. We have shown that 16K hPRL blocks MAPK activation by blocking the activation of Ras, an upstream signaling event [14]. Future studies will address where TGF-β1 acts to block the activation of MAPK.

An interesting finding was that treatment with TGF-β1, but not 16K hPRL, inhibited proliferation of BBE cells cultured in 0.5% CS. One hypothesis to explain why TGF-β1 but not 16K hPRL inhibits basal cell proliferation is that basal growth is regulated by an autocrine factor, or a serum growth factor whose action is only blocked by TGF-β1. This would suggest that TGF-β1 activates an additional pathway to suppress basal cell growth. Consistent with this hypothesis TGF-β1 treatment had no effect on basal MAPK activity in BBE cells, suggesting that its inhibitory effect on basal cell proliferation is downstream from or does not involve MAPK. One caveat in these studies was that the concentration of 16K hPRL used in our experiments may not have been high enough to inhibit basal cell proliferation. The highest concentration of 16K hPRL used (10 nM) caused only a 61% inhibition of the bFGF-induced cell proliferation, while 10 pM TGF-β1,

which inhibited about half of the bFGF stimulation, also did not have a significant effect on basal cell growth. We did not test higher concentrations of 16K hPRL, since the increase in the amounts of endotoxin in higher concentrations of recombinant 16K hPRL could begin to contribute to the antiangiogenic effects.

Both 16K hPRL and TGF-β1 increase PAI-1 protein levels in the cell associated and secreted fractions of BBE cells. The initial events in activation of the TGF-β receptor complex is binding to TβR-II subunit which in turn activates the serine/threonine kinase activity of the TβR-I subunit [31]. It was previously shown that the stimulation of PAI-1 production by TGF-β1 could be blocked with the non-specific serine/threonine kinase inhibitor H-7 [24]. We confirmed this observation, and in addition showed that treatment with H-7 blocked the stimulatory effect of 16K hPRL. The inhibitory effect of H-7 was not mediated via PKA and PKC, two serine/threonine kinases involved in hormone signaling, since specific pharmacological inhibition of these kinases had no effect on the response to either TGF-β1 or 16K hPRL. These observations were consistent with 16K hPRL and TGF-β1 increasing PAI-1 expression via the same transactivating factor, a hypothesis which we then tested.

Both 16K hPRL and TGF-β1 stimulate PAI-1 production at the transcriptional level [11, 32]. The response elements for the TGF-β1 response have been localized within the first 800 bp of the 5' flanking sequence of the human PAI-1 gene [25]. Multiple TGF-β1 response elements have been identified in the human PAI-1 promoter [25, 33, 34]. We asked whether the response element for the 16K hPRL response was within the same region of the promoter. In the first series of experiments luciferase reporter genes driven by overlapping regions of the human PAI-1 5' flanking region were transfected in BBE cells. Although the levels of expression were low, a statistically significant stimulation of -800 bp luciferase reporter gene was observed with TGF-β1, while treatment with 16K hPRL had no effect. A small but statistically significant stimulation was observed with 16K hPRL of the -6400 bp reporter gene. To extend and confirm these findings we

infected BBE cells with an adenovirus vector which contained the -800 bp of human PAI-1 promoter upstream of a β galactosidase reporter gene [23]. Confirming the transfection studies, TGF-β1 treatment resulted in a 4-fold increase in β gal expression, while 16K hPRL, at two different concentrations, had no effect. Although, TGF-β1 caused greater than a 20-fold increase in reporter gene expression in CPAE cells infected with the Ad vector [23], only a 4-fold stimulation was observed in BBE cells. The low level of expression with the Ad vector is consistent with the transfection results, and reflects the difficulties in performing transfection studies in primary cell cultures. These data clearly show that the response elements for 16K hPRL and TGF-β1 are located in different regions of the human PAI-1 promoter, and they do not support the idea that the same transactivating factor is involved in mediating the actions of the factors.

Apoptosis has been suggested to play an important role in the regulation of angiogenesis [35-38]. We first asked whether both TGF-β1 and 16K hPRL were capable of activating apoptosis in BBE cells. 16K hPRL increased DNA fragmentation in BBE cells 5-fold above that seen with serum deprivation, but to our surprise we found no effect of TGF-β1. TGF-β1 was reported to induce programmed cell death in several different cell types, including: hepatocytes [39]; uterine epithelial cells [40]; and glomerular endothelial cells [41], although not in all cell types studied such as cortical neurons [42], or bone marrow (BM) progenitor cells and Lin-BM cells [43]. One explanation is that serum deprivation increases the endogenous production of TGF-β1, masking any further effect of the addition of TGF-β1. Therefore, it appears that 16K hPRL increases DNA fragmentation via a signaling pathway different from those activated by TGF-β1. Reinforcing this conclusion, we showed that while 16K hPRL treatment resulted in the cleavage and activation of caspase 1 [6], TGF-β1 had no effect on caspase 1. A recent study showed that caspase 2, but not caspase 1, played a crucial role in TGF-β1-induced apoptosis in FaO rat hepatoma cell line [44]; however, caspase 3 appears to be activated by both TGF-β1 and 16K hPRL [6, 45]. As previously described the Bcl 2/Bax ratio

appeared to be an important regulatory element of programmed cell death induced by serum deprivation [46, 47], however, the induction of apoptosis by 16K hPRL was not mediated via this pathway.

PAI-1 has been shown to inhibit endothelial cell tube formation [16] and migration [17]. It also inhibits smooth muscle cell migration by blocking integrin αvβ3 binding to vitronectin [48]. Furthermore, the blockade of αvβ3 by antibodies induced apoptosis in endothelial cells [49]. We demonstrated that PAI-1 is not involved in the regulation of apoptosis in BBE cells, since neither the addition of PAI-1 nor TGF-β1, which increases PAI-1 levels without affecting apoptosis, had any effect. Consistent with our observations, Chen et. al. (1997) established that PAI-1 gene expression and programmed cell death are induced by TGF-β1 via separate pathways in human hepatoma cell line Hep3B.

In conclusion, *in vitro* 16K hPRL and TGF-β1 inhibit mitogen-induced proliferation and stimulate PAI-1 expression in BBE cells via respectively, convergent and parallel signaling pathways. 16K hPRL and TGF-β1 appear to inhibit the proliferative effects of VEGF and bFGF by preventing MAPK activation. Both factors stimulated the production of PAI-1 through a serine/threonine kinase dependent pathway, however, the stimulation of PAI-1 expression was mediated via different response elements located in the 5' flanking region of the human PAI-1 gene. Most significantly only 16K hPRL stimulated DNA fragmentation via activation of caspace 1, demonstrating a clear difference in the signaling pathways involved in their actions. These findings provide clear evidence in highly differentiated capillary endothelial cells that considerable pleiotropy exists in the signaling pathways mediating antiangiogenic factors.

FIGURE LEGENDS

Fig. 4-1. Effect of TGF-ß1 or 16K hPRL on bFGF stimulated or basal growth of BBE cells.

BBE cultures were serum-starved for 24 hrs prior to addition of 16K PRL (A, B) or TGF-β1 (C, D) in the presence (A, C) or absence (B, D) of 50 pM bFGF. 3H-thymidine was added 20 hrs after addition of 16K PRL, TGF-ß1 and bFGF. Cells were harvested for liquid scintillation counting 4 hrs later. Data are represented as mean± SD of triplicate wells for a representative experiment.

Fig. 4-2. Inhibition of VEGF-induced MAPK Activation by 16K hPRL and TGF-β1. Quiescent BBE cells were left untreated (control) or stimulated for 5 min with 2 nM VEGF and 2 nM 16K hPRL, 2 nM VEGF, 2 nM 16K hPRL, 2 nM VEGF and 80 pM TGF-β1, or 80 pM TGF-β1 as indicated. (A) detergent lysates were immunoprecipitated with anti-MAPK antibody, and kinase activity was assayed against MBP in the immune complexes. Reaction mixtures were separated by SDS/PAGE (15% gel) and subjected to autoradiography. (B) ^{32}P in MBP was quantitated by phosphoimaging. Values are the average of two experiments. Data were corrected for background radioactivity in the MAPK immune complexes in the absence of MBP. Corrected incorporation (VEGF) was set to 100%.

Fig. 4-3. Analysis of PAI-1 Production

(A). Equal amounts of conditioned media (secretory) or cell lysates (cell associated) from BBE cells treated with 10 nM or 20 nM of human 16K hPRL (16K), or 1 ng/ml of TGF-β1 were separated by SDS/PAGE (10% gel) and transferred to nitrocellulose membrane and PAI-1 protein expression was detected using a monoclonal anti-bovine PAI-1 antibody. (B). To compare the signaling pathway used by 16K hPRL and TGF-β1, BBE cells were

cotreated as in (A) with 50 μM H7, 10 μM H89 or 100 nM Calphostin C for 16 h. Cell lysates collected from the treated cells were analyzed by PAI-1 Western blot as in (A).

Figure 4-4. Effect of 16K hPRL and TGF-β1 on Expression of Transfected Human PAI-1 Reporter Constructs

(A). PAI-1 reporter vectors used in calcium phosphate transfection experiments. Luciferase reporter p19LUC (van Zonneveld, 1988) containing -800 bp (P800LUC), -1500 bp (P1500LUC) or -6400 bp (P6400LUC) of overlapping human PAI-1 promoter in the multiple cloning sites were used in our studies. For P800LUC, a -800 bp HindIII-EcoRI fragment was subcloned into the multiple cloning site of p19LUC. For P1500LUC, a -1500 bp KpnI-EcoRI fragment was used. For P6400LUC, a -6400 bp SmaI-EcoRI fragment was used. pBR322 amp, amplification site of pBR322; PA, SV40 polyadenylylation site; SV40 Splice, splice site of SV40; MCS, multiple cloning site. (B). BBE cells were transfected with P800LUC, P1500LUC or P6400LUC plasmid using a calcium phosphate method and treated with 10 nM 16K hPRL or 80 pM TGF-β1 for 16 h. Cell lysates were assayed for luciferase activity and values were corrected for the enzyme activity generated by the co-transfected prLucCMV plasmid. Values are mean±SD of 4 samples.

Fig. 4-5. Effect of 16K hPRL and TGF-β1 on Expression of an -800 bp PAI-1 Adenovirus Expression Vector

(A). Structure of AdIAPβGal: Vector was made by homologous recombination between shuttle vector plasmid and the large fragment of Ad5-dl327 mutant. This recombination results in insertion of the -800 bp human PAI-1 promoter at the site of the E1 gene deletion. Structural features of the vector and expression cassette are indicated: ITR, inverted terminal repeat; SV40 pA, polyadenylation signal from SV 40; nLac Z, nuclear targeted E. Coli Lac Z gene; PAI800,

-800 bp of the promoter region of human PAI-1 gene inserted 3' to 5' relative to the adenovirus genome. Direction of transcription is indicated by the arrow. The location of the TATA box and of putative binding sequences for the transcription factors AP-1 and NF-1 in PAI-1 promoter are shown. (B). BBE cells were infected with AdIAP800βGal. The infected cells were treated with 5 or 10 nM of 16K hPRL or 80 pM of TGF-β1 for 16 h. Cell lysates from treated cells were assayed for β galactosidase activity and the enzyme activities were corrected to the amount of protein in the samples. Values are mean±SD of 4 samples.

Fig. 4-6. Effects of 16K hPRL and TGF-β1 on Apoptosis

Quiescent BBE cells were left untreated (10% CS), serum deprived (0.5% CS) or stimulated in 0.5% CS with 1 nM or 10 nM 16K hPRL, 0.5 IU Endotoxin, 80 pM TGF-β1 or 50 μM of PAI-1 as indicated. (A) bar graph showing the relative amount of soluble nucleosomes determined by ELISA. Each bar represents the mean±SD for 4 independent experiments. Basal DNA fragmentation of quiescent cells was set to 100%. *$p<0.01$ and **$p<0.001$ vs. serum deprived cells (0.5%). (B) Processing of apoptotic protease caspase 1 (ICE). Equal amounts of protein (50 μg) from each sample were subjected to SDS/PAGE (15%) and immunoblotted with a rabbit polyclonal antibody raised against a synthetic peptide of the p20 subunit of human ICE. Arrows denote inactive proform of caspase 1 (p45) and its active subunit (p20). Note that the p20 subunit of active caspase 1 was only observed in presence of 10 nM 16K hPRL. (C) Expression of Bcl-2/Bax/Bak proteins. Equal amounts of protein (50 μg) from each sample were subjected to SDS/PAGE (15% gels) and immunoblotted with the corresponding antibody. (D) Ratio of the relative expression of Bcl-2 and Bax. Data were obtained by scanning autoradiographs in (C) and analyzing by densitometry. Each bar represents the average value of two separate experiments.

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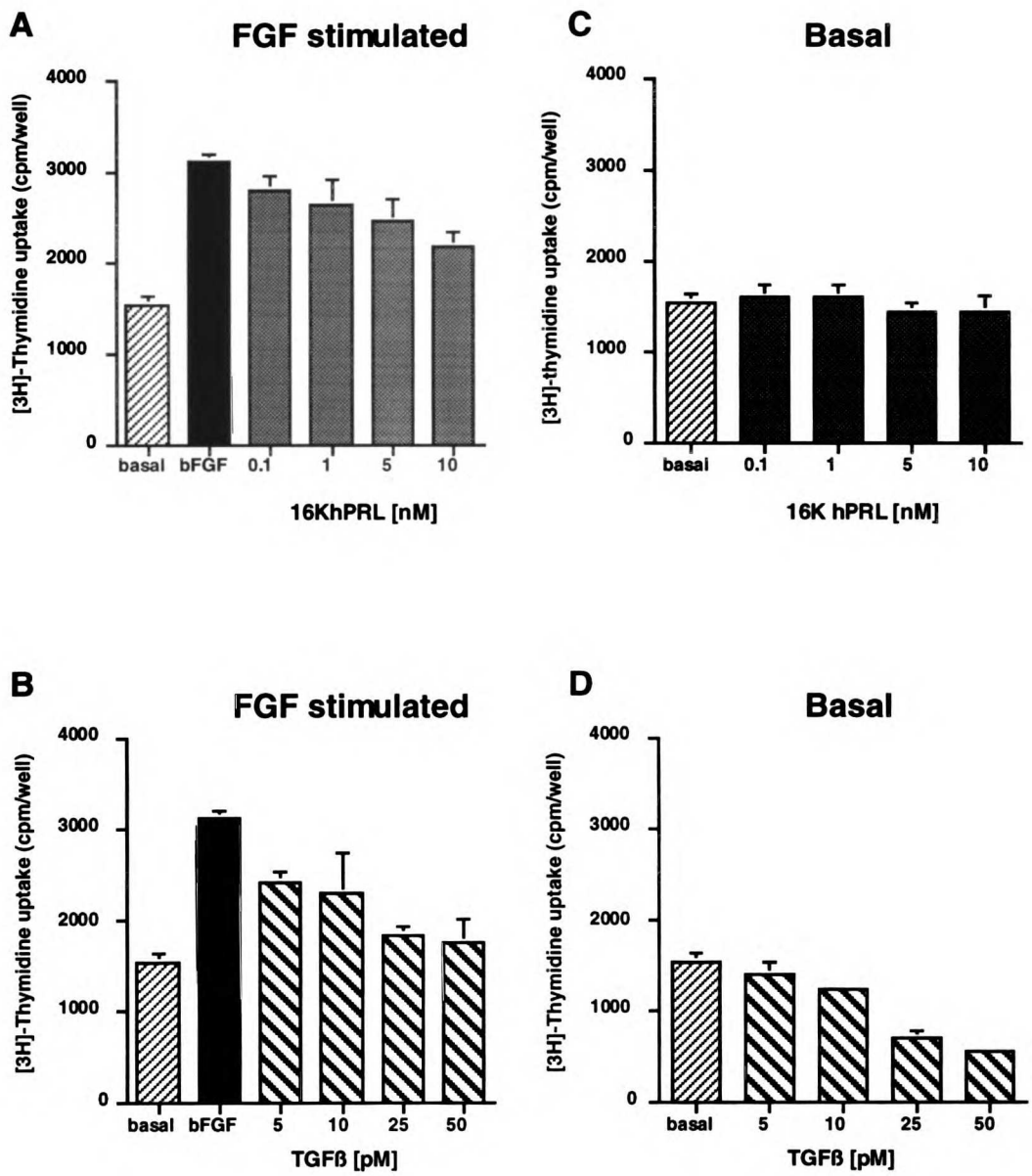
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[3H]-Thymidine uptake (cpm/well)
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2000
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16KhPRL [nM]
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[3H]-thymidine uptake (cpm/well)
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FGF stimulated
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TGFß [pM]
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[3H]-Thymidine uptake (cpm/well)
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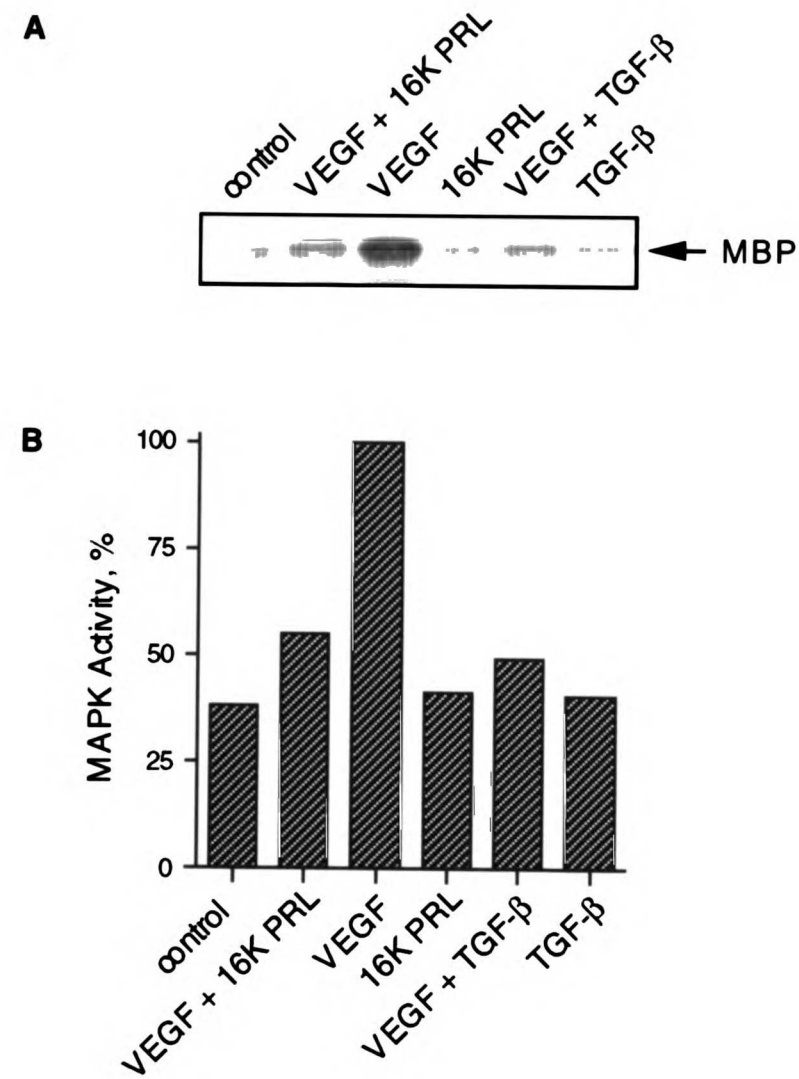
FIGURE 4-1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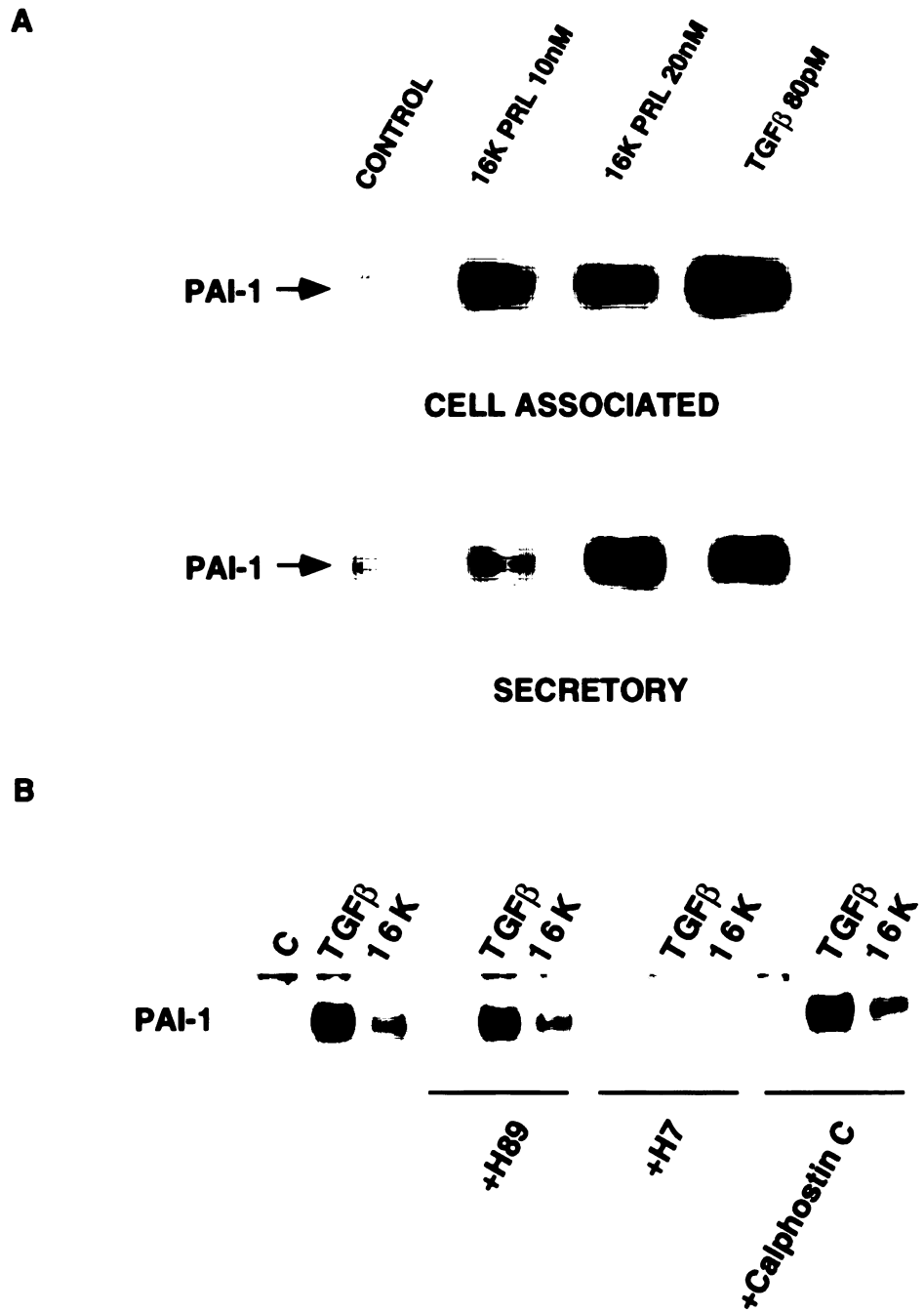
VEGF + TGF-β
TGF-β

FIGURE 4-2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PAI-1
CELL ASSOCIATED
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C
TGFβ
16K
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16K
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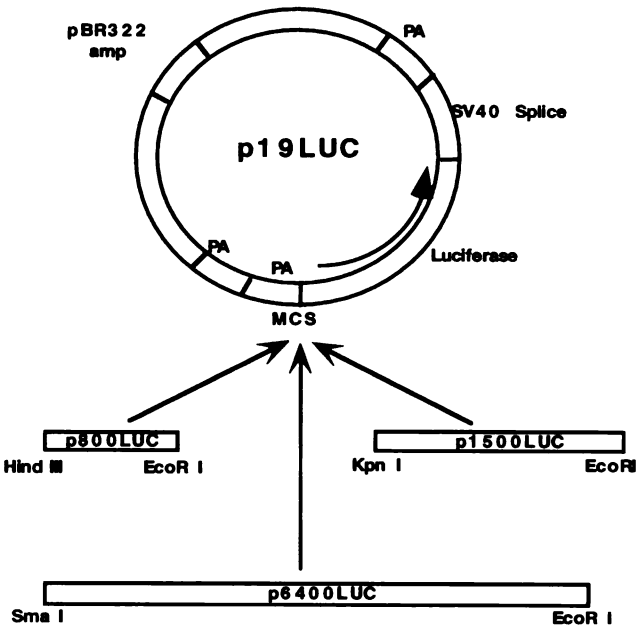
16K
TGFβ
16K
PAI-1
+H89
+H7
+Calphostin C

FIGURE 4-3

A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EcoR I

B

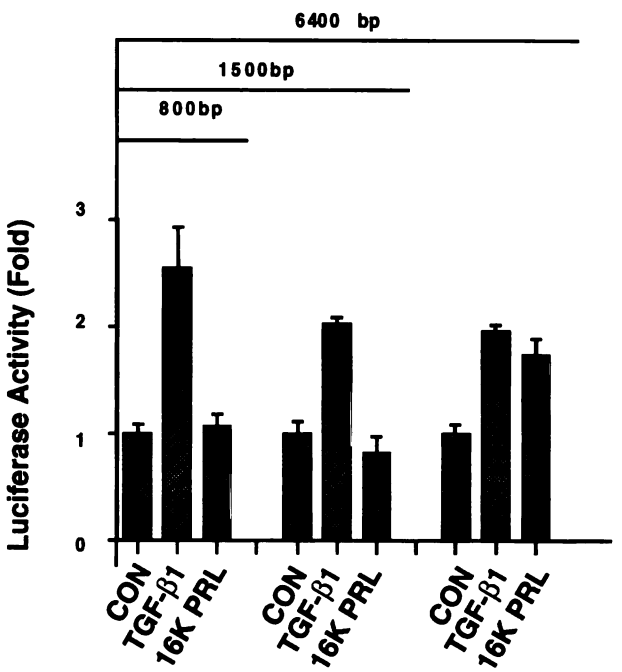
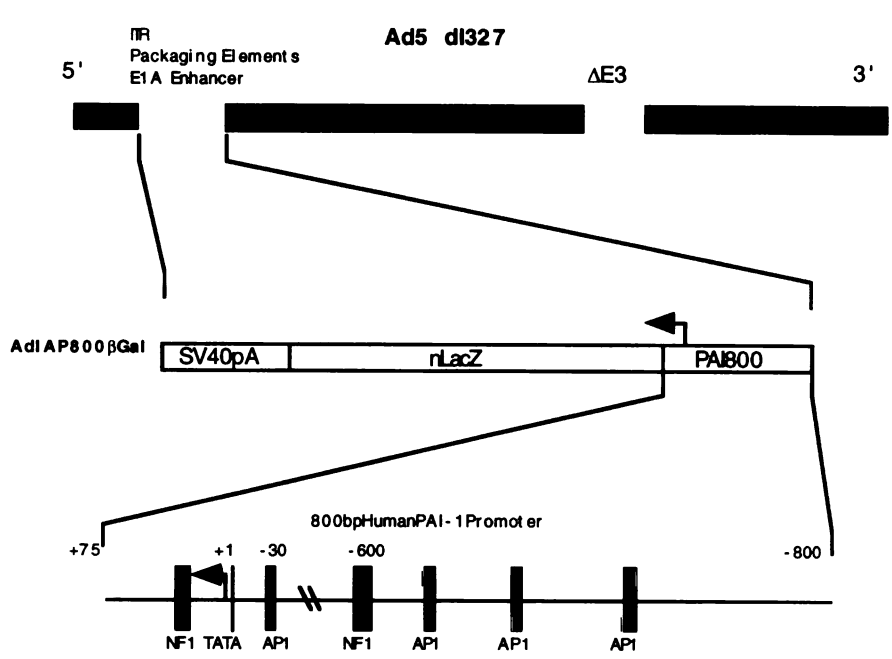

6400 bp
1500bp
800bp
Luciferase Activity (Fold)
0
1
2
3
CON
TGF-β1
16K PRL
CON
TGF-β1
16K PRL
CON
TGF-β1
16K PRL

FIGURE 4-4

A


ITR
Packaging Elements
E1A Enhancer
Ad5 dl327
5'
ΔE3
3'
AdlAP800βGal
SV40pA
nLacZ
PAI800
800bpHumanPAI-1Promoter
+75
+1
-30
-600
-800
NF1
TATA
AP1
NF1
AP1
AP1
AP1

B

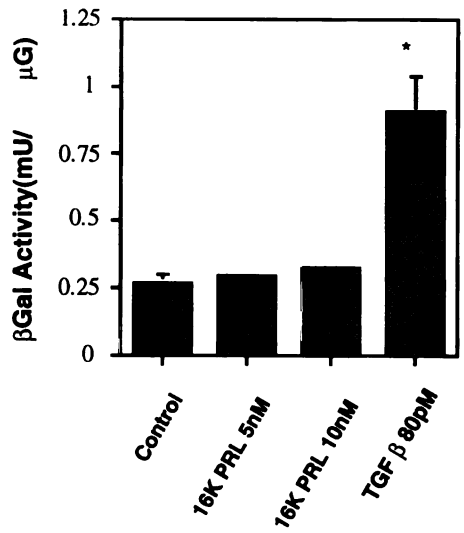

βGal Activity(mU/ μG)
1.25
1
0.75
0.5
0.25
0
*
Control
16K PRL 5nM
16K PRL 10nM
TGF β 80pM

FIGURE 4-5

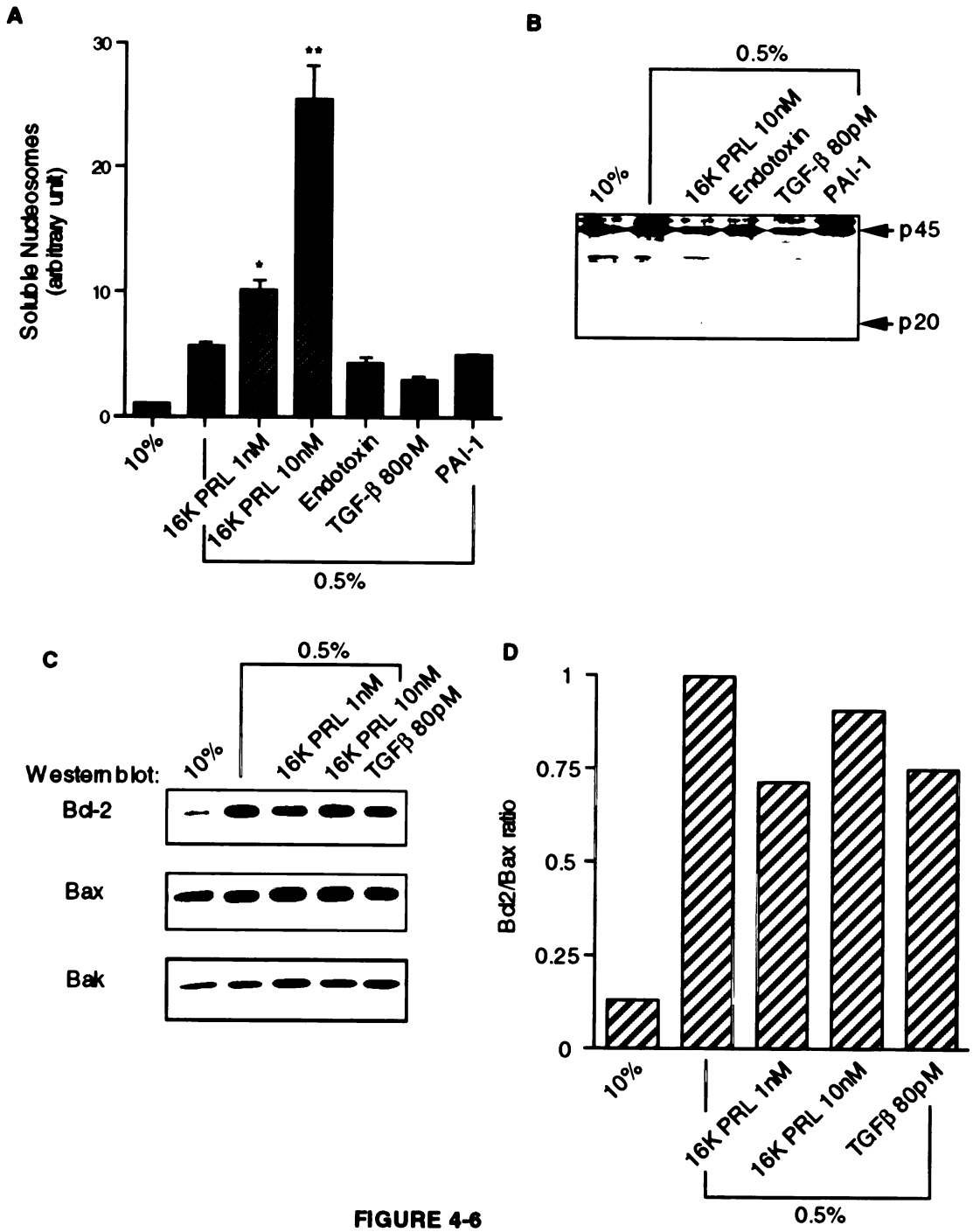

A
Soluble Nucleosomes (arbitrary unit)
30
20
10
0
*
**
10%
16K PRL 1nM
16K PRL 10nM
Endotoxin
TGF-β 80pM
PAI-1
0.5%
B
0.5%
10%
16K PRL 10nM
Endotoxin
TGF-β 80pM
PAI-1
p45
p20
C
0.5%
10%
16K PRL 1nM
16K PRL 10nM
TGFβ 80pM
Western blot:
Bcl-2
Bax
Bak
D
Bcl2/Bax ratio
1
0.75
0.5
0.25
0
10%
16K PRL 1nM
16K PRL 10nM
TGFβ 80pM
0.5%

FIGURE 4-6

Chapter 5. 16K hPRL Family Members Stimulate PAI-1 Production in BBE Cells

Introduction

In chapter 2, we showed that 16K PRL stimulates PAI-1 production in BBE cells. In this chapter, we invetigate if 16K PRL family members also regulate PAI-1 production. This chapter includes data from collaborative studies between Drs. Richard Weiner and Joseph Martial's laboratories. This publication shows that human PRL family members including: human prolactin (hPRL), growth hormone (hGH), growth hormone varient (hGHV) and placental lactogen (hPL) stimulate angiogenesis, while their 16 kDa N-terminal fragments are antiangiogenic factors. The 16K fragments all stimulated PAI-1 production while the full length molecules from which they were derived have no effect. The experiments described in chapter 6 show that all the 16K fragments utilize a serine/threonine kinase dependent pathway to stimulate PAI-1. Taken together with their similar biological activity, the data are consistent with the 16K fragments acting at the same receptor or multiple receptors which signal in a similar fashion.

MANUSCRIPT 3

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Abbreviations : hPRL, human prolactin ; 16K hPRL, 16 kDa N-terminal fragment of hPRL ; hGH, human growth hormone ; 16K hGH, 16 kDa N-terminal fragment of hGH ; hPL, human placental lactogen ; 16K hPL, 16 kDa N-terminal fragment of hPL ; hGH-v ; human placental variant of growth hormone ; 16K hGH-V, 16 kDa N-terminal fragment of hGH-V ; MAPK , mitogen-activated protein kinase ; PAI-1, type 1 plasminogen activator inhibitor ; PLF, proliferin ; PRP, proliferin-related-peptide ; BBCE cell, bovine brain capillary endothelial cell ; DMEM, low glucose Dulbecco's modified Eagle's medium ; bFGF, basic fibroblast growth factor ; BSA, bovine serum albumin ; MBP, myelin basic protein ; VEGF, vascular endothelial growth factor.

ABSTRACT

Angiogenesis, the process of development of a new microvasculature, is regulated by a balance of positive and negative factors. We show both *in vivo* and *in vitro* that the members of the human prolactin/growth hormone family i.e. human prolactin (hPRL), human growth hormone (hGH), human placental lactogen (hPL), human growth hormone variant (hGH-V) are angiogenic, while the respective 16 kDa N-terminal fragments are antiangiogenic. The opposing actions are regulated in part via activation or inhibition of mitogen-activated protein kinase (MAPK) signaling pathway. In addition the N-terminal fragments stimulate expression of type 1 plasminogen activator inhibitor (PAI-1), while the intact molecules have no effect, an observation consistent with the fragments and intact

molecules acting via separate receptors. The concept that a single molecule encodes both angiogenic and antiangiogenic peptides represents a new model for regulating the balance of positive and negative factors controlling angiogenesis. This hypothesis has potential physiological importance for the control of the vascular connection between the fetal and maternal circulations in the placenta where hPRL, hPL and GH-V are expressed.

INTRODUCTION

Prolactin (PRL), growth hormone (GH), and placental lactogen (PL) are homologous protein hormones believed to have arisen from a common ancestral gene (1). PRL participates in the regulation of reproduction, osmoregulation, and immunomodulation (2-3), while GH is involved in regulating growth and morphogenesis (4). Human (h) GHs, unlike other mammalian GHs, bind to the PRL receptor and thus display PRL-like activity; however, hPRL does not bind to the hGH receptor (5).

PRL and GH are produced mainly by the anterior pituitary in all vertebrates. PRL is also expressed in lymphocytes and in the decidua (6). The human placenta expresses two structural homologs of hGH, hPL and a variant of hGH, hGH-V (7). hGH-V rather than pituitary hGH is believed to regulate maternal metabolism during the second half of pregnancy. hPL is somatotropic in fetal tissues and contributes to stimulating mammary cell proliferation (8). Rodent placentas express and secrete several proteins whose biological activities are more PRL-like than GH-like: these include proliferin (PLF) and a proliferin-related peptide (PRP) (9).

Members of the PRL/GH family and derived peptides have been reported to both stimulate and inhibit angiogenesis. PLF expressed during the first half of pregnancy in the mouse is angiogenic, while PRP expressed later in gestation is antiangiogenic. These findings suggest that PLF and PRP might play a role in initiating and stopping placental neovascularization (9). Human GH is reported to be angiogenic *in vitro* (10) while both bovine and chicken GH were shown to be angiogenic *in vivo* (11). We have shown that

the 16 kDa N-terminal fragments of rat PRL (16K rPRL) and human PRL (16K hPRL) are antiangiogenic both *in vitro* (12) and *in vivo* (13). Rat PRL is cleaved by cathepsin D (14) to yield a 16 kDa N-terminal fragment and a 7 kDa C-terminal fragment linked by a disulfide bridge (cleaved PRL). After reduction, the free N-terminal fragment is released (15). Cleaved PRL has been observed in mouse, rat and human serum (16), while free 14 kDa and 16 kDa PRL appear to be secreted in the hypothalamo-neurohypophyseal system of the rat (17). Human GH is cleaved by plasmin, thrombin and subtilisin, yielding similar fragments (18). Human 16K PRL and 16K hGH were observed in serum under non-reducing conditions(19) and the free 14.5 kDa hPRL fragment was observed in amniotic fluid and in maternal serum (20).

We now show that the members of the human PRL/GH family, i.e. hPRL, hPL, hGH, and hGH-V, stimulate vessel formation, while their 16 kDa N-terminal fragments are antiangiogenic both *in vivo* and *in vitro*. We hypothesize that the intact and N-terminal fragments of the PRL/GH family members, through their opposite actions, are important physiological modulators of neovascularization.

MATERIALS AND METHODS

Production of Recombinant Proteins

The cDNAs encoding hPRL, hPL, hGH, or hGH-V minus the corresponding signal peptide were inserted into the pT7L expression vector (21). An ATG was genetically engineered 5' to the first codon of each cDNA. hPRL and hGH were previously cloned in our laboratory (22,23). hGH-V was cloned by screening a placental cDNA library (Clontech). hPL was cloned by RT-PCR performed on RNA extracted from a primary culture of human syncytiotrophoblast cells. The intact proteins were produced and purified essentially as described (24). In brief, after induction of protein expression by treatment with IPTG, the cells (E. coli BL21 DE3) were disrupted and the inclusion bodies were isolated. After washing, the inclusion bodies were solubilized in denaturation buffer

(8 M urea, 20 mM ethanolamine pH 10, 1% 2-mercaptoethanol, 0.5 mM PMSF) for 10 min at 55° C and overnight at room temperature. After renaturation by dialysis against 20 mM ethanolamine pH 9, the proteins/peptides were purified and for some fragments proteolytically cleaved and repurified as described below.

The 16 kDa N-terminal fragments of the various hormones were produced either by cleavage (introduction of a specific cleavage site (hPRL, hPL), use of a natural cleavage site (hGH-V)) or by introducing a stop codon into the coding sequence (hGH)). The DNA sequences from which the 16 kDa fragments were produced, were constructed as follows. For the production of the 16 kDa fragment of hPRL (16K hPRL), the codon for Cys 58 (TGC) was mutated to a Ser codon (TCC) and the nucleotide sequence coding for amino acids 139-144 (Pro-Glu-Thr-Lys-Glu-Asn : CCT-GAA-ACC-AAA-GAA-AAT) in wt hPRL was replaced with the nucleotide sequence coding for the specific cleavage site of IgA protease (Pro-Arg-Pro-Pro-Thr-Pro : CCT-AGA-CCC-CCA-CAC-CCT). Cleavage occurs between Pro 142 and Thr 143. For 16K hPL, Cys 53 (TGC) was mutated to Ser (TCT) and Arg 133 (CGC) to Pro (CCC), to introduce a thrombin-specific cleavage site. A natural thrombin-specific cleavage site is present in 16K hGH-V at position Pro133-Arg134. Cleavage occurs after arginine. For 16K hGH, the codon for Cys 53 (TGT) was mutated to a Ser codon (TCT) and the codon for Arg 134 (CGG) to a stop codon (TAG). To generate 16K hPRL, 16K hPL and 16K hGH-V ; mutated hPRL, hPL and intact hGH-V were produced and purified as described above and then enzymatically cleaved. For 16K hPRL, cleavage was performed with IgA protease (0.05%, 25°C, overnight; *Boehringer*) ; for 16K hPL and 16K hGH-V, cleavage was performed with thrombin (0.3%, 25°C, overnight; *Sigma*). 16K hGH and all the 16 kDa fragments obtained by cleavage were purified by ion-exchange chromatography (HiTrap Q, *Pharmacia*) except for 16K hPL which was purified by hydrophobic chromatography (Phenyl Sepharose 6 Fast Flow, *Pharmacia*). The purity of each recombinant protein exceeded 95%. The *Limulus*

amoebocyte lysate assay (E-Toxate kit, *Sigma*) was used to detect and quantify endotoxin levels.

The restriction and other modification enzymes were obtained from *Gibco BRL.*. The oligonucleotides were obtained from *Eurogentec*. Mutagenesis experiments were performed using oligonucleotide site-directed mutagenesis kits purchased either from *Amersham* or *Strategene*. All mutations were verified by sequencing.

Cell Culture

Bovine Brain Capillary Endothelial (BBCE) cells were isolated as described (25). The cells were grown and serially passaged in low glucose Dulbecco's modified Eagle's (DMEM) medium supplemented with 10 % fetal calf serum. Human recombinant basic fibroblast growth factor (bFGF) (*Promega*) was added (1 ng/ml) to the culture every other day. Experiments were initiated with confluent cells between passages 5 to 13.

In Vitro Endothelial Cell Proliferation Assay

On day 1, confluent cell cultures were dispersed and plated at a density of 1 x 10^4cells per well (in 24-well plates) in 0.25 ml DMEM containing 10% fetal calf serum, 1 ng/ml bFGF and increasing concentrations of the purified recombinant proteins as indicated. Wells containing cells + medium + serum without bFGF were included as basal-growth controls. On day 3, bFGF (1 ng/ml) and the purified recombinant were added once again to the dishes. On day 4, the cells were incubated with 500,000 cpm of [^{3}H] thymidine for 4 h, washed in 5% trichloroacetic acid, solubilized in NaOH, and counted as previously described (13).

In Vivo Early-stage Chick Chorioallantoic Membrane (CAM) Assay

On day 3 of development, fertilized chick embryos were removed from their shells and placed in plastic Petri dishes. On day 6, 5 mm disks of methylcellulose (0.5%, *Sigma*)

containing 20 μg recombinant purified protein and 2 μg bovine serum albumin (BSA) were laid on the advancing edge of the chick CAM as previously described (26). After a 48 h exposure, white India ink was injected into the chorioallantoic sac for photographic purposes.

In Vivo Late-stage CAM Assay

On day 10 of development, a window 1 cm wide was removed from the shell of the fertilized chick embryos and the membrane allowed to drop as previously described (27). 5 mm disks of methylcellulose (0.5%, *Sigma*) containing one of the purified recombinant proteins (10 μg of 16 kDa fragment or 20 μg of full-length hormone) and 2 μg bovine serum albumin (BSA) were placed on the chick CAM. After closing the shell with tape, the egg was returned to the incubator. On day 14, white India ink was injected into the chorioallantoic sac for visualization of the vasculature.

Preparation of Cell Extracts

Cell lysates were prepared as previously described (28). Confluent BBCE cell cultures were dispersed and plated at a density of 1 x 10^5 cells per 60-mm culture plate in 1 ml DMEM containing 10% calf serum. 24 h after plating, cells were serum starved in DMEM containing 0.5 % calf serum for 48 h. Cells were treated for 5 min with 10 nM purified 16 kDa fragments and 250 pM bFGF or for 10 min with 10 nM intact hormone but without bFGF or left untreated for control.

Western Blotting Analysis

Cellular proteins were resolved by SDS/PAGE and transferred to PVDF membrane (*Boehringer*). Western blots were probed with an anti-phosphotyrosine mouse monoclonal antibody (4G10, *UBI*, 1:2000 dilution) and an anti-MAPK polyclonal antibody that recognizes both p42 and p44 MAPKs (erk1-CT, UBI, 1:10000 dilution). Western blots

were incubated with the appropriate antibody and then washed in Tris-buffered saline containing 0.5% Nonidet P-40 and 0.1 % Tween 20. Antigen-antibody complexes were detected with horseradish peroxidase-coupled secondary antibodies and the enhanced chemiluminescence system (ECL ; *Amersham*). Western blots were " stripped " for reprobing with other primary antibodies by incubation for 30 min at 22°C in buffer containing 0.2 M glycine (pH 2.5) followed by two washes in PBS.

In-gel MAPK Assay

Approximately 20 µg of protein from cell lysates were resolved by SDS-PAGE in gels containing 0.5 mg/ml myelin basic protein (MBP) copolymerized in the running gel. After electrophoresis, the gel was washed in buffer A (50 ml of Tris/HCl (pH 8), 5 mM 2-mercaptoethanol) containing 20% isopropyl alcohol and denatured in buffer A containing 6 M guanidine hydrochloride. After the guanidine hydrochloride wash, the proteins in the gel were allowed to renature at 4 °C by extensive washing in buffer A containing 0.04% Tween 40. Renatured MBP kinase activity was detected by incubating the gel for 60 min at room temperature in a reaction buffer containing 40 mM HEPES (pH 7.4), 2 mM dithiothreitol (DTT), 15 mM $MgCl_2$, 300 µM sodium orthovanadate, 100 mM EGTA, 25 µM ATP and 100 µCi of [γ-^{32}P] ATP. Unincorporated radioactivity was removed by extensive washing in 5 % trichloroacetic acid containing 1% tetrasodium pyrophospate. The gels were then dried and autoradiographed.

Assay for type 1 plasminogen activator inhibitor (PAI-1) Expression

Confluent BBCE cell cultures were dispersed and plated at a density of 1 x 10^5 cells per well (in 24 well plate) in 1 ml DMEM containing 10% calf serum. Twenty-four hours after plating, the cells were treated for 16h with the purified 16 kDa fragments or the full-length hormones (10 nM) in serum-free DMEM. Untreated wells were left for controls. Cell lysates were prepared as described above. Cellular proteins (20 µg of cell

lysate) were resolved by SDS/PAGE (4-10%) and transferred to a nitrocellulose membrane (*Schleicher & Schuell*). The blots were blocked for 1 h with 5% milk in Tris-buffered saline with 0.1% Tween 20 and probed for 2 h with mouse anti-bovine PAI-1 monoclonal antibody (*Gibco*) at 1:2000 dilution. The antigen-antibody complexes were detected with horseradish-peroxidase-conjugated secondary antibody and an enhanced chemiluminescence system (ECL ; *Amersham*).

RESULTS

Production of recombinant proteins

We produced and purified recombinant full-length (intact) hPRL, hPL, hGH, and hGH-V and the corresponding recombinant 16 kDa fragments (16K hPRL, 16K hPL, 16K hGH, and 16K hGH-V). Endotoxin expressed by E. coli is known to inhibit endothelial cell proliferation. We thus analyzed the endotoxin level for all our recombinant protein preparations. The endotoxin level for 1 ng protein (EU) ranged from 0.000012 to 0.00125 EU. These levels are well below the lowest concentration capable of inhibiting bFGF stimulation of BBCE cells (13).

Capillary Endothelial Cell Proliferation

We examined *in vitro* the effects of the full-length hormones and 16 kDa fragments on the proliferation of BBCE cells induced by bFGF. As shown in Fig. 1A, all four 16 kDa peptides had a dose-dependent inhibitory effect on bFGF-induced BBCE cell proliferation. The concentration required for half-maximal inhibition (IC_{50}) ranged from 1 to 2 nM for various fragments. In contrast, both hGH and hGH-V stimulated cell proliferation with a maximum of twice the level obtained with bFGF alone (EC_{50}= 3 to 4 nM, Fig. 1B). Intact hPL and hPRL had no significant effect.

In Vivo Formation of Capillaries

The *in vivo* activity of the intact molecules and fragments was examined in two different CAM assays. The CAM appears in the yolk sac at 48 h, grows rapidly over the next 6-8 days, and stops growing on day 10 (29). We performed an early-stage CAM bioassay (days 6-8) to assess each molecule's effect on developing capillaries and a late-stage bioassay (days 10-14) to test their effect on non-growing quiescent CAM. Experiments were performed by laying methylcellulose disks containing purified

recombinant protein/peptide on the CAM of embryos on day 6 (early stage-CAM assay) or day 10 (late-stage CAM assay) of development. In the early-stage CAM assay, an avascular area is clearly present surrounding the disks containing the 16 kDa fragments (Fig. 2). The full-length hormones had no effect in this bioassay. In the late-stage bioassay, the intact proteins stimulated new capillary and blood vessel formation which could be observed emerging from the protein-containing disks, while the 16 kDa fragments had no effect (Fig. 3).

Activation of the MAPK Signaling Pathway

The signaling pathway by which 16K hPRL blocks the mitogenic activity of bFGF or vascular endothelial growth factor (VEGF) has been partially characterized (28). 16K hPRL inhibits the activation and tyrosine phosphorylation of MAPKs, downstream of bFGF or VEGF receptor phosphorylation. Therefore, we examined the effects of 16 kDa fragments on MAPK activation and phosphorylation. Western blot analyses and *in vitro* enzyme assays (Fig. 4) revealed that all the 16 kDa fragments inhibited tyrosine phosphorylation and activation of MAPKs induced by bFGF. As shown in figure 4, treatment with all the intact hormones induced the tyrosine phosphorylation and activation of MAPK in capillary endothelial cells. Although the JAK/Stat cascade is presumably the major signaling pathway for the PRL and GH receptors, activation of the MAPK pathway by GH and PRL has also been reported in several biological systems (30,31).

Regulation of PAI-1 Expression

16K PRL was recently shown to stimulate the expression of PAI-1 in capillary endothelial cells (32). This is consistent with the action of an antiangiogenic factor, since PAI-1 limits degradation of the extracellular matrix by urokinase plasminogen activator, thus preventing angiogenesis (33). Activation of PAI-1 expression by 16K PRL is independent of the prior treatment of the cells with bFGF. Therefore, we asked whether

the other N-terminal fragments or intact molecules would also affect PAI-1 expression. All four 16 kDa fragments stimulated the cellular content of PAI-1 protein approximately 4-fold in BBCE cells, while the intact molecules had no effect on PAI-1 levels (Fig. 5A). A similar response was observed for PAI-1 secreted into the medium (data not shown). As shown in Fig. 5B, a 5-fold excess of hGH failed to inhibit the stimulation of PAI-1 expression induced by 16K hGH, providing direct evidence that the receptors(s) mediating the actions of the intact molecules and N-terminal fragments are different.

DISCUSSION

Our findings demonstrate that angiogenic activity is a general property of members of the human PRL/GH family with the intact molecules being angiogenic and their N-terminal fragments being antiangiogenic. In vivo, intact and N-terminal fragments show opposing actions in two separate bioassays performed at different stages of CAM development. On growing capillaries, the 16 kDa fragments prevent angiogenesis and the full-length hormones have no effect, while on the quiescent vasculature, intact hormones stimulate blood vessel formation, and the 16 kDa fragments have no effect. *In vitro*, all the four 16 kDa fragments inhibit BBCE cell proliferation, while hGH and hGH-V were stimulatory. Interestingly, intact hPRL and hPL were not stimulatory. The inability of observing an effect in BBCE cells could be explained by the recent preliminary observation that BBCE cells express and secrete a prolactin-like molecule and antibodies against bPRL inhibit BBCE cell growth (34). The presence of the endogenous hormone would mask the effects of the added hPRL and hPL. This explanation is consistent with the actions of hGH and hGH-V being mediated by GH receptors present on the BBCE cells, while the actions of hPRL and hPL would be expected to be mediated via PRL receptors (5).

The opposite actions of the full-length and cleaved hormones appear to be mediated by independent receptors rather than by competition for binding to the same receptor. A saturable, high-affinity binding site for ^{125}I-labeled rat 16K PRL has been described on BBCE cell membranes. Intact GH and PRL do not compete for this receptor (35). On the other hand, 16K hPRL does not compete with hPRL for binding to the prolactin receptor (data not shown). Data showing that the addition of excess hGH had no effect on the stimulation of PAI-1 expression by 16K hGH further supports the hypothesis that the N-terminal fragments act at separate receptors. To date we have no data as to whether the 16K fragments act at one receptor or multiple receptors. It appears that if multiple receptors

for the fragments exist they signal via similar pathways, since they all stimulate PAI-1 expression and inhibit the proliferative action of bFGF.

We have obtained preliminary data that the MAPK signaling pathway may mediate the related actions of the intact molecules and N-terminal fragments. 16K hPRL has been shown to inhibit activation of the MAPK signaling pathway by bFGF (28). The inhibition of MAPK activation by 16K hPRL appears to occur at the level of Ras activation (36). PRL and GH have been shown to activate the MAPK signaling pathway confirming the current findings in BBCE cells (30,31). Stimulation of PAI-1 expression by the N-terminal fragments does not require the activation of bFGF receptors, and would appear to be mediated by a more proximal signaling event no involving MAPK activation. This hypothesis is consistent with the finding that the intact molecules which activate MAPK have no effect on PAI-1 expression.

Angiogenesis is essential in several physiological processes, such as the formation of embryonic tissues or placental development, where induction and cessation of angiogenesis appear to be highly regulated through the expression/repression of angiogenic/antiangiogenic factors (37). It has been suggested that in the mouse placenta, initiation and cessation of vascularization correlates with the sequential expression of PLF and PRP (9). These related members of the PRL/GH family have been shown respectively to stimulate and inhibit angiogenesis.

Unlike the opposite actions of PLF and PRP, both the angiogenic and antiangiogenic actions of the PRL/GH family reside within the same molecule. The idea that a fragment of a larger molecule is antiangiogenic agrees with studies using several other peptides (38). However, the peptides of the PRL/GH family are novel in that in addition to the N-terminal fragments being antiangiogenic the intact molecules are angiogenic. The ratio of intact to cleaved molecules would constitute an angiogenic switch regulated by the level of the expression of the protein and of the proteases responsible for their processing.

The angiogenic factors (intact hormones) can be cleaved by several enzymes (14,18) to generate antiangiogenic factors (the 16kDa fragments). The hormone and protease responsible for formation of the fragment should be located in the same compartment, and their levels should be correlated with angiogenic activity. Consistent with this hypothesis hPRL, hGH-V, and hPL and cathepsin D, an enzyme that cleaves rat PRL(14) and hPRL (39) are produced at the deciduoplacental interface (6-8). We hypothesize that regulation of the production and proteolytic cleavage of the PRL/GH family members could constitute a mechanism for modulating vascularization of the human placenta. Regulation of placental vascularization has important clinical implications, as impairment of vascular development is observed in pregnancies complicated by preeclampsia and intrauterine fetal growth retardation (40).

Angiogenesis is essential for tumor growth and metastasis. The production of angiogenic factors by a tumor is essential for tumor progression. Numerous cancers secrete proteases e.g. cathepsin D (41) which, according to our model, will lead to the formation of the antiangiogenic factors including 16K hPRL. Simultaneous production by a tumor of an angiogenic factor and an antiangiogenic factor is not inconsistent (42). It is now assumed that neovascularization, an essential component of tumor progression, requires secretion of an angiogenic molecule. However, the simultaneous production of an antiangiogenic factor, may be involved in regulating the dormancy of micrometastases (43). The inhibition of tumor progression by antiangiogenic factors is being widely tested as a therapeutic approach for the treatment of cancer. In addition to 16K PRL, this study identified three new antiangiogenic factors which are also potential therapeutic agents.

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FIGURE LEGENDS

Figure. 1 BBCE cell proliferation

Cells were treated with bFGF (1 ng/ml) and the recombinant proteins. Proliferation was assess by incorporation of 3H-thymidine into the DNA. Wells containing cells + medium + serum without bFGF were included as basal-growth controls. The data are expressed as percentages of the stimulation obtained with bFGF alone, 0 % being the basal growth level. (A) Inhibition of BBCE cell proliferation. Symbols : (■) 16K hPRL; (◆) 16K hPL ; (●) 16K hGH ; (▲) 16K hGH-V. (B) Stimulation of BBCE cell proliferation in presence of bFGF. Symbols : (□) hPRL ; (◇) hPL ; (O) hGH; (Δ) hGH-V. Each point represents the mean of triplicate wells. The experiments were repeated at least three times, with similar results.

Figure. 2. Early-stage CAM assay

Inhibition of angiogenesis in the CAM. (A) The disks are visible by light reflection and the black arrow shows the border of the disk or the border of the avascular area if present. (B) Antiangiogenic index representing the percentage of eggs testing positive, calculated from three separate sets of experiments done with 5 to 7 eggs per experiment and 20 μg protein. Results were considered positive only if an avascular zone was observed 5 mm in diameter or greater.

Figure. 3. Late-stage CAM assay

Stimulation of blood vessel formation in the CAM. (A) The black arrow shows the border of the disk. (B) Angiogenic index representing the percentage of eggs testing positive in two separate sets of experiments with at least 5 to 7 eggs per experiment. Eggs were counted as positive when the number of blood vessels within 1 cm^2 around the disk was at least three times the number obtained with BSA alone.

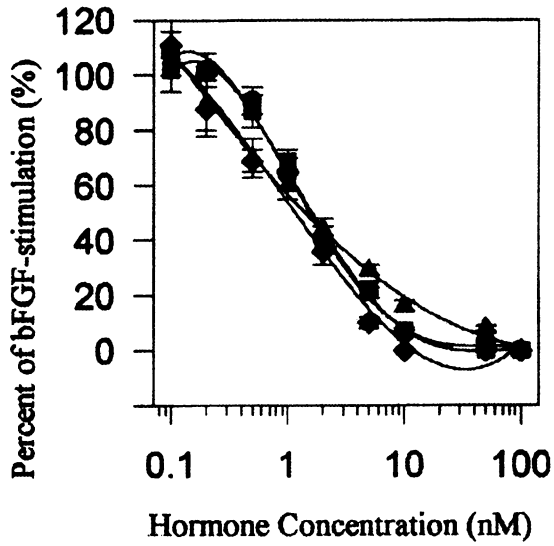
Figure. 4. 16 kDa fragments inhibit bFGF-dependent MAPK tyrosine phosphorylation and activity, while full-length hormones stimulate these processes in the absence of bFGF

BBCE cells were treated for 5 min with 10 nM purified 16 kDa fragments and 250 pM bFGF or for 10 min with 10 nM intact hormone but without bFGF or left untreated for control. (A), P-Tyr panel : tyrosine phosphorylation of MAPK p42 and p44. Cell lysate proteins were separated by SDS-PAGE, transferred to PVDF membranes, and probed with an anti-phosphotyrosine antibody. MAPK panel : the Western blot shown in the P-Tyr panel, stripped and reprobed with an anti-MAPK antibody. (B), In-gel MAPK activity: cell lysates were resolved by SDS-PAGE in gels containing 0.5 mg/ml myelin basic protein (MBP). After electrophoresis, the gel proteins were denatured, renaturated, and subjected to an in situ kinase reaction with 100 μCi of [γ-^{32}P] ATP. The gels were then dried and autoradiographed. ^{32}P incorporated into MBP was quantified by a phosphorImager. Numbers at the bottom of the histograms represent the number of experiments performed.

Figure. 5. Stimulation of PAI-1 expression in BBCE cells by the 16 kDa fragments

Mouse anti-bovine PAI-1 western blotting performed on BBCE cells prestimulated by 20 nM of recombinant protein or left untreated for control. (A) 16 kDa fragments stimulate PAI-1 expression, full-length hormones do not. Lower panel : Quantification. (B) Competition between hGH (22K hGH) and 16K hGH. Mousse anti-bovine PAI-1 western blotting performed on BBCE cells left untreated (control lanes), or treated with 20 nM of 16K hGH (16K hGH lanes), with 20 nM of hGH (22K hGH lanes) or with 20 nM of both 16K hGH and hGH (16K hGH + 22K hGH lanes).

A.


120
100
80
60
40
20
0
Percent of bFGF-stimulation (%)
0.1
1
10
100
Hormone Concentration (nM)

B.

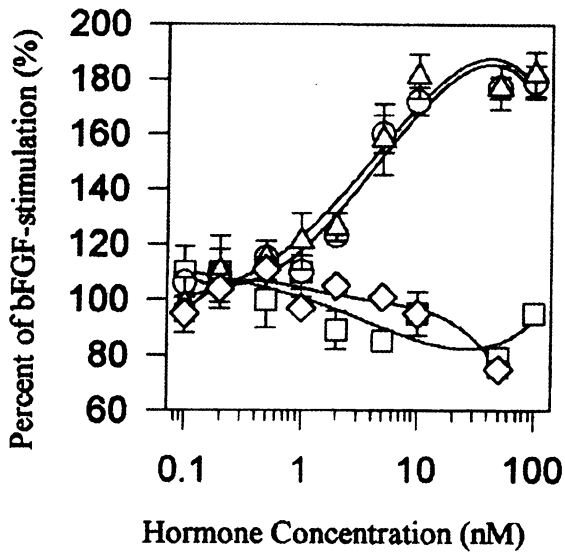

200
180
160
140
120
100
80
60
Percent of bFGF-stimulation (%)
0.1
1
10
100
Hormone Concentration (nM)

FIGURE 5-1

5-2A

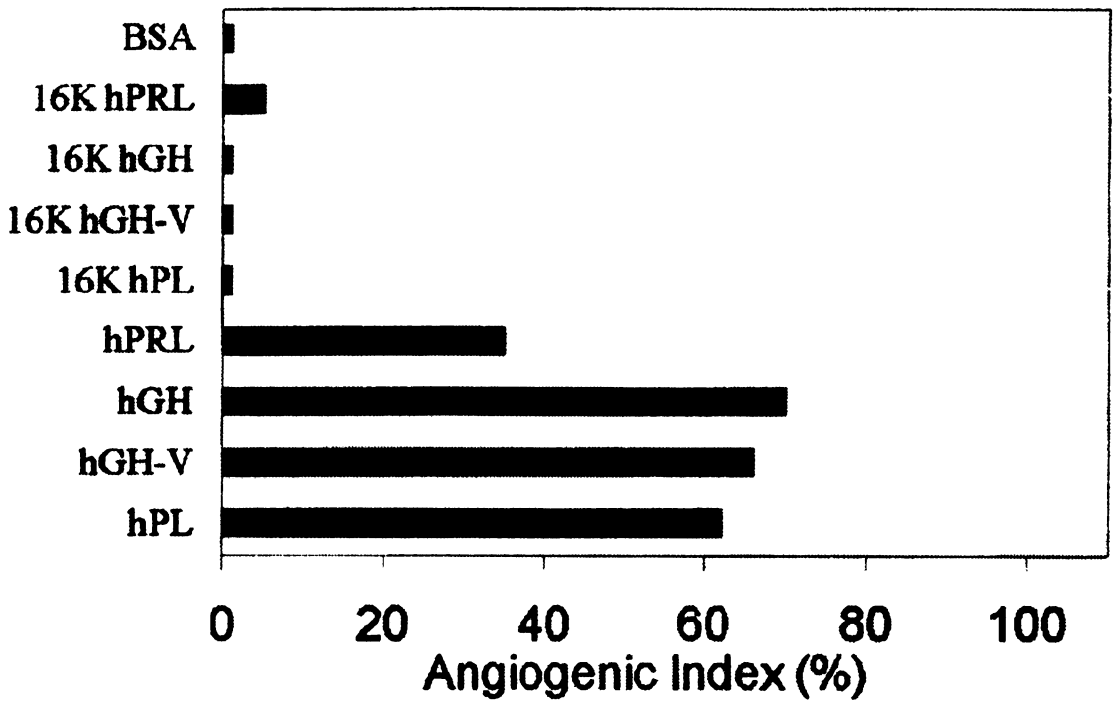
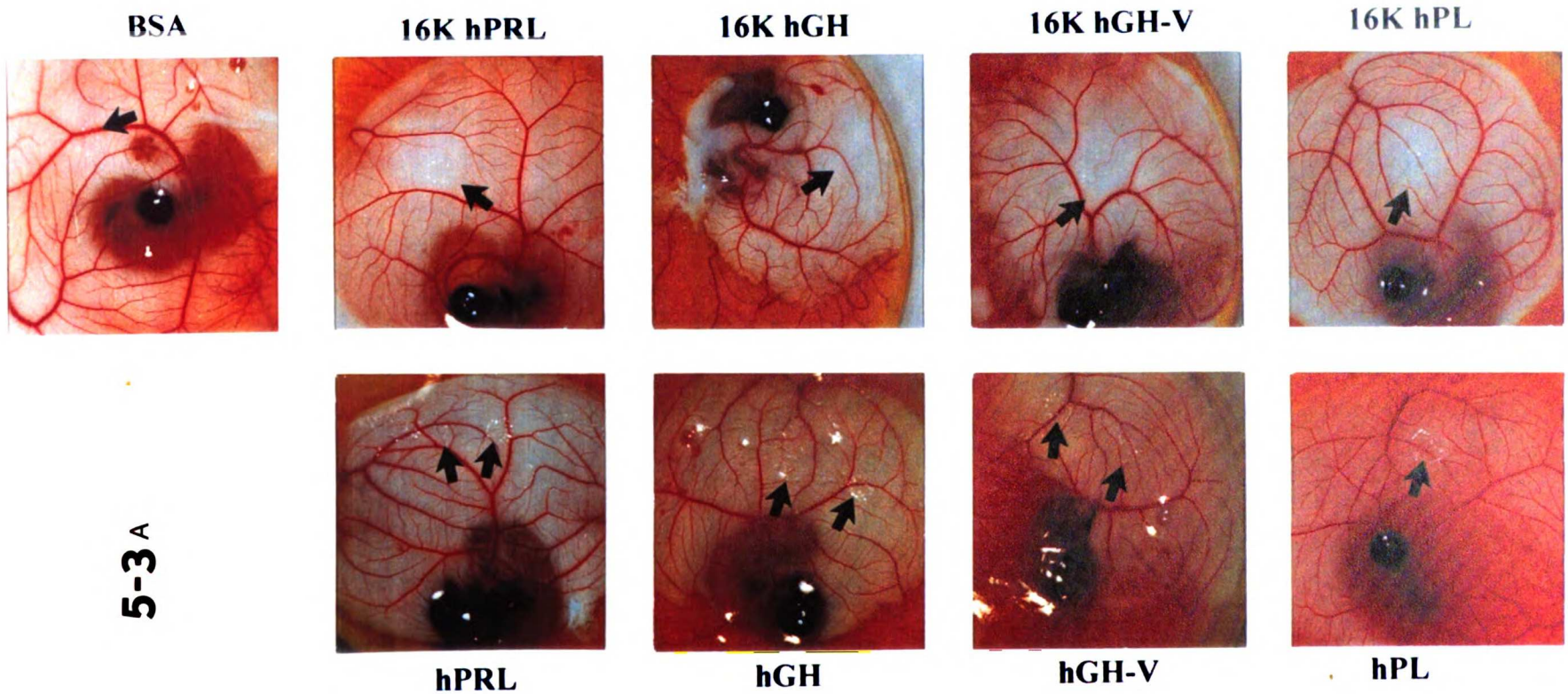

BSA
16K hPRL
16K hGH
16K hGH-V
16K hPL
hPRL
hGH
hGH-V
hPL
0
20
40
60
80
100
Angiogenic Index (%)

FIGURE 5-2B



5-3A

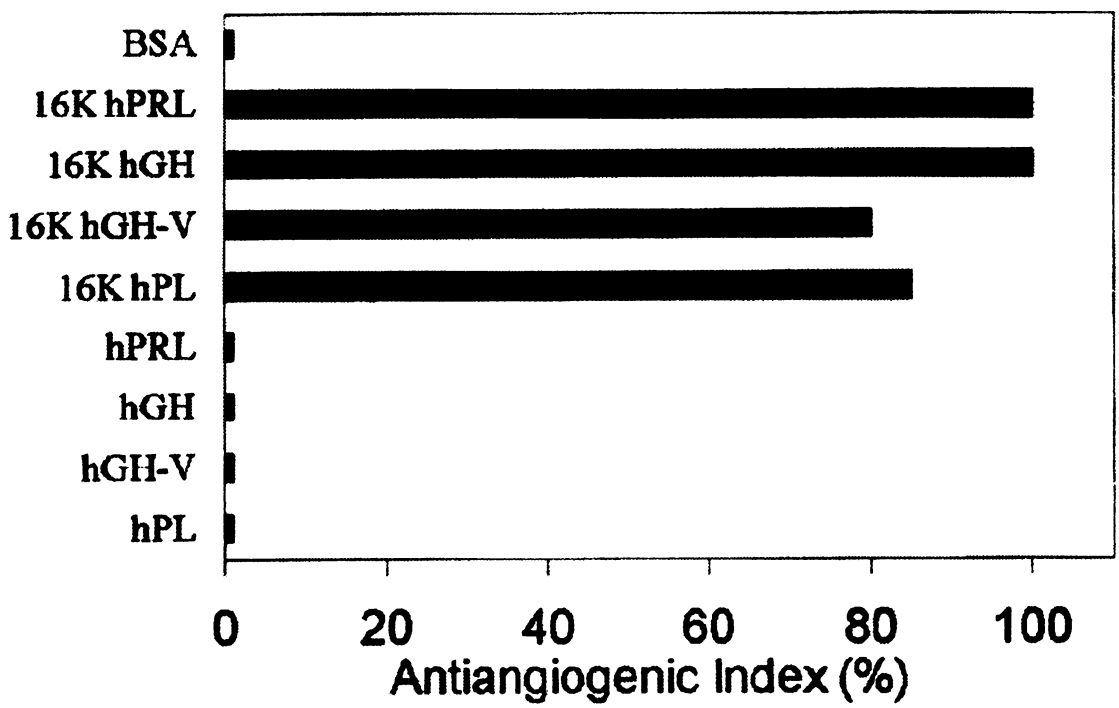

BSA
16K hPRL
16K hGH
16K hGH-V
16K hPL
hPRL
hGH
hGH-V
hPL
0
20
40
60
80
100
Antiangiogenic Index (%)

FIGURE 5-3B

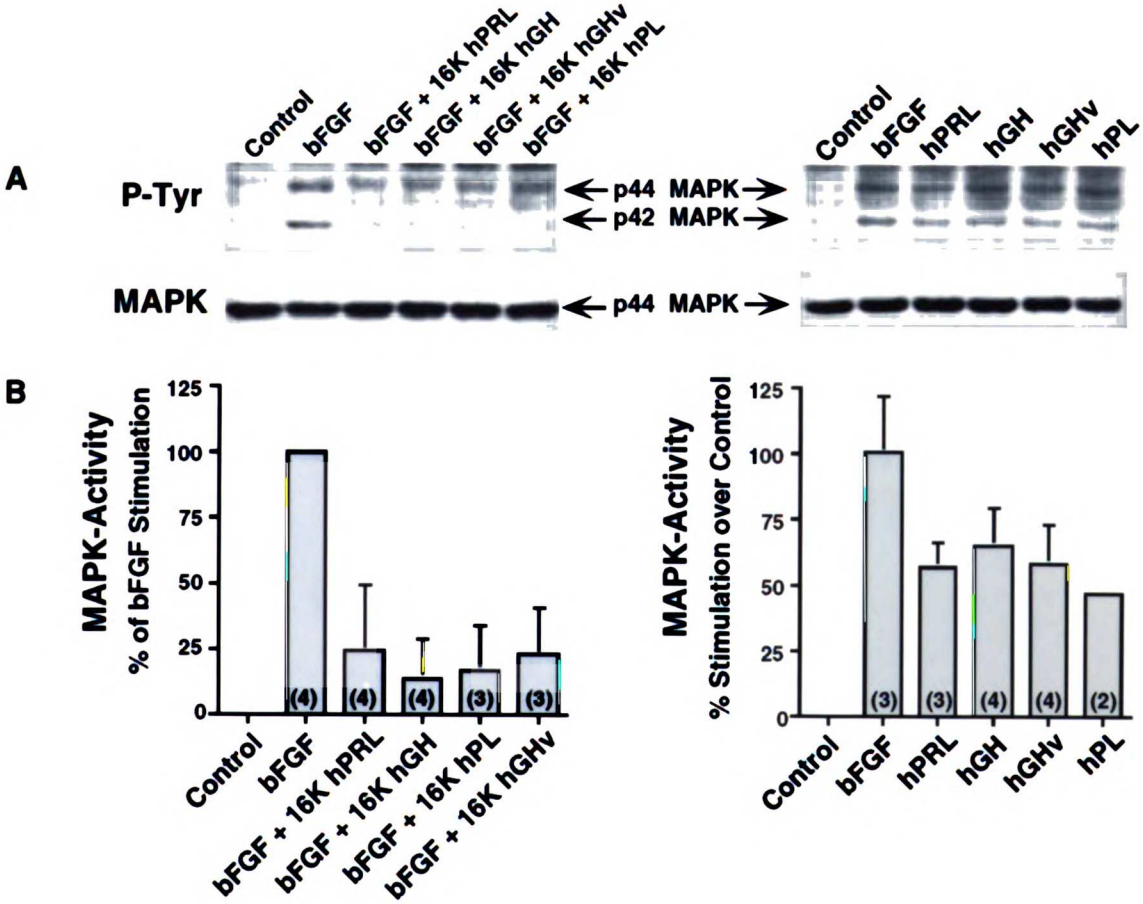
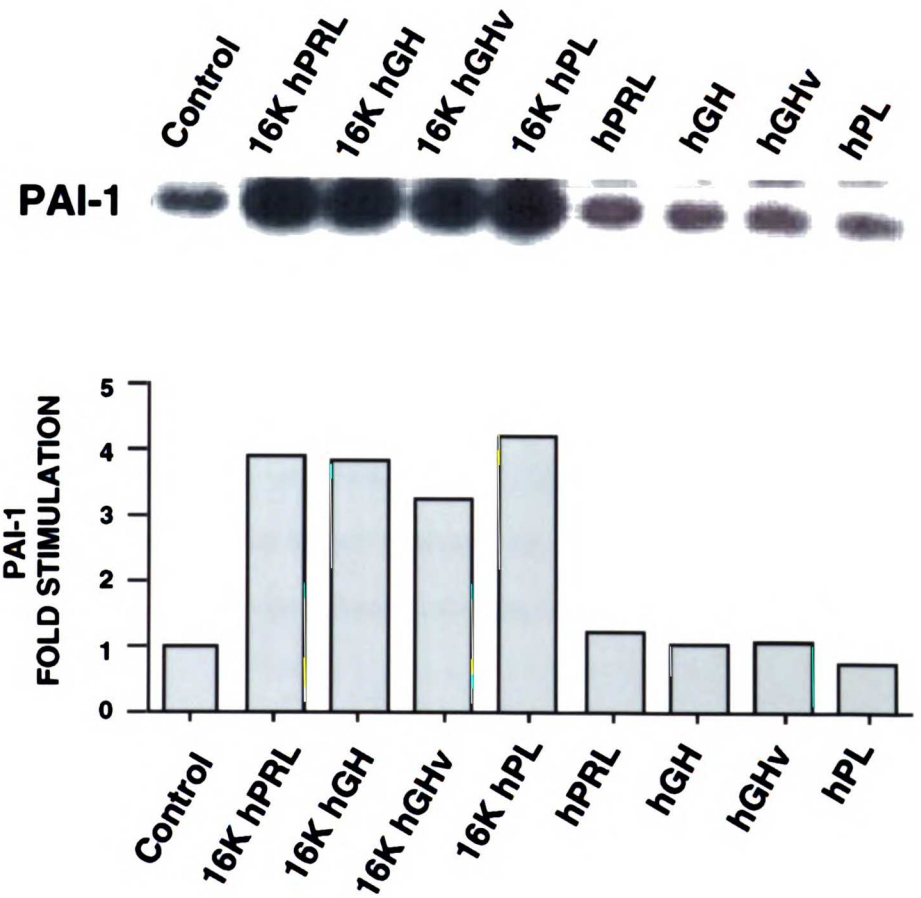

A
P-Tyr
MAPK
Control
bFGF
bFGF + 16K hPRL
bFGF + 16K hGH
bFGF + 16K hGHv
bFGF + 16K hPL
p44 MAPK
p42 MAPK
hPRL
hGH
hGHv
hPL
B
MAPK-Activity
% of bFGF Stimulation
% Stimulation over Control
125
100
75
50
25
0
(4)
(3)
(2)

FIGURE 5-4

A.


Control
16K hPRL
16K hGH
16K hGHv
16K hPL
hPRL
hGH
hGHv
hPL
PAI-1
PAI-1
FOLD STIMULATION
5
4
3
2
1
0
Control
16K hPRL
16K hGH
16K hGHv
16K hPL
hPRL
hGH
hGHv
hPL

B.

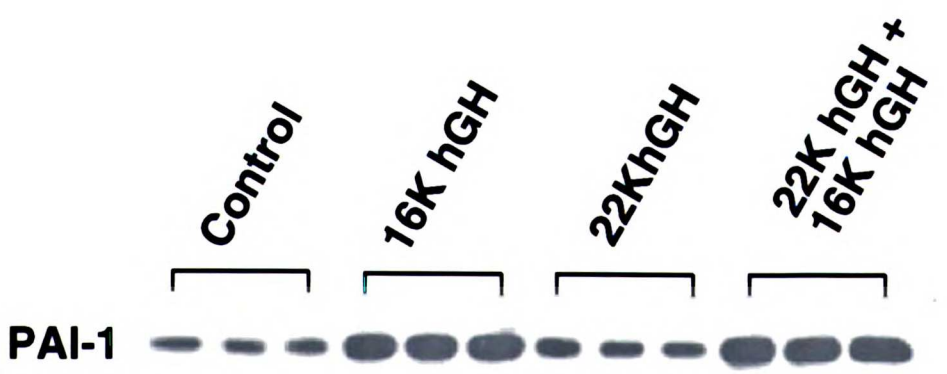

Control
16K hGH
22KhGH
22K hGH +
16K hGH
PAI-1

FIGURE 5-5

Chapter 6. 16K hPRL Family Members Stimulate PAI-1 through a Serine/threonine Kinase Dependent Pathway

INTRODUCTION

In previous chapters, we showed that 16K hPRL stimulated PAI-1 production through a serine/threonine kinase dependent pathway. Since 16K hPRL family members all stimulate PAI-1 production, we tested whether 16K hGH in addition to 16K hPRL stimulated PAI-1 production through a similar signaling pathway. We showed that the stimulatory effect of 16K PRL and 16K GH on PAI-1 production were blocked by treatment with the serine/threonine kinase inhibitor, H7. These results suggest that both 16K hPRL and 16K hGH either signal through the same receptor or receptors signaling via a similar mechanism.

MATERIALS AND METHODS

To nonspecifically inhibit serine threonine kinases, cells were treated 50 μM H7. BBE cells treated with 10 nM of 16K hPRL or 16K hGH alone or together with the inhibitor were harvested and the cell lysates were subjected to PAI-1 Western analysis. The blots were stripped and reprobed with a MAPK antibody to control for sample loading.

RESULTS and DISCUSSION

We have previously shown that 16K hPRL stimulates PAI-1 by a serine/threonine kinase dependent mechanism. H7, a serine/threonine kinase inhibitor, blocked increased PAI-1 production stimulated by 16K hPRL. We asked if 16K hGH utilized a similar mechanism to stimulate PAI-1 production in BBE cells. BBE cells were treated with 10nM of 16K hPRL, 16K hGH alone or together with 50 uM of serine/threonine kinase inhibitor H7 for 16 hrs. Cell lysates were collected and assayed by PAI-1 Western analysis. 16K hPRL and 16K hGH both stimulated PAI-1 production in BBE cells as described in

previous sections. H7 blocked the stimulation of PAI-1 in BBE cells by both 16K hPRL and 16K hGH, Fig. 1. Western analysis of MAPK levels showed that protein synthesis was not nonspecifically affected by H7 treatment (lower panel). We also showed that H7 blocked the stimulation by 16K hGHV and 16K hPL of PAI-1 secreted into the medium (data not shown). These results suggest that a similar signaling mechanisms mediates the stimulation of PAI-1 production by all the 16K hPRL family members.

Since all 16K PRL family members stimulate PAI-1 production and a similar mechanism is utilized for signal transduction, a reasonable hypothesis is that a common or a related receptor might be used by the four 16K fragments. This hypothesis is also supported by the observation that all these fragments have similar biological functions: they all inhibit bFGF-induced cell proliferation, stimulate PAI-1 production and inhibit angiogenesis in the CAM *in vivo* assay. Cloning of these receptors will give us a better understanding of the similarities of these signaling mechanisms.

FIGURE LEGEND

Figure 6-1. Effect of H7 on The Stimulation of PAI-1 protein levels by 16K hPRL and 16K hGH:

BBE cells were treated with 10nM of 16K hPRL or 16K hGH alone, or in addition with 50 uM of H7 for 16 hrs. The lysates were collected and subjected to PAI-1 Western analysis (upper panel). The blots were stripped, and reprobed with MAPK antibody to show the even loading of the samples (lower panel). Treatment with concentrations of H7 up to 100 uM had no obvious effect on basal PAI-1 or MAPK protein level.

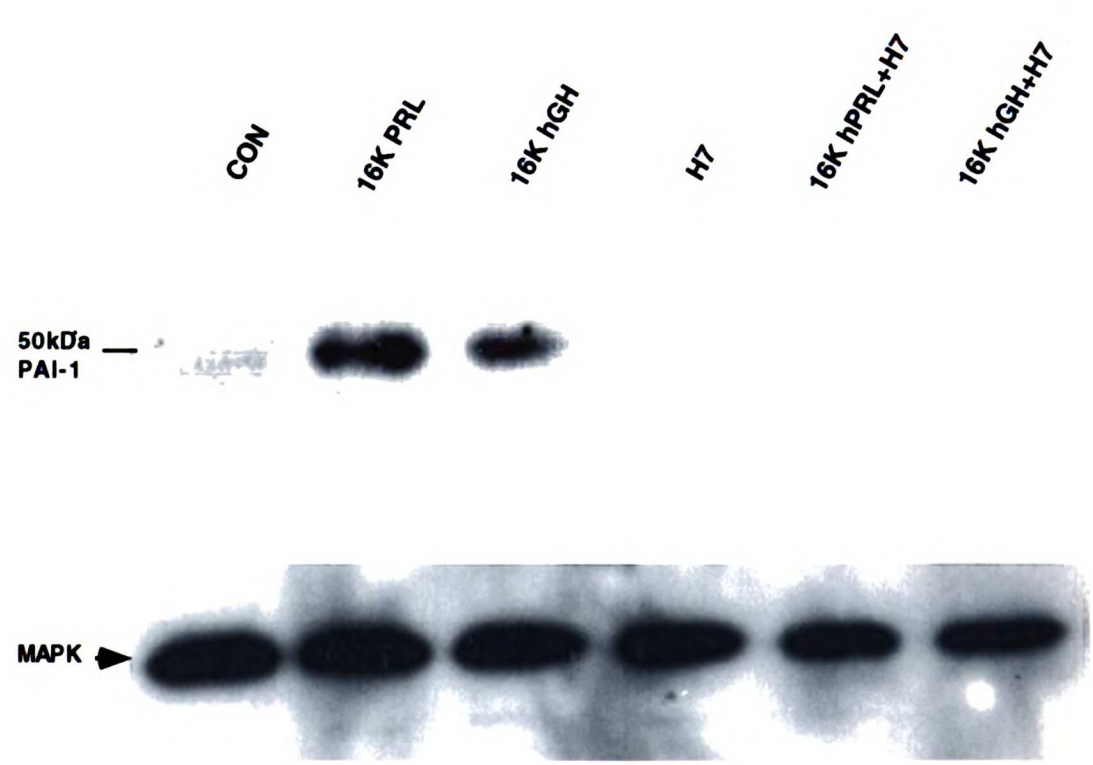


FIGURE 6-1

Chapter 7. General Discussion

The formation of new blood vessels is a complicated, multistep process involving many levels of regulation (Auerbach, 1994; Findlay, 1986; Klagsbrun, 1991, Folkman, 1997; Hanahan, 1996). In this report, we show that the antiangiogenic factor 16K PRL inhibits multiple components of angiogenesis. Most importantly, we showed that 16K PRL inhibited uPA activity stimulated by bFGF, an angiogenic factor, in capillary endothelial cells. The inhibition of uPA activity by 16K PRL is indirect via the stimulation of the expression of PAI-1. We compared the signaling pathways mediating the stimulation of PAI-1 by 16K hPRL and another *in vitro* antiangiogenic factor TGF-β. Finally, we showed that 16 kDa N-terminal fragments of the PRL/GH family all have antiangiogenic activity and stimulate PAI-1 production.

The clinical evidence that many diseases, including cancer, were dependent on angiogenesis stimulated research on antiangiogenic compounds (Folkman, 1992; Leibovich, 1987). During recent years, many antiangiogenic substances have been described. These materials either inhibit angiogenesis *in vitro* or *in vivo*. Platelet factor IV (PF4) (Maione, 1990) and endostatin (O'Reilly, 1997) inhibit tumor progression, supporting the hypothesis that antiangiogenic factors might serve as therapeutic agents for the treatment of cancer. Another important potential benefit of antiangiogenic therapy is that it avoids the problem of acquired resistance observed with traditional anti-cancer treatments. This is related to the assumption that antiangiogenic agents are acting on capillary endothelial cells, rather than on genetically unstable cancer cells (Barinaga, 1997). Several antiangiogenic factors are currently being used in stage 1 and stage 2 clinical trials.

Despite the muti-laboratory effort on antiangiogenic research, the mechanisms through which most of the antiangiogenic factor work remain unknown. 16K PRL is one of the most carefully characterized antiangiogenic factor described at this time (Ferrara, 1991; Clapp, 1992; Clapp, 1993; D'Angelo, 1995). It inhibits multiple cellular

mechanisms of angiogenesis including: endothelial cell proliferation; activation of proteolytic enzymes; endothelial cell migration; and organization of endothelial cells into capillary-like structures. We showed that the inhibition of cell proliferation by 16K hPRL is mediated through the inhibition of mitogen-induced activation of the MAPK signaling cascade. The inhibition of MAPK appears to occur upstream in the signaling pathway by preventing Ras activation (D'Angelo, G., unpublished results). In this report, we described the mechanisms by which 16K hPRL inhibited bFGF-induced activation of uPA activity. The inhibition of uPA activity was shown to be indirect via the stimulation of PAI-1 production. The stimulation of PAI-1 is mediated via a signaling pathway which includes a serine/threonine kinase which is not PKA and PKC.

In chapter 2, we presented data showing that 16K PRL inhibits uPA activity by stimulating the expression of the PAI-1 gene. The blockade of uPA activity affects multiple cellular mechanisms necessary for angiogenesis to occur. The inhibition of uPA activity could result in a decrease in the degradation of components of extracellular matrix, thereby, retarding penetration of endothelial cells through the basement membrane barrier. Furthermore, it may also prevent the release of matrix-bound growth factors, thus further slowing the angiogenic process. The increase of PAI-1 has been correlated with inhibition of endothelial cell migration (Petzelbauer, 1996). Moreover, exogenously added PAI-1 can inhibit endothelial cells from forming tube-like structures in collagen gels (Schnaper, 1995). These findings are consistent with the conclusion that the inhibitory effect of 16K PRL on endothelial cell migration and tube formation may be the result of increased PAI-1 production.

The report by Blei et al (Blei, 1993) showed that antiangiogenic steroid medroxyprogesterone acetate (MPA) inhibited uPA activity by stimulating PAI-1 expression. However, the inhibition of uPA was only observed in the secretory fraction and not in the cell-associated fraction of bovine aortic endothelial cells (BAE). They hypothesized that the secreted uPA was mostly in the two-chain form, while the cell-

associated uPA was mostly in the single-chain form. Only the activated two-chain form of uPA was inhibitable by PAI-1, thus no inhibition was observed in cell-associated fraction of BAE cells. These findings do not agree with our results. In the current studies, both cell-associated and secreted uPA were inhibited by 16K hPRL treatment of BBE cells. Additionally, in long term exposures of PAI-1 Western blots of BBE cell lysates from 16K hPRL treated BBE cells, we showed that PAI-1-uPA complexes could be observed. Exogenously added human PAI-1 inhibited uPA activity of bFGF-treated BBE cells in a dose dependent manner. When the same samples were separated by PAGE and immunoblotted with anti-human PAI-1 antibody, PAI-1-uPA complexes were observed. These results suggested that the 16K hPRL-induced increase in PAI-1 resulted in a decrease in uPA activity. The decrease of uPA activity is likely due to the increased formation of complexes between PAI-1 and uPA. These results also suggested that most of the cell-associated uPA was in the activated two-chain structure.

We consistently observed that bFGF treatment had little or no effect on PAI-1 protein and mRNA levels. These findings are in contrast to earlier reports that bFGF stimulated PAI-1 production in endothelial cells (Pepper, 1990; Saksela, 1987). These discrepancies are possibly due to differences in the cell type used in the experiments. However, BBE cells do respond to bFGF to increase cell proliferation and migration. Moreover, 16K PRL stimulated PAI-1 production in both BAE and BBE cells. An explanation to the inability of bFGF to stimulate PAI-1 in BBE cells could be related to the number or subtype of FGF receptors expressed on the cells.

LPS has been shown to stimulate PAI-1 production in endothelial cells (Colucci, 1985; Sawdey, 1989). However, the 16K hPRL effect on PAI-1 does not appear to be due to LPS contamination in the recombinant preparations. First, 16K rPRL prepared by cleavage of rat prolactin has a low level of LPS, but stimulated PAI-1 production in BBE cells. Secondly, a 100-fold excess of LPS present in the 16K hPRL preparations only mildly stimulated PAI-1 production in BBE cells. Recombinant 23K hPRL containing

similar amounts of LPS to that of the 16K hPRL preparation had no effect on PAI-1 production in BBE cells. Furthermore, antibodies raised against 16K hPRL block the stimulatory effect of 16K hPRL on PAI-1 (data not shown). All these results are consistent with the conclusion that the stimulatory effect of 16K PRL is not due to LPS contamination.

PAI-1 binds to many different molecules including uPA, tPA, vitronectin (Salonen, 1989), and LRP (Nykjaer, 1992). Increases in PAI-1 might change the binding patterns between these molecules. Vitronectin is a ligand for αvβ3 integrins. αvβ3 has been shown to be involved in the regulation of endothelial cell apoptosis (Brooks, 1994; Drake, 1995). The increase of PAI-1 by 16K PRL might affect these interactions and thus affect the survival of endothelial cells. These results brought out the hypothesis that PAI-1 stimulated by 16K PRL might inhibit angiogenesis in two respects: by inhibiting urokinase activity and by inducing apoptosis of endothelial cells through disturbing the αvβ3 interaction with ECM. In chapter 4, we show evidence that 16K PRL does induce apoptosis in BBE cell. However, exogenous added PAI-1 did not induce apoptosis, suggesting that the apoptotic effect of 16K hPRL is not an indirect effect of increasing PAI-1.

TGF β is a potent *in vitro* antiangiogenic factor (Muller, 1987; Pepper, 1993). It inhibits endothelial cell proliferation (Frater-Schroder, 1986) as well as uPA activity. However, TGF β stimulated angiogenesis in an *in vivo* assay (Roberts, 1986). This discrepancy is likely due to the chemoattractant action of TGF β on macrophages which release angiogenic factors. The action of TGF β is similar to 16K PRL in many aspects, including inhibition of cell proliferation, inhibition of MAPK activity, inhibition of endothelial cell migration, inhibition of protease activation and stimulation of PAI-1 production. The similarities between 16K PRL and TGF β prompted us to compare the mechanisms involved in the signaling of the two hormones. In chapter 4, we present data that 16K PRL and TGF β act in a distinct fashion. TGF β inhibited basal BBE cell

proliferation, however, 16K PRL had no effect. Moreover, 16K PRL stimulated apoptosis in BBE cells, while TGF β had no effect. The reporter gene expression data in BBE cells also showed that the response element for 16K PRL and TGF β were different. The response element for 16K PRL is located within -6.4 kb and -1.5 kb region of human PAI-1 promoter, while response element for TGF β is located within -800 bp region of the promoter (Westerhausen. 1991).

However, the signaling mechanism for increasing PAI-1 expression by 16K hPRL and TGF β have some similarities. The ability of both molecules to stimulate PAI-1 can be inhibited by H7, a non-specific serine/threonine kinase inhibitor. This observation is consistent with the hypothesis that the two molecules might signal through receptors of the same family. TGF β signals through a transmembrane serine/threonine kinase receptor system (Attisano, 1993; Bassing, 1994; Ebner, 1993; Wrana, 1994). Many of the TGF β family receptors have been described in vertebrates (Miyazono, 1994), including receptors for TGF β, activin (Mathews, 1991; ten Dijke, 1993), Mullerian inhibitory substance (MIS; Teixeira, 1996), BMP (ten Dijke,1994; Liu, 1995) and some orphan receptors (Matsuzaki, 1993) with unknown ligands. Further experiments are required to show if 16K PRL signals through these receptors.

We attempted to clone the 16K PRL receptor by expression cloning in Cos cells. A BBE cell library constructed in the PCDNA 1 expression vector was screened using radioligand binding. Over two million recombinants were screened with no positive colonies being identified. The inability to isolate the receptor could be explained by the requirement for two molecules for receptor binding. The chance of having both molecules expressed in any pool of recombinants would be very low. In crosslinking studies we also showed that 16K rPRL bound to multiple proteins of approximately 50 and 30 kDa. It is interesting that members of the TGF β receptor family are composed of heterodimers.

By genetic analysis, Smad family proteins have recently been shown to be involved in signaling of TGF β family (Liu, 1995; Liu, 1996; Massague, 1996). Smad 2, 3 and 4

are signaling intermediates that are involved in TGF β stimulation of PAI-1 expression. TGF β likely induces complexes composed of Smad 2, Smad3 and Smad 4 that translocate to the nucleus and stimulate transcription (Nakao, 1997). A recent report showed that Smad 4 could bind to DNA directly (Yingling, 1997), supports the notion that Smad proteins might act as transcriptional activators. Two Smad family members, Smad6 and Smad7, are expressed specifically in endothelial cells. They are involved in the modulation of the TGF β response in this cell type (Topper, 1997). Further experiments are required to show whether 16K PRL signals through these or undiscovered members of the Smad family. The observation that the response elements mediating the actions of 16K hPRL and TGF β on PAI-1 expression are different, suggests that different Smads will be involved.

One discrepancy in the argument that the inhibitory effect of 16K PRL on angiogenesis is due to an increase in PAI-1, is that both uPA and PAI-1 levels were increased in *in vivo* angiogenic processes (Bacharach, 1992; Landau, 1994; Pedersen, 1994). In these histological studies, the parallel increase in uPA and PAI-1 is likely due to the sampling from different populations of endothelial cells. Urokinase activity is increased during matrix degradation and endothelial cell migration. On the other hand, PAI-1 is likely increased in the final steps of the process to prevent the excess degradation of the extracellular matrix and allow the organization of endothelial cells into tube-like structures.

Angiogenesis is a carefully regulated physiological process. A single angiogenic stimulator like bFGF or VEGF can orchestrate all components of angiogenesis (Presta, 1992), suggesting that a finely tuned program is controlled by these factors. The local increase of PAI-1 stimulated by 16K PRL might disturb the initial events of angiogenesis. Similar to the angiogenic factors, 16K PRL also effects multiple components of angiogenesis. The extensive actions of 16K PRL result in the antiangiogenic phenotype *in vivo* .

The high degree of homology between members of the PRL/GH family led us to investigate whether 16K fragments of hGH, hGHV and hPL would also have

antiangiogenic activity. Intact hormones stimulated MAPK activity, while 16 kDa fragments inhibited bFGF-induced stimulation of MAPK. Intact hormones and 16K fragments showed opposite actions in two separate CAM *in vivo* assays performed at different stages of CAM development. The 16K fragments inhibited angiogenesis in growing capillaries, and the intact hormones had no effect. However, full length hormones stimulated angiogenesis in the quiescent vasculature of later stage CAMs, while 16K fragments have no effect. These results suggested that the intact and 16K fragments might have opposite effect on angiogenesis. Using PAI-1 as an marker, we showed that 16K hGH did not signal via the hGH receptor. Furthermore, the 16K fragments utilized similar signaling mechanism in stimulating PAI-1. Together with their similar biological activity, it is likely that the 16K fragments interact with the same receptor or receptors belonging to the same family.

The physiological roles of 16K hPRL family members remain unknown. Some evidence suggests that these hormones might be involved in the development of the vascular connection of the placenta. Angiogenesis is essential in placental development (Folkman, 1992). It appears to be regulated through the expression of both positive and negative angiogenic factors. In mouse placenta, sequential expression of PLF and PRP is correlated with the initiation and termination of angiogenesis (Jackson, 1994). Both hormones belong to PRL family. The placenta is a major source of PRL production outside of the pituitary (Golander, 1978; Handwerger, 1991; Rosenberg, 1980). Furthermore, large amounts of hPRL, hGHV and hPL are produced at the deciduoplacental interface (Ben-Jonathan, 1996; Walters, 1983). High levels of 16K PRL binding can be observed in second and third trimester human placental membrane preparations, but not in first trimester membranes (Bentzien, F., unpublished data). These observations are consistent with the idea that the formation of the 16K fragments could play a regulatory role in development of the vascular connections between the fetus and mother. Antiangiogenic

activity would be low during the early rapid phase of vascular development and be slowed in later stages of pregnancy.

The formation of several antiangiogenic factors appears to involve proteolytic processing of precursor molecules. These factors include PRL (Ferrara, 1991), thrombospondin (Tolsma, 1993), fibronectin (Homandberg, 1985), platelet factor-4 (Gupta, 1995), EGF (Nelson, 1995), angiostatin (Gately, 1996; O'Reilly, 1994) and endostatin (O'Reilly, 1997). These findings raise the possibility of a general mechanism of generating antiangiogenic factors locally by specific proteolytic cleavage of a larger protein. The angiogenic effects of hPRL, hGH, hPL and hGHV and antiangiogenic effects of 16K hPRL, 16K hGH, 16K hPL and 16K hGHV represents a new concept for the regulation of angiogenesis. A single molecule can stimulate or inhibit angiogenesis depending on its molecular form: the full-length molecule stimulating angiogenesis and the proteolytically processed molecule inhibiting angiogenesis. Regulation of angiogenesis could be controlled by both the production of the full length molecules and the processing of the fragments by proteases.

Proposed further studies

From these results, we showed that the antiangiogenic factor 16K PRL inhibited uPA activity by stimulating PAI-1 production. The signaling mechanism utilized by 16K hPRL to stimulate PAI-1 involved a serine/threonine kinase, which was neither PKA nor PKC . In order to understand the initial events in the signaling mechanisms for 16K hPRL it is imperative that the receptor is identified. We unsuccessfully attempted to clone the receptor by expression cloning in COS cells with a BBE cell cDNA library. Since, 16K PRL and TGF β share signaling pathways in activating PAI-1 expression, one could hypothesize that 16K hPRL signals via a TGF β receptor family member. Therefore, an additional strategy for cloning the 16K PRL receptor could be by homology to the TGF β receptor family. An alternative approach would be by protein purification of solubilized 16K PRL receptor from BBE cells by affinity purification. Recombinant 16K hPRL containing a His tag on the C-terminus would be utilized as the affinity ligand, and would be separated on nickel columns.

To better understand the signaling mechanisms mediating the actions of 16K PRL, we could study the 16K PRL response element in the promoter region of PAI-1. The Adenovirus system used in our studies worked reasonably well. An Adenovirus vectors containing overlapping regions of the PAI-1 promoter region are necessary to clearly define the response element for 16K PRL. Once a response element is isolated, we could further investigate the transcription factor involved and how these factors are activated by 16K PRL. These findings in conjunction with the receptor studies, would provide substantial information of the signaling mechanisms regulating the biological actions of 16K PRL. This information would help in the development of agonists and antagonist to the 16K PRL receptor, which would be potential therapeutic agents.

The ultimate goal of studies involving 16K hPRL is to develop antitumoral agents. We are currently preparing mg amounts of the recombinant 16K hPRL produced in bacterial and mammalian cells. This will permit detailed animal studies using a variety of tumor models to test the efficacy. The physiological role of the 16K PRL/GH family members in the control of the vascularization of the placenta is an additional area of potential medically related importance. The rate and time course of the formation of the 16K antiangiogenic factors could be an important mechanism in the etiology of diseases of pregnancy.

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