AN ABSTRACT OF THE THESIS OF

Adel Abdulla Al-Hozab for the degree of Doctor of Philosophy in Animal Science presented on April 27, 1990.

Title: The Effects of Hormones and Inducers of Intracellular Messengers on Bovine Embryo Development In Vitro: Plasminogen Activator Production and Changes in Embryonic Size.

Abstract approved: Redacted for Privacy
Alfred R. Menino, Jr.

The effects of several hormones and inducers of intracellular messengers on plasminogen activator (PA) production and changes in embryonic size by cultured bovine embryos were evaluated. Day 8 embryos were cultured in Ham's F-12 with 1.5 mg/ml bovine serum albumin (BSA) containing different levels of progesterone (P), estradiol-17β (E_2), dexamethasone (Dex), retinoic acid (RA), dibutyryl cyclic AMP (dbcAMP), or phorbol myristate acetate (PMA) for 5 days under paraffin oil in a humidified atmosphere of 5% CO_2 in air at 37°C. The concentrations of PA in the conditioned media were determined by a caseinolytic assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography were used to determine the molecular weight of PA in the medium and in the embryo homogenate. Changes in embryonic size were determined by measuring overall embryo diameter (OD) at 24-h intervals. None of the hormones and agents tested herein had a significant effect on PA production. Dimethyl sulfoxide (DMSO) which was used to dissolve PMA significantly inhibited PA production during the first 72 h of

culture. Time of culture, however, exerted a significant effect on PA production by cultured embryos. The production of this protease was low during the first 48 h, increased during 72 and 96 h, and either remained high or slightly decreased toward the end of the culture period. Furthermore, the peak production of PA was attained 48 h after hatching. The molecular weight of PA in the conditioned medium and embryo tissues suggested that the bovine embryo at this developmental stage produced an urokinase-type PA. With the exception of dbcAMP and PMA, the hormones tested in this study did not affect embryonic size. While dbcAMP decreased OD later in culture, PMA enhanced OD throughout culture. The mechanism by which dbcAMP and PMA modulated embryonic size is not clear. These results suggest that cultured bovine embryos produce urokinase-type PA in a time dependent manner and the production of this enzyme is independent of exogenous hormonal regulation.

The Effects of Hormones and Inducers of Intracellular Messengers on Bovine Embryo Development In Vitro: Plasminogen Activator Production and Changes in Embryonic Size

by

Adel Abdulla Al-Hozab

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed April 27 1990

Commencement June 1990

APPROVED:

Redacted for Privacy

Professor of Animal Science in charge of major

Redacted for Privacy

Head of Department of Animal Science

Redacted for Privacy

Dean of Graduate School

Date thesis is presented April 27, 1990

Typed by Cindy Withrow for Adel Abdulla Al-Hozab

DEDICATION

To my mother and father, to my wife and sons, to all my family and friends, I dedicate this manuscript.

ACKNOWLEDGMENT

I thank Dr. Alfred Menino, my major professor, for his continued support and encouragement every step of the way. His scientific excellence and humorous personality inspired me to work hard, and yet enjoy and appreciate it. He taught me how to think scientifically and how to harness technical problems. Special thanks are also extended to my committee members for their time and effort. Thanks to my laboratory colleagues: Jane Williams, Hai Yan Li, Arie Dyk, N. Y. Kim and Linda Kjerulf for their technical contributions and above all their friendship. I would also like to thank my friend, Dr. Abdullah Al-Massomi, for his valuable help in preparing graphs and figures. Thanks to my brother, Ibraheem, for his constructive criticism during the course of this manuscript preparation. Lastly, I am most grateful to my wife, Hoda, for her endless support, her many sacrifices, and for giving me the reason to work hard: my three sons Abdulla, Abdulaziz and Ibraheem.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

The Effects of Hormones and Inducers of Intracellular Messengers on Bovine Embryo Development In Vitro: Plasminogen Activator Production and Changes in Embryonic Size

INTRODUCTION

Plasminogen activators (PA) are serine proteases that convert the zymogen plasminogen to the active protease plasmin by the cleavage of an Arg-Val peptide bond. Two types of PA have been identified by functional differences, molecular weight (MW), and immunologic activities: urokinase-type (uPA) and tissue-type (tPA) with MW of 30-55 and 70 kD, respectively (Dano et al., 1985). The PA are products of two distinct genes which have been isolated and sequenced (Degen et al., 1987; Rajput et al., 1987). Tissue-type PA displays a high affinity for fibrin, thus it is considered as the key enzyme in thrombolysis (Astrup, 1978). Urokinase-type PA has been suggested to participate in the localized proteolysis of the extracellular matrix accompanying tissue remodeling and cellular migration (Blasi et al., 1987).

Beside many types of normal and malignant cells, PA is produced by cultured embryos of mice (Strickland et al., 1976), rats (Liedholm and Astedt, 1975), swine (Mullins et al., 1980), cattle (Menino and Williams, 1987), and sheep (Menino et al., 1989). Although the function of PA in early embryo development is obscure, it was implicated in cellular migration and implantation in mouse embryos (Sherman et al., 1976; Strickland et al., 1976), tissue remodeling and proliferation in swine embryos (Fazleabas et al., 1983), and facilitating embryo hatching in cattle and sheep (Menino and Williams, 1987; Menino et al., 1989).

The extracellular proteolytic activity of the PA/plasminogen system is regulated by a delicate balance between PA, plasminogen, and their endogenous inhibitors. Several agents have been shown to modulate plasminogen activation in a variety of cell types. Gonadotropins stimulated PA activity in rat granulosa cells (Strickland and Beers, 1976; Reich et al., 1985; Canipari and Strickland, 1986), cultured human cytotrophoblasts (Queenan et al., 1987), and rat and bovine Sertoli cells (Lacroix et al., 1981; Hettle et al., 1986; Jenkins et al., 1988). The effects of gonadotropins were presumably mediated by cyclic adenosine monophosphate (cAMP). Estrogen stimulated production of PA in human breast carcinoma cell lines (Dickerman et al., 1989) and enhanced plasminogen uptake by the mouse uterus (Finlay et al., 1983). Glucocorticoids, such as dexamethasone, inhibited PA activity in rat granulosa cells (Harlow et al., 1987) and bovine Sertoli cells (Coombs and Jenkins, 1988) by stimulating the production of PA-inhibitors (PAI). Similarly, progesterone was found to stimulate production of protease inhibitors in mouse and pig uteri (Harpel et al., 1966; Mullins et al., 1980), thus inhibiting fibrinolytic activity within the uterus during specific stages of the estrous cycle. Phorbol myristate acetate (PMA) and retinoic acid (RA) are also potent inducers of PA production in a number of different cells and tissues (Strickland and Mahdavi, 1978; Wilson and Reich, 1978; Opdenakker et al., 1983; Santel and Levin, 1988).

Hormonal regulation of embryonic PA production has not been studied. Therefore, the objective of this study was to evaluate the effects of progesterone (P), estradiol-17β (E_2), dexamethasone (Dex), retinoic acid (RA), dibutyryl cyclic AMP (dbcAMP), and phorbol myristate

acetate (PMA) on PA production in cultured bovine embryos. Changes in embryonic size were also evaluated to assess embryo viability in response to the hormonal treatments.

LITERATURE REVIEW

The significance of proteolytic enzymes in biological control mechanisms has been recognized since the turn of the century when Bayliss and Starling (1902) described the role of secretin in stimulating the production of pancreatic juice. Several proteases have been discovered in the gastrointestinal tract of higher animals which facilitate digestion and absorption of nutrients. Another class of proteolytic enzymes resembling the gastrointestinal proteases was found in blood plasma, sperm, and many other tissues. These enzymes participate in various control functions such as coagulation, fibrinolysis, tissue remodeling and cellular migration.

Plasminogen activators (PA) are serine proteases that cleave the inactive zymogen, plasminogen, to the active enzyme plasmin. Plasminogen activators and plasmin are the major components of the fibrinolytic system and the main physiological role for this system is the dissolution of fibrin clots formed in blood vessels. Several other functions of PA have been proposed in a variety of normal and abnormal processes such as tissue remodeling, ovulation, tumor proliferation, and cellular migration.

This literature review will be divided into three parts. The first part will be devoted to the biochemical properties of plasminogen, PA, and their inhibitors. The second part will cover the various functions of these enzymes including their physiologic role in the reproductive system. The final part will discuss the regulatory mechanisms controlling PA activity.

I. Biochemical Properties of Plasminogen and Plasminogen Activators

1. Plasminogen and Plasmin

Plasminogen is the inactive precursor of the proteolytic enzyme plasmin. Highly purified plasminogen can be obtained from several species by a combination of affinity binding to agarose L-lysine columns and ion-exchange chromotography on carboxymethyl-cellulose or diethylaminoethyl-Sephadex. Human plasminogen isolated by such methods has a molecular weight (MW) of 83-92 kD. It is composed of a single chain glycoprotein consisting of 790 amino acids, with a glutamine residue at the amino-terminus and an asparagine residue at the carboxy-terminus. Two major forms of plasminogen have been identified: one containing glutamine at the amino-terminus (Glu-plasminogen), and the other having lysine at the amino-terminus (Lys-plasminogen) (for a review, see Christman et al., 1977). Glu-plasminogen has an electrophoretic mobility similar to plasminogen from fresh plasma, whereas Lys-plasminogen has a higher isolectric point, suggesting that Lys-plasminogen has been proteolytically modified during the purification procedure (Wallen and Wiman, 1970). When purified Glu-plasminogen is treated with plasmin, a small peptide of about 8 kD is cleaved from the amino-terminal to yield a modified plasminogen (Lys-plasminogen) with Lys-77 as the new amino-terminus (Ryan, 1987).

Plasmin is generated from either Glu-plasminogen or Lys-plasminogen by proteolytic cleavage of a single peptide bond between Arg-560 and Val-561. Plasmin is a two chain molecule in which the polypeptide chains are held together by two disulfide bonds. The heavy (A) chain has a molecular weight of 48-63 kD and

contains the carbohydrate units as well as the lysine binding sites. The light (B) chain of plasmin has a molecular weight of 20-25 kD and contains the serine protease portion of the molecule. The light chain has amino acid sequence homologies with other serine proteases, such as trypsin and chymotrypsin, and has an active site composed of Ser, His and Asp. Plasmin has typical trypsin-like specificity and hydrolyzes proteins at Lysyl and arginyl bonds. Plasmin cleaves many substrates such as casein, protamine, cell membrane proteins and some hormones. It displays a high specificity toward fibrin and fibrinogen and it has been shown that plasmin is 50-100 times faster than trypsin in clot lysis. The difference in the rate of hydrolysis is due mainly to the presence of lysine-binding sites, which are not present in trypsin. (For reviews, see Dano et al., 1985; Christman et al., 1977; Ryan, 1987).

There is a high degree of homology in plasminogen and plasmin among the different mammalian species. Summaria and coworkers (1973) compared highly purified preparations of cat, dog, rabbit, and bovine plasminogens with human plasminogen, and found that the molecular weights among plasminogens were similar. Moreover, they found that the heavy and light chains of plasmin from these different species were similar to the human. The complete amino acid sequence of human, bovine, and porcine plasminogen have been established and demonstrate an overall homology of 78%. Highly conserved sequences were found among the light chains (80% homology) and heavy chains (83%). Despite the similarities in sequence and molecular features, plasminogens of various species

differ in their activation with streptokinase. Human plasminogen is efficiently activated by trace amounts of streptokinase, whereas bovine, ovine, and porcine plasminogens are not activated by such meager amounts. This is probably due to differences in the amino acid sequence in certain regions of the plasminogen from these species (Schaller and Rickli, 1988; Schaller et al., 1985).

The heavy (A) chain of plasmin is characterized by five triple loop structures known as kringles. Kringle domains are also present in prothrombin, urokinase, and several other fibrinolytic proteins. The kringle structure has a molecular weight of about 10 kD, and it is crosslinked internally by three cystine linkages. These domains play a very important role in specific protein-protein interactions. Several workers suggested that kringles mediate the attachment of plasminogen or plasmin to the fibrin matrix of blood clots. Moreover, these kringles are able to bind lysine, and thus facilitate fibrin-binding capability of plasminogen (Petros et al., 1988; Thewes et al., 1988).

Receptors for plasminogen and plasmin have been identified in human tumor cell line SW 1116. These receptors are the same for both proteins, but exhibit higher affinity for plasmin. Bound plasmin retains its enzymatic activity, and may activate pro-urokinase resulting in the formation of urokinase, which in turn increases the binding of plasminogen and plasmin. Ultimately, this process increases the concentration of plasmin at the surface of tumor cells, enabling them to degrade the connective tissues and component proteins of the basement membrane (Burtin and Fondaneche, 1988).

Plasma contains several protease inhibitors which regulate the fibrinolytic system. α_2-antiplasmin (AP) and α_2-macroglobulin are the most important inhibitors of plasmin. α_2-antiplasmin is characterized by rapid inactivation of plasmin, while α_2-macroglobulin exerts its action only when AP levels are depleted (Dano et al., 1985). The fast-reacting property of AP is attributed to the ability of AP to bind Lys-binding sites of plasmin. This association brings together the active site of plasmin with the reactive site of AP, resulting in a covalent complex which is enzymically inactive. A recent study showed that the C-terminal lysine residue of AP plays a major role in binding AP to plasmin. α_2-antiplasmin will lose its ability to inhibit plasmin when the C-terminal of AP is cleaved by carboxypeptidase-B (Hortin et al., 1988). α_2-antiplasmin is homologous with antithrombin III, α_2-antitrypsin, and other serine-proteinase inhibitors (serpins). It is a single-chain glycoprotein with a MW of 70 kD, and consists of 452 amino acids (for review see Rijken et al., 1988).

2. Plasminogen activators

Christensen (1945) was the first to demonstrate that plasminogen can be converted into plasmin by a streptococcal factor (for review, see Christman et al., 1977). Two major plasminogen activators have been identified by functional differences and by differences in molecular weight and immunological reactivities: Urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) with molecular weights of 30-55 and 70 kD, respectively (Dano et al., 1985).

a. Urokinase-type plasminogen activator.

Urokinase-type (uPA) plasminogen activator is the best characterized of the endogenous PA. It is a serine protease isolated from human urine, as the name suggests. Urokinase is a glycoprotein synthesized as an one-chain proenzyme (MW=55 kD) with little or no PA activity. The zymogen is converted to the active enzyme (MW=55 kD) by limited proteolysis with plasmin (Vassalli et al., 1984). Active urokinase consists of two polypeptide chains bridged by a disulfied bond, and exists in high and low molecular weight forms (55 and 33 kD, respectively). The high molecular weight form consists of a catalytic subunit (B-chain) with a MW of about 33 kD which is homologous to the catalytic subunit of other serine proteases. The non-catalytic unit (A-chain) of the high MW form contains one kringle domain and a domain required for interaction with cellular receptors. The low MW form is a proteolytic product of the two-chain uPA lacking most of the A chain but retaining the active site-containing the B chain (Dano et al., 1985; Hart and Rehemtulla, 1988; Vassalli et al., 1985).

Recent studies indicate the presence of a single-chain form of uPA (scuPA), and it has been purified from several natural sources, including urine (Lijnen et al., 1986). This form, in contrast to one-chain pro-urokinase, has considerable interinsic plasminogen-activating activity. When scuPA and plasminogen are combined, two-chain uPA (tcuPA) and plasmin were formed. Addition of plasmin inhibitors to this mixture abolishes the generation of tcuPA but not the activation of

plasminogen to plasmin, suggesting that scuPA activates plasminogen directly (Lijnen et al., 1986). A model has been proposed to explain the role of scuPA in plasminogen activation. In the first reaction scuPA directly activates plasminogen to plasmin, plasmin then converts scuPA to tcuPA, and in the final reaction, plasminogen is converted to plasmin by tcuPA (Lijnen and Collen, 1988). Lijnen and his colleague (1988) also used site-directed mutagenesis of certain amino acids comprising recombinant scuPA (rscuPA) to study the enzymatic properties of the scuPA and tcuPA. Their findings suggested that the enzymatic properties of rscuPA are dependent on the amino acids in position 158 and 159. Substitution of the basic amino acid in position 158 resulted in a 10-20 fold reduction of the catalytic efficiency of scuPA, but yielded a fully active tcuPA. The presence of Ile in position 159 is the primary determinant for the activity of the single and two-chain derivatives. They concluded that scuPA constitutes the primary active principle; however, the catalytic efficiency of tcuPA is higher than scuPA. Single chain uPA can be converted by thrombin to an inactive two-chain derivative (thrombin-derived tcuPA), which can be converted to active two-chain uPA by treatment with plasmin. The active tcuPA generated from thrombin-derived tcuPA is similar to tcuPA obtained by digestion of intact scuPA with plasmin (Lijnen et al., 1987).

The previous findings of Lijnen and his colleagues (1986) have been challenged by several workers. Petersen and his

coworkers (1988), believed that human and murine cells released uPA as a single-chain proenzyme that has little or no plasminogen-activation capacity. They compared the plasminogen activating capacity of single and two-chain uPA using ^{125}I-plasminogen conversion assay in the presence of high amounts of plasmin inhibitor. Single-chain uPA has a maximal intrinsic activity of about 0.4% of the two-chain uPA activity. They noted that one-chain uPA has a variety of other features similar to the one chain proenzyme forms of other serine proteases. Therefore, they classified scuPA as a genuine proenzyme form of uPA.

The complete amino acid sequence of human and porcine uPA has been established. Moreover, the mRNA of uPA has been identified and characterized, and cDNA have been constructed. Using this information, the human uPA gene was identified to be in the long arm of chromosome 10 (for review, see Dano et al., 1985). Comparisons of the murine uPA gene to porcine and human uPA genes revealed a high degree of evolutionary sequence conservation of the base pair constituents of exons and introns (Degen et al., 1987).

Urokinase type PA is synthesized and released by a wide variety of cell types, such as monocytes, macrophages, polymorphonuclear leukocytes, implanting trophoblasts, and malignant tumor cells (Vassalli et al., 1984; Strickland et al., 1976; Markus et al., 1980). Specific uPA receptors were detected in human monocytes and cells of monocyte line U937. These membrane receptors bind the high MW uPA (55 kD) with

high affinity, but do not bind the low MW uPA (33 kD). These findings demonstrate the importance of the non-catalytic A-chain of 55 kD uPA, and suggest the presence of a cellular interaction domain within the A-chain of uPA. Enzyme-bound uPA has a great importance in initiating localized lysis of extracellular matrix, thereby facilitating monocyte migration (Vassalli et al., 1985).

Urokinase catalyzes the conversion of plasminogen to the active protease, plasmin, by the hydrolysis of Arg-560-Val-561 peptide bond. Plasminogen is the only well-documented protein substrate for uPA (Dano et al., 1985); however, Keski-Oja and Vaheri (1982) found that purified urokinase cleaved a 66 kD protein component of pericellular matrix of cultured human lung fibroblasts. The resulting 62 kD protein remained attached to the matrix, suggesting that it was derived from the 66 kD protein. Therefore, they concluded that the 66 kD protein was the first known extracellular target for urokinase. Baron-Van Evercooren and coworkers (1987) showed that the proliferation of Schwann cells from newborn rat dorsal root ganglia is stimulated in a dose-dependent manner by urokinase. This mitogenic effect was observed in the absence of plasminogen, suggesting that plasminogen was not the substrate of urokinase in the developing nervous system.

Plasminogen activation by uPA is species specific to some degree. Chicken uPA fails to activate human plasminogen, but human uPA activates chicken plasminogen (Dano et al., 1985).

b. Tissue-type plasminogen activator.

Tissue-type PA has been identified and isolated from several different tissue extracts: human uterus, porcine kidney, heart and ovary, and many other normal, as well as malignant, tissues (Dano et al., 1985). Tissue-type PA is a serine protease synthesized as a proenzyme with a MW of 70 kD and composed of 530 amino acids and 3-4 N-glycosylation sites. Limited proteolysis by plasmin, kallikrein, or factor Xa cleaves the Arg-278-Ile-279 bond of the proenzyme yielding two polypeptide chains linked by disulfied bonds. The heavy chain (A-chain) is derived from the amino-terminal, and has a MW of 40 kD, whereas the carboxy-terminal of the molecule comprises the light chain (B-chain) and has a MW of 30 kD. (For review, see Degen et al., 1986).

The catalytic site of tPA is located in the B-chain, and is composed of His-322, Asp-371, and Ser-478 residues. The heavy (A) chain consists of several structural domains and shares a high degree of homology with other hemostatic proteins. These domains are categorized as the fibronectin finger domain, the epidermal growth factor (EGF) domain, and the two kringle domains (I and II) (Gerard et al., 1986).

The nucleotide sequence of human tPA has been established, and compared with urokinase; an evolutionary relationship of the two human plasminogen activators is apparent (Degen et al., 1986). The tPA gene is located on chromosome 8 in the human and mouse (Rajput et al., 1985; Rajput et al., 1987). Complementary DNA (cDNA) of human tPA had been constructed and

several deletion mutants lacking specific functional domains have been generated (Van Zonneveld et al., 1986). These mutant tPAcDNA provide a powerful tool for studying the physiological significance of the structural domains of tPA.

Tissue-type PA like uPA, catalyzes the conversion of plasminogen to plasmin by cleaving the Arginyl-Valyl peptide bond; however, a major difference between the two PA is the high affinity of tPA for fibrin. In the absence of fibrin, tPA is a poor plasminogen activator due to its low affinity for the substrate, but the activity of tPA is strikingly enhanced with fibrin. Fibrin provides a surface for tPA and plasminogen yielding a ternary complex, thus enhancing plasminogen activation (Hoylaerts et al., 1982). The binding site of fibrin is located in the non-catalytic (A) chain. Van Zonneveld and his colleagues (1986) used tPA mutants with specific deletions of the heavy A-chain domains. They found that the deletion of Kringle-II reduces plasminogen activation by tPA in the presence of fibrin, while deletion of both Finger and Kringle-II domains abolished the enzyme activity. Therefore, the Finger domain and Kringle-II are responsible for the interaction of tPA with fibrin. A very interesting study was conducted by Dirk and his colleagues (1987) who used a recombinant fusion molecule of the Finger domain of tPA with scuPA, and demonstrated that the chimeric protein did not acquire the fibrin affinity of tPA. Fusion of the amino-terminal (A-chain) of tPA, which contains the structures responsible for fibrin binding, with the carboxy-terminal of

uPA, which contains the catalytic region of the molecule, resulted in improve fibrin-mediated plasminogen activation (Nelles et al., 1987). The results of these two studies suggest that the functional domains of the heavy- (A) chain may not be entirely independent.

Tissue-type PA is a glycoprotein containing approximately 7% carbohydrate. The carbohydrate moieties may not influence the activity of the enzyme, however, glycosylation plays an integral part in the transport of the protein out of the cell (Hart and Rehemtulla, 1988). Hansen and coworkers (1988) studied the role of Asn-linked oligosaccharide in non-glycosylated and glycosylated tPA. The non-glycosylated tPA showed higher fibrin binding, as well as greater fibrinolytic potency than the glycosylated form. Their results indicated a significant role for Asn-linked oligosaccharide in fibrin binding and fibrinolytic potency of tPA.

Several reports have documented the existence of several variants of native tPA. Two variants have been isolated from the Bowes melanoma that differ by 3 kD from the native tPA. This MW difference was localized in the amino-terminal of the molecule. Differences in glycosylation pattern also lead to the appearance of type I and II variants, with an apparent MW difference of about 3 kD in the A-chain of tPA (Hart and Rehemtulla, 1988; Grossbard, 1987). Immunoblot analysis, using antibodies against tPA identified different types of tPA variants (I to IV) in melanoma cell culture fluids, indicating

that the composition of tPA is more complex and heterogenous (Opdenakker et al., 1988).

Cell surface receptors, specific for tPA, have not been identified as yet; however, the existence of an efficient clearing system in the liver may suggest the presence of tPA receptors. The rate and site of clearance of uPA is similar to tPA. Urokinase receptors were identified in the liver, and it is possible that a common receptor for both plasminogen activators might exist in this organ (Hart and Rehemtulla, 1988). The clearance rate of a mutant tPA lacking the growth factor domain was compared with normal tPA. Mutant tPA was cleared much more slowly than normal tPA, indicating a role for the growth factor domain in recognition of tPA by the hepatic clearance system. Therefore, the growth factor domain may constitute a determinant recognized by a receptor in the liver (Browne et al., 1988). Two types of recognition sites for tPA have been identified in the liver: a high affinity specific tPA site on parenchymal cells and the mannose receptor on endothelial cells which recognizes the carbohydrate moiety of tPA. These sites are involved in the clearance process of tPA from the circulation (Kuiper et al., 1988).

3. Plasminogen activators inhibitors.

Inhibitors of PA can be divided into two classes. The first class comprises the general protease inhibitors, such as the macromolecular inhibitors, metal ions, and organic molecules. These molecules are not specific for PA and their inhibitory

effects may include a wide variety of plasma proteases. The second class of PA inhibitors includes newly purified proteins that exhibit high specificity toward PA.

a. Non-specific plasminogen activator inhibitors.

Diisopropyl fluorophosphate (DFP), a reagent which binds to the active site of serine proteases, inhibits both uPA and tPA as well as plasmin. While bovine pancreatic trypsin inhibitor (Trasylol, aprotinin), soybean trypsin inhibitor, and lima bean trypsin inhibitor exert a strong inhibitory effect on plasmin, they fail to measurably inhibit PA. Zinc is the most potent inhibitor of PA of all metal ions studied. ω-Aminocarboxylic acids affect PA-mediated fibrinolysis in a complex manner. (For review, see Dano et al., 1985).

b. Specific plasminogen activator inhibitors (PAI).

Extracellular PA activity is mainly regulated by specific high affinity inhibitors. Four distinct PA inhibitors (PAI) have been identified: endothelial type PAI (PAI-1), placental type PAI (PAI-2), urinary type PAI (PAI-3), and protease nexin-1 (Laiho et al., 1987)1.

Plasminogen activator inhibitor-1 is the main PAI present in plasma and serum. Plasminogen activator inhibitor-1 inhibits uPA, as well as tPA by forming stable complexes. Plasminogen activator inhibitor-1 has been purified and characterized, a cDNA has been cloned, and the amino acid sequence deduced from the nucleotide sequence. These data have clearly demonstrated that PAI-1 belongs to the

superfamily of serpins (Lambers et al., 1987). Plasminogen activator inhibitor-1 is a glycoprotein with a MW of 50 kD, and is encoded by a gene located on the long arm of chromosome 7 (Mayer et al., 1988). Several human cells produce large amounts of PAI-1 such as endothelial cells, platelets and fibroblasts. Immunological studies using monoclonal antibodies showed that PAI-1 from different cell types is highly cross-reactive (Hart and Rehemtulla, 1988). Plasminogen activator inhibitor-1 forms SDS-stable complexes with both PA yielding proteinase-inhibitor complexes detectable by zymography (Hart and Rehemtulla, 1988).

Plasminogen activator inhibitor-1 appears to exist in two immunologically similar forms, an inherently active form and a latent form which has no inhibitory activity. Treatment with denaturants such as SDS or ammonium thiocyanate activate the latent form. Cell-associated PAI-1 has similar molecular and immunological characteristics with the latent form of PAI-1, but is not affected by SDS treatment, suggesting that PAI-1, synthesized as the active form, is a precursor to the latent form (Eugene et al., 1987). Mimuro and coworkers (1987) identified PAI-1 in the extracellular matrix (ECM) of cultured bovine aortic-endothelial cells (BAE). They concluded that PAI-1 is produced and released into the medium where it is rapidly inactivated or into subendothelium where it binds to ECM. The ECM-associated PAI-1 is active and stable, indicating that ECM protects PAI-1 from inactivation. In a subsequent study Mimuro and Loskutoff (1989a) demonstrated the

presence of protease-sensitive structures on ECM of BAE that bind active PAI-1 tightly and selectively, stabilizing PAI-1 against the spontaneous loss of activity. Bovine plasma contains protein(s) that bind PAI-1 and prevent its interaction with ECM. The PAI-1-binding protein has three polypeptides with MW of 65 kD, 80 kD, and 57 kD, and share immunological properties similar to vitronectin (Mimuro and Loskutoff, 1989b). Another binding protein for PAI-1 was isolated from human plasma, with a MW of 150 kD, and it reacted only with the active PAI-1. This binding protein may play an important role in regulating the activity of PAI-1 (Declerck et al., 1988).

Masson and Angles-Cano (1988) used radioisotropic and spectrophotometric analysis to study the kinetics of inhibition of tPA by active PAI-1 in homogeneous (plasma) and heterogeneous (solid-phase fibrin) systems. The tissue-type PA soluble phase is rapidly inhibited by PAI-1, whereas fibrin-bound tPA is protected from inhibition. Furthermore, PAI-1 interferes with the binding of fibrin to tPA in a competitive manner. These data suggest the existence of an equilibrium distribution of tPA between the amount of fibrin generated and the concentration of circulating inhibitor, which participate in a control mechanism for the fibrinolytic activity of human plasma.

Plasminogen activator inhibitor-2 has been purified from several cell types including human trophoblastic epithelium, leukocyte-derived cell lines and fibrosarcoma cells.

Plasminogen activator inhibitor-2 is immunologically distinct from PAI-1. It exists in two different forms; a 47 kD, non-glycosylated form and a 60 kD, glycosylated form, both sharing identical inhibitory effects of PA. The cDNA of PAI-2 has been cloned, and the amino acid sequence deduced from this cDNA confirmed that this PAI belongs to the serpin family. Plasminogen activator inhibitor-2 also can form an SDS-stable complex with PA, and may be involved in regulating extracellular PA activity (for review, see Kruithof, 1988; Hart and Rehemtulla, 1988).

Plasminogen activator inhibitor-3 was first identified and purified from human urine. It is a glycoprotein with an apparent MW of 50 kD, and forms an SDS-stable complex with mainly urokinase. Plasminogen activator inhibitor-3 is antigenically distinct from PAI-1, PAI-2 and protease nexin. Plasminogen activator inhibitor-3 purified from plasma has the same MW, amino acid composition, and PA inhibitory effect as the urinary protein. Kinetic analysis showed that PAI-3 has an intermediate rate with urokinase and slow activity with tPA (Stump et al., 1986).

Protease nexin (PN) is a glycoprotein with a MW of 47 kD. It was first isolated from the conditioned medium of fibroblasts and other cells. Protease nexin, unlike other PAI, is not specific for PA; PN also binds to plasmin, thrombin, and trypsin (Kruithof, 1988).

II. Physiological Role of Plasminogen Activators

Plasminogen activators are found in a wide variety of normal and neoplastic cells and tissues, suggesting these proteases have a physiological role in several biological processes. Both enzymes have been implicated in the degradation of extracellular matrices in several normal and tumor-associated processes. It is beyond the scope of this review to list and discuss all biological functions of PA, however, a fairly thorough literature review will be provided for the role of PA in various reproductive processes.

1. Fibrinolysis.

As stated by Ranby and Brandstrom (1988), "Fibrinolysis is an integrated part of hemostasis, a collective name for the process involved in keeping the circulatory tract intact yet free from occlusive deposits." The role of PA has been most extensively studied in fibrinolytic processes because of their potential use in therapeutic treatment of some pathological conditions associated with this process. Fibrin is the major component of clots and it enhances the activity of tPA. The concentration of tPA in blood increases after clot formation and tPA, not uPA, has been found in the venous endothelial cells. These findings strongly point to tPA, rather than uPA, as the key enzyme in the fibrinolytic system (Dano et al., 1985). Besides tPA, the fibrinolytic system has several components such as plasminogen, α_2-AP, and PAI-1. The control mechanism for tPA-mediated fibrinolysis is based on the synthesis, release, and clearance of other fibrinolytic components in the plasma (Ranby and Brandstrom, 1988).

Circulating platelet aggregates are important in the formation of thrombi. It has been shown that platelets provide a surface for tPA thereby enhancing plasminogen activation. Plasmin, once generated, degrades fibrin and promotes platelet disaggregation (Schafer and Adelman, 1985; Deguchi and Shirakawa, 1988). Lascalzo and Vaughan (1987) studied the disaggregation of human platelets by tPA and found that fibrinogen induced platelet aggregation by maintaining cohesion between platelet surfaces. Tissue-type PA induced fibrinogenolysis through selective proteolysis by plasmin and promoted platelet disaggregation. They concluded that tPA may be important in vivo for lysis of thrombi containing both fibrin and platelets.

Heparin and other sulphated polysaccharides may potentiate the fibrinolytic activity by promoting the release of tPA from endothelial cells. Heparin can bind to both plasminogen and PA resulting in greater PA activity. Fears (1988) found that heparin promoted the activity of PA and this effect was attenuated in the presence of soluble fibrin. The binding site for heparin was located on the A-chain of PA. If the effects of heparin occur in vivo, then in the presence of heparin the enhancement effect of fibrin on tPA will be diminished resulting in an increased systemic activation of tPA.

2. Malignancy

The relationship between proteases and tumor cells was reported as early as the first decade of this century. Several investigators in that period noted that explants of cultured cancerous tissue consistently caused dissolution of plasma clots

(for a review, see Dano et al., 1985). This phenomenon was explained a decade ago by Ossowski and her colleagues (1973). They demonstrated that the transformation of normal fibroblasts by oncogenic viruses caused an increase in extracellular proteolytic activity due mainly to the release of PA. Ossowski and coworkers (1973) compared the growth of normal and SV-40-transformed hamster embryo fibroblasts in the presence and absence of plasminogen. They discovered that the growth of normal and transformed cells proceeded at the same rate in the absence or presence of plasminogen, but the mobility and aggregation of transformed cell were retarded in the absence of plasminogen. They concluded that the fibrinolytic activity associated with malignancy was responsible for cell aggregation and migration. The morphological characteristics of transformed fibroblasts were altered by plasmin and these changes did not develop in the presence of soybean trypsin inhibitor. These data indicated the requirement of the fibrinolytic system for the morphological changes accompanying transformation of hamster fibroblasts (Ossowski et al., 1974).

Plasminogen activator activity was detected in many tumors such as breast cancer, lung cancer, endometrial carcinoma, and human prostatic cancer. Malignant tumors have a complex ECM composed of collagen, soluble glycoproteins (such as fibronectin and laminin) and fibrin. The interaction between malignant cells and the extracellular matrix is important to the invasion and metastatic processes. During metastasis, tumor cells first adhere to ECM components, then initiate degradation with matrix and migrate through the degraded products. High levels of PA activity

have been localized extracellularly suggesting that fibrinolytic activity is indeed involved in the degradation of extracellular matrix surrounding cancerous cells, thus facilitating their invasiveness and metastasis. The best support for this hypothesis comes from the inhibition of metastasis of human HEP-3 tumor in chicken embryos by antibodies raised against uPA (Dano et al., 1985).

Christman and coworkers (1975) used Brad-U sensitive melanoma B-16 to study the functional relationship between expression of PA and tumorigenicity. Melanoma cells produced appreciable amounts of PA in the absence of Brad-U. However, tumorigenicity and the expression of PA reversibly and concurrently disappeared when melanoma cells were grown in the presence of this drug. The precise mechanism by which Brad-U suppresses PA and tumorigenicity in these cells is not known.

The relationship between brain tumors and PA has not been fully investigated. Extracts of human brain tumors induce fibrinolysis. Varani and coworkers (1987) showed that different types of human brain tumor produced high levels of PA. These enzymes were capable of degrading ECM and influenced tumor-cell adhesion to ECM moieties. Specific MW patterns ranging from 36 kD to 100 kD were identified in five brain tumors suggesting a cell-specific origin of various PA forms. Malignant tumors showed a greater PA activity and a larger number of MW patterns when compared to normal brain tissues and benign tumors. Exposing tumor cells to radiation significantly reduced PA activity. Brain edema,

associated with brain tumors, correlated slightly with PA activity (Sawaya et al., 1988).

3. Inflammatory reactions.

Monocytes and macrophages are the major phagocytes participating in inflammatory reactions. These cells produce and release PA. Inflammatory agents enhance PA production, while anti-inflammatory agents, such as glucocorticoids suppress it. The physiological role of phagocytic PA is not fully understood, however, it may be involved in the proteolytic digestion of ECM necessary for these cells to migrate (Vassalli et al., 1984). Plasminogen activator secretion is a differentiation-linked property, with tPA being produced by primitive cells, such as macrophage progenitors, and uPA by more differentiated cells, like neutrophils and macrophages (Wilson and Francis, 1987).

Peritoneal macrophages, induced with inflammatory agents such as endotoxins, produced significant amounts of PA, whereas non-activated macrophages do not. Differences in PA production by the two cell populations may be due to cell age (Unkeless et al., 1974). Vassalli and coworkers (1974) proposed the following hypothesis to explain the relevance of PA to macrophages and inflammation. They suggested that macrophage precursors require an enzymatic mechanism to hydrolyze some of the supporting structures of blood vessels and allow their migration from the bone marrow to the circulation and from the circulation to the peritoneal cavity. Plasmin generated by PA released from macrophages will provide a localized proteolysis of the connective tissue structures of small

vessels, thereby facilitating the migration between various body compartments (Vassalli et al., 1976).

A recent study by Gudewicz and Gibola (1987) examined the effect of PA on the migration of human neutrophils. Their results indicated that human uPA exhibited chemoattractant activity in human neutrophils. Incubation of uPA with anti-uPA antibody completely abolished the chemotactic activity of uPA. Human tPA, with similar concentration as uPA, showed no chemotactic activity. They concluded that the chemotactic-stimulating activity of uPA plays a role in recruiting circulating phagocytes to the inflammatory site.

4. Nervous system.

Cell migration is necessary for the development and maintenance of several parts of the nervous system. Plasminogen activators have been identified in various neural cells, including Schwann cells, astrocytes, neuroblastoma cells, and sensory neurons. Plasminogen activator activity has been implicated in granule cell migration in the cerebellum, Schwann cell proliferation and migration, and memory storage (for review, see Pittman, 1985; Krystosek et al., 1988).

Pittman (1985) studied the patterns and properties of PA released by neurons from neonatal rat superior cervical, dorsal root, and trigeminal ganglia. Pittman (1985) found that neurons spontaneously released PA with similar immunological and physiochemical properties as uPA. Most PA was released by distal processes and/or growth cones suggesting that this enzyme may regulate growth cone function. Verrall and Seeds (1988) found that

neonatal mouse cerebellar cells release mainly tPA. Fibrin overlays localized PA to granule neurons, and the activity of this enzyme was blocked by anti-tPA antibodies. Verrall and Seeds (1988) also demonstrated that granule cell-associated tPA can be displaced by pH shock and exogenous mouse-tPA binds specifically to granule neurons. Accordingly, Verrall and Seeds (1988) claimed that these results supported the presence of a specific receptor for tPA on granule neurons where the enzyme retained its activity and may function in cell migration.

Neonatal rat dorsal root ganglia release both uPA and tPA. Urokinase-type PA is secreted by neurons while tPA is released by Schwann cells. Only uPA was shown to have a mitogenic effect on astrocytes and Schwann cells. This effect was observed in serum-free medium without plasminogen, suggesting the presence of an unknown substrate for PA other than plasminogen (Baron-Van Evercooren et al., 1987).

5. Other functions of PA.

Urokinase-type PA has been implicated in the migration and tissue remodeling in the bursa of Fabricicus. The production of PA by pancreatic islets of Langerhans has been correlated with the conversion of proinsulin to insulin. A similar correlation was found between PA and anterior pituitary hormones, suggesting that PA is involved in prohormone processing. Furthermore, PA have been related to angiogenesis, thymocyte maturation, development of neonatal skeletal muscles, and many other functions (Dano et al., 1985; Hantai et al., 1989; Fulton and Hart, 1987).

6. Reproduction.

Extracellular proteolysis and tissue degradation are involved in several reproductive phenomena. The distribution of PA in many reproductive organs is well documented and numerous studies have been conducted to further investigate the role of PA in male and female reproduction. The review of this subject will be subdivided into several categories describing the role of PA in female reproduction, male reproduction, and embryogenesis.

a. Female reproduction.

Involution of the mammary glands is characterized by considerable tissue degradation and remodeling. A strong correlation has been reported between mammary gland involution in mice and the activity of PA. Plasminogen activator activity in mammary glands tissue is inhibited in the presence of combinations of lactogenic hormones such as insulin, prolactin and hydrocortisone. Only uPA was identified in tissue extracts obtained from involuting mammary gland. Histological changes of mammary tissue during involution have some resemblance to the changes observed in metastatic growth of cancerous tissues. All these findings clearly demonstrate the importance of PA during involution of mammary glands which is necessary for preparing the udder for the next lactation (for review, see Dano et al., 1985).

Ovulation in mammals is a unique biological phenomenon because it requires the disruption of a healthy tissue at the surface of the ovary. This process involves many morphological changes at the site of rupture such as (1)

disruption of the dense layers of connective tissue surrounding the mature follicle, (2) extravasation of blood, (3) release of the ovum, (4) subsequent formation of a blood clot, and (5) wound healing (Espey, 1975). The changes that accompany ovulation necessitate the existence of highly efficient proteolytic and fibrinolytic mechanisms in order for these changes to occur. Plasminogen activators have been detected in large quantities during the ovulatory process indicating a close correlation between ovulation and PA.

Several workers studied the involvement of PA in the degradation of the follicular wall during ovulation. A close temporal correlation has been found between PA activity and the disruption of rat granulosa cells. Low PA activity was shown to be associated with granulosa cells obtained from immature animals. Comparative data have shown that granulosa cells from preovulatory follicles have high PA activity, while granulosa cells isolated from nonpreovulatory follicles in the same ovary exhibited low PA activity. Plasminogen, PA, and PAI have been detected in copious amounts in the follicular fluids. The activation of follicular plasminogen to plasmin by PA decreases the tensile strength of the follicular wall. The above findings led to the conclusion that PA plays a significant role during mammalian ovulation (Beers, 1975; Beers et al., 1975; Strickland and Beers, 1976).

Although the biochemical basis of ovulation is not firmly established, the critical role of gonadotropins and enzymatic activity are well documented. Beers and coworkers (1975)

attempted to elucidate the enzymatic basis of the events preceding ovulation. They proposed that granulosa cells elaborate increasing amounts of PA in response to the increased levels of circulating LH. This enzyme acts on follicular plasminogen, generating plasmin within the follicular wall. Plasmin, in turn, activates another protease, collagenase, which nicks the collagen helices and allows further degradation of collagen and the follicular wall by nonspecific proteases. The enzymatic degradation of the basement membrane will lead to the disruption of follicular integrity necessary for the release of the ovum. The ability of several inhibitors of PA and collagenase to block ovulation supports this hypothesis. Treatment of proestrous rats with ϵ-amino-caproic acid (CA) and benzamidine, inhibitors of serine proteases, including PA and plasmin, blocked ovulation and collagenolysis. Serine protease inhibitors significantly inhibited ovulation and collagenolysis only during the first two hours after hCG administration (Reich et al., 1985). By contrast, inhibition of ovulation and collagenolysis by collagenase inhibitor such as cysteine, was effective even when administered 7 h after hCG. These temporal relations support the role of PA and plasmin in the early events of the ovulatory process (Reich et al., 1985).

Gonadotropins, having a central role in ovulation, have been identified as the major controlling factor for follicular-associated PA activity (Beers et al., 1975). Gonadotropins stimulate the release of PA from inactive

ovarian granulosa cells in a time- and dose-dependent fashion. Follicle-stimulating hormone (FSH) was more effective in inducing PA activity than luteinizing hormone (LH) and prostaglandins E_1 and E_2 and cAMP effectively stimulated the production of PA by granulosa cells (Strickland and Beers, 1976). Canipari and Strickland (1986) compared the effect of FSH and LH on granulosa cells in culture and found that both hormones stimulated PA secretion; however, a lag phase in the pattern of enzyme secretion was noted with LH. They suggested that LH induction of PA is mediated by prostaglandins (PG). Evidence to support their claim came from the observation that LH stimulates PG synthesis and that treatment with PG inhibitors such as indomethacin suppresses PA secretion. These results may explain the ability of indomethacin to inhibit ovulation. The above findings contrasted the results presented by Shimada and coworkers (1983). They found that PG were not involved in the synthesis of PA in the rat ovary during the preovulatory period. This contrast can be explained by the difference in tissue extracts and analytical methods employed in these studies.

Two types of PA (uPA and tPA) have been demonstrated in granulosa cells. Liu and Hsueh (1987) studied the molecular patterns and secretion of PA in rat cumulus-oocyte complexes. They found that both uPA and tPA were present in the cumulus-oocyte complexes, while denuded oocytes contained only tPA. Human chorionic gonadotropin (hCG) treatment induced a time-dependent increase in tPA activity in both cumulus-oocyte

complexes and denuded oocytes. Urokinase-type PA activity was also detected after hCG injection. Morphological signs of cumulus cell expansion and dispersion have been observed after the increase in oocyte tPA activity. They concluded that oocyte tPA may be involved in oocyte maturation and cumulus cell expansion and dispersion. Immunohistochemical studies supported the conclusion that tPA induction by gonadotropin is correlated with follicle rupture (Liu et al., 1987). Substantial evidence for the role of tPA in ovulation was recently presented by Tsafriri and colleagues (1989) who examined the direct relationship between PA and ovulation using α_2-AP and an antibody raised against tPA. They found that anti-tPA and α_2-AP significantly inhibited ovulation only when injected at the time of hCG treatment. These results confirmed the earlier finding that PA participates in the early follicular events of the ovulatory process.

Species differences with regard to the types of PA secreted by granulosa cells were reported. While rat granulosa cells secrete mainly tPA, mouse cells secret predominantly uPA. Similar differences between mouse and rat PA were observed in the follicular fluids. Mouse uPA production in granulosa cells is stimulated by gonadotropins, prostaglandin E_2, and cAMP (Canipari et al., 1987). The follicular wall and follicular fluid obtained from the sow were shown to have both uPA and tPA. The activity of these enzymes exhibited the highest level at the time of ovulation,

and specifically in the area of the follicular rupture (Smokovitis et al., 1988).

b. Male reproduction.

Sertoli cells are found along the basement membrane of the seminiferous tubules. These cells have a central role in the maintenance of spermatogenesis. They form the major component of the blood-testis barrier, which is required for the maintenance of the microenvironment necessary for developing germinal cells. Sertoli cells have been shown to secrete several proteins including PA in response to hormonal stimulation (Fritz et al., 1976; Lacroix et al., 1981). The morphological development of spermatogonium to spermatozoa occurs in a cycle known as the cycle of the seminiferous epithelium. Fourteen successive stages have been identified in the rat cycle (Clermont, 1972).

Lacroix and coworkers (1981) studied PA secretion in tubule segments at different stages of the cycle of the seminiferous epithelium of the rat. Plasminogen activators were observed to increase 100-fold at stages VII and VIII of the cycle. These stages are the regions in which spermiation, and movement of Sertoli cell cytoplasmic processes around leptotene spermatocytes take place. They concluded that PA was involved in the localized restructuring of the seminiferous tubule which allows the translocation of spermatocytes in meiosis into the adluminal compartment.

Primary cultures of immature rat Sertoli cells maintained under basal conditions secrete predominantly uPA; however,

these cultures predominantly produce tPA when stimulated with FSH or cAMP. A similar trend, with regard to types of PA, has been observed at defined stages of the cycle of the seminiferous epithelium. Tubule segments induced by FSH showed an increased secretion of PA, predominantly tPA. Nevertheless, higher levels of uPA were observed particularly during stages VII and VIII. These observations further confirmed the significant role of PA in the restructuring of the seminiferous tubules during spermatogenesis (Hettle et al., 1986).

In addition to the secreted forms of PA, cultured Sertoli cells expressed a membrane-associated PA with a molecular weight of 38-40 kD. The membrane-associated PA may function in the restructuring of discrete membrane components at the cellular interface (Marzowski et al., 1985). Immunohistochemical studies show the presence of uPA exclusively at stages VII and VIII in Sertoli cells. On the other hand, tPA immunoreactivity was maximal during stages IX-XIII. These studies identified uPA as the main PA in the process of seminiferous tubule restructuring (Vihko et al., 1988). Coombs and coworkers (1988) used monospecific antibodies to determine the type of PA produced by bovine Sertoli cells. In contrast to the rat, bovine Sertoli cells produce only tPA, indicating a species difference between rat and bovine cells.

c. Embryo development.

The role of PA in oocyte physiology has been explored by several workers. Huarte and coworkers (1985, 1987) found that

ovulated rat and mouse secondary oocytes contain tPA, whereas primary oocytes isolated from ovaries do not. Tissue-type PA in primary oocytes matured in vitro became detectable 5 h after germinal vesicle breakdown, and this induction was blocked by protein translational inhibitors and cAMP. These results suggest that tPA mRNA is present in primary oocytes in a dormant state. Translation of tPA mRNA is triggered upon resumption of meiosis, and is accompanied by an elongation of the poly (A) tail. Finally, they postulated that PA may participate in early biochemical events such as fertilization, proacrosin activation and the zona reaction that blocks polyspermy.

Liedholm and Astedt (1975) provided the first evidence of the crucial role of PA in the developing embryo. Their results demonstrated that the fibrinolytic activity of the fertilized rat ova was high during tubal passage but this activity disappeared during implantation. The high fibrinolytic activity during tubal passage may help to prevent the adhesion of the embryo to the tubal wall. In contrast, the low fibrinolytic activity during implantation may be required for embryo attachment to the endometrium.

The extensive tissue remodeling of the endometrium and embryonic cell migration during the early periods of embryo development demonstrate the importance of PA. Strickland and coworkers (1976) examined the production of PA during the early stages of mouse embryo development. Plasminogen activator was produced by cultured blastocysts in a biphasic

pattern. In the first phase, enzyme activity started at the sixth day and reached a maximum at the eighth or ninth day, then started to decline. In the second phase, PA activity started at the eleventh day, persisted until the fifteenth day and was 5-fold higher than the activity exhibited during the first phase. This temporal pattern of PA production suggests the participation of at least two cell populations. To identify the source of PA, they separated the blastocyst into trophoblast which constitutes the outer cellular layer and the inner cell mass (ICM). They found that trophoblast cells contributed to PA production during the first phase. The enzymatic activity of trophoblast was correlated with the invasive capacity of these cells suggesting that PA participated in embryo implantation. On the other hand, the second phase of PA production was correlated with the differentiation of parietal endoderm from ICM. The migratory properties of parietal cells implicate PA in cellular migration and tissue remodeling. The overall conclusion of this study was that PA may play a role in embryo morphogenesis. Plasminogen activator production by parietal endoderm led Sherman and coworkers (1976) to suggest the possibility of using PA as a biochemical marker to distinguish and identify parietal endoderm in the early embryo.

Bode and Dziadek (1979) further investigated the types of embryonic cells which could synthesize and release PA. They dissected midgestation mouse embryos into their major component tissues. Their results demonstrated that the

development of mouse embryos from the seventh to the tenth day of gestation was associated with a progressive increase in PA secretion in different tissues. By day 10 all tissues tested (parietal endoderm, visceral yolk sac endoderm, mesoderm, and amnion) secreted PA. The widespread pattern of embryonic PA restrict its usefulness as a tissue-specific biochemical marker. Nevertheless, the presence of this protease in various parts of the midgestation embryo indicates an important function in embryonic growth.

Sherman (1980) compared the fibrinolytic activity in zona-enclosed embryos, empty zonae, zona-stripped embryos, and cultured embryos. He found that the fibrinolytic activity of preimplantation mouse embryos was restricted to the zona pellucida, indicating that mouse embryos at this stage do not secrete PA. Furthermore, he showed that this enzyme was not involved in either hatching from the zona pellucida or the acquisition of trophoblast adhesiveness. However, the observations that blastocysts produce PA at the same time when trophoblast cells acquire their migratory properties is consistent with the view that PA plays a pivotal role in trophoblast invasiveness during implantation.

Two types of PA have been identified in mouse embryos. The parietal endoderm PA has a predominant MW of 79 kD and this enzyme is inhibited only by antibodies raised against t-PA. The visceral endoderm and extraembryonic mesoderm PA have MW of 48 kD and are inactivated by anti-uPA antibodies. The specific cell-associated PA can be used as a diagnostic marker

to study parietal endoderm differentiation during the early stages of mouse embryogenesis (Marotti et al., 1982). On the other hand, cultured human cytotrophoblasts synthesize and secrete uPA which may account for the high capacity of these cells to degrade extracellular matrix proteins (Queenan et al., 1987).

The attachment of the porcine embryo to the endometrium does not involve a high degree of trophoblast invasion; however, pig trophoblast becomes highly invasive when ectopically transplanted to the spleen or lens capsule (Samuel, 1971). Mullins and coworkers (1980) examined the absence of the invasive characteristic of porcine blastocysts during the period of uterine attachment. Plasminogen activator activity in the uterine flushings was high during the early and late stages of the estrous cycle. In contrast, the activity of the enzyme became low during the luteal phase and at day 12 of gestation. A time-dependent increase in PA activity was observed when blastocysts were grown in culture, indicating that the enzyme activity was inhibited in vivo. A hormonally-induced protease inhibitor was found in the flushings of cycling gilts during the luteal phase and of progesterone-treated ovariectomized gilts. They concluded that the lack of the invasiveness in porcine blastocyst may have been due to the secretion of a progesterone-induced inhibitor by the uterus. The potential ability of porcine blastocysts to produce PA was confirmed by Fazleabas and coworkers (1983). A biphasic pattern of PA production by Day-

10 and Day-16-cultured porcine blastocysts was detected. The first phase (Days 10-12) coincided with the period of tissue remodeling, while the second phase (Days 14-16) occurred during the stage of tissue proliferation. Moreover, the release of protease inhibitor by pregnant animals rose to 7-fold between days 10-12. A similar effect can be initiated in cycling gilts by a single injection of estradiol valerate on Day 11 of the estrous cycle. Thus, it is reasonable to conclude that the initiation of estrogen production by elongating pig blastocyst triggers the release of plasmin inhibitor by the endometrium (Fazleabas et al., 1983).

Plasminogen has been detected in uterine flushings from both the pig and mouse and the source of this zymogen is probably the circulation (Fazleabas et al., 1983; Finlay et al., 1983). The ability of the embryo to produce PA, which in turn converts plasminogen to plasmin, suggests that the fibrinolytic system may be involved in the regulation of various aspects of early embryo development. Menino and O'Claray (1986) compared the development of mouse embryos in media supplemented with plasmin or plasminogen to media lacking these enzymes or containing trypsin and pronase. More embryos developed to blastocysts, and escaped from the zona pellucida in media supplemented with plasmin or plasminogen. Furthermore, the incidence of attachment to the substratum and trophoblastic growth was greater in the presence of plasmin and plasminogen. Therefore, they concluded that plasmin and

plasminogen may exert a trophic effect during the early stages of embryonic development.

Our laboratory has been inspired by the numerous reports associating PA with early embryo development. Several studies have been designed to characterize PA production, and to elucidate the function of this enzyme in different farm animal species. Menino and Williams (1987) evaluated plasminogen activation by cultured bovine embryos. They found that plasminogen had no effect on hatching rate, but high concentrations of this zymogen accelerated hatching. The production of PA was low for the first two days of culture, increased between Days 2 and 5, and plateaued thereafter. They speculated that plasminogen activation by the bovine blastocyst may facilitate hatching by inducing a sublysis of the zona pellucida. However, data from zona dissolution experiments showed that plasminogen had no effect on bovine zona pellucida solubility (Menino and Williams, unpublished results). The latter finding may indicate that plasminogen is not involved in hatching. Kaaekuahiwi and Menino (1989) demonstrated that PA activity was positively correlated with the viability of bovine embryos. Dyk and Menino (unpublished data) found that bovine hatched blastocysts produce two types of PA associated with the trophoblast, an uPA (MW=49 kD) and a tPA (MW=85 kD).

Cultured ovine embryos produced PA at Day 5; however, enzyme activity was first low, then peaked approximately 2-3 days prior to hatching, and remained high throughout the

culture period. Unlike the cow, increasing levels of plasminogen in the culture media increased both the hatching rate and the solubility of the ovine zona pellucida. These findings support the hypothesis which implicates plasminogen and PA in embryo hatching (Menino et al., 1989). Plasminogen has also been shown to stimulate cell division and hatching in cultured rabbit embryos (Grobner and Menino, unpublished results).

III. Regulation of Plasminogen Activator Activity

Total PA activity is determined by a balanced interaction between plasminogen, PA, and their inhibitors. Regulation of the extracellular proteolytic activity of PA occurs at various levels: (1) transcriptional, (2) translational, (3) cellular release of the proenzyme, (4) activation of proenzyme to active PA, (5) plasminogen activation, and (6) proteolytic activity of plasmin (Dano et al., 1985). Several hormones and factors have been shown to modulate PA activity either directly or indirectly by affecting the availability of the substrate or inducing protease inhibitors. Some of the well-characterized PA modulators will be discussed here with a brief description of the mechanisms by which they exert their action.

1. Gonadotropins and cyclic AMP.

Gonadotropins stimulate the activity of PA in rat granulosa cells (Beers et al., 1975; Strickland and Beers, 1976; Shimada et al., 1983; Espey et al., 1985; Reich et al., 1985; Canipari and Strickland, 1986; Canipari et al., 1987; Ny et al., 1987; Liu et al., 1987), rat cumulus-oocyte complexes (Liu and Hsueh, 1987; Liu

et al., 1987; Ny et al., 1987), cultured human cytotrophoblasts (Queenan et al., 1987), rat Sertoli cells (Lacroix et al., 1981; Marzowski et al., 1985; Hettle et al., 1986; Guillou et al., 1987; Bergh et al., 1987; Hettle et al., 1988), and bovine Sertoli cells (Jenkins et al., 1988).

The stimulatory effect of gonadotropins on PA activity was mimicked by cAMP in most of these studies suggesting that gonadotropin action is mediated by cAMP. This is in agreement with the generally accepted hypothesis that cAMP mediates the response of many polypeptide hormones. The increase of cellular cAMP can increase de novo synthesis of PA by promoting transcription of the PA gene (Nagamine et al., 1983). A parallel increase in uPA synthesis and uPA mRNA was observed in cAMP-treated human cytotrophoblasts (Queenan et al., 1987).

The molecular mechanism of PA gene regulation by cAMP is not yet known; however, the gene regulatory effect of cAMP is usually mediated by cAMP-dependent protein kinase A (cAMP-PK-A). These enzymes are made up of two regulatory (R) and two catalytic (C) subunits. The binding of cAMP to the regulatory subunits results in the release and subsequent activation of the catalytic subunits. Three hypothetical mechanisms for intracellular transmission of cAMP-mediated gene regulatory signals have been presented by Nigg and coworkers (1988). Pathway I postulates that PK-A regulatory subunits, activated by bound cAMP, translocate to the nucleus and interact with cis-regulatory DNA sequences of the PA gene. Pathway II postulates that transcriptional activation involves the phosphorylation of trans-acting-regulatory factor by the activated

catalytic subunit (C) of PK-A. Pathway III postulates that cAMP-dependent phosphorylation of a cytoplasmic mediator will trigger cascade events leading to the transcriptional signal. Immunocytochemical analysis of subcellular distribution of PK-A subunits indicates that the second pathway is indeed the mechanism by which cAMP regulates and induces the transcription of the PA gene (Nigg et al., 1988). Jans and coworkers (1987) evaluated the induction of uPA by reagents activating cAMP-PK-A. Their results excluded the involvement of the regulatory (R) subunit of cAMP-PK-A in promoting gene transcription. They suggested that the cAMP-PK-A catalytic subunit was the primary regulator for PA gene expression. Degen and his colleagues (1987) isolated the complete nucleotide sequence of the murine uPA gene and identified a short sequence homologous to the proposed 5′-flanking cis-acting regulatory elements which mediate cAMP induction of gene expression.

Gonadotropin-releasing hormone (GnRH) induces the production of tPA and tPA mRNA by cultured granulosa cells and cumulus-oocyte complexes. These effects were inhibited by co-treatment with a GnRH antagonist suggesting the presence of steriospecific receptors in these cells. Moreover, the GnRH stimulatory effect was mimicked by phorbol myristate acetate (PMA), a stimulator of protein kinase C. These results indicate that the GnRH effect may be mediated by specific receptors through the activation of the protein kinase C pathway. This is in contrast to other gonadotropins which mediate their effects by elevating cellular cAMP and activating the protein kinase A pathway (Ny et al., 1987).

2. Ovarian steroids.

Estrogen stimulated production of PA in human breast carcinoma cell lines MCF-7 (Dickerman et al., 1989), and ZR-75-1 (Huff and Lipmann, 1984). The enhancement of PA activity in MCF-7 cell lines was suppressed by 2,3,7,8,-tetrachlorodibenzo-p-dioxin (Gierthy et al., 1987). In addition, estrogen stimulated the uptake of plasminogen by the mouse uterus (Finlay et al., 1983); however, PA activity of mouse uterine fluid was suppressed by estrogen (Harpel et al., 1966, 1967). Estrogen has been shown to induce the production of a plasmin-inhibitor by pig endometrium during the period of implantation, thus preventing the proteolytic degradation of the uterine wall (Fazleabas et al., 1983).

Progesterone has been reported to induce protease inhibitors in mouse (Harpel et al., 1966) and pig uteri (Mullins et al., 1980; Fazleabas et al., 1982). The progesterone-induced inhibitor is secreted during the luteal phase and Day 12 of pregnancy which coincide with the period of embryo attachment to the endometrium.

Steroid hormones are generally believed to influence protein synthesis by changing the rate of gene transcription. Actinomycin-D blocked the induction of PA by estrogen from MCF-7 breast carcinoma cell line. This conforms with the assumption that estrogen induction of PA requires de novo synthesis of mRNA (Dano et al., 1985).

3. Glucocorticoids.

Glucocorticoids have been reported to suppress the activity of PA in a variety of cultured cells and tissues, including rat granulosa cells (Harlow et al., 1987), bovine Sertoli cells (Coombs

and Jenkins, 1988), primary cultures of rat hepatocytes (Heaton et al., 1989), rat hepatoma cells (Gelehrter et al., 1987; Heaton and Gelehrter, 1989), human tumor-derived cells (Roblin and Young, 1980; Andreasen et al., 1986; Amin et al., 1987; Andreasen et al., 1987; Rehemtulla and Hart, 1987), and murine macrophages (Vassalli et al., 1976; Chow et al., 1987). Glucocorticoids had no effect on PA activity expressed by human melanoma cells (Roblin and Young, 1980).

There are three possible mechanisms by which glucocorticoids suppress PA activity. First, glucocorticoids can operate at the transcriptional and/or translational levels to suppress de novo synthesis of PA. Second, glucocorticoids could reduce cellular PA activity by inducing synthesis and release of protease inhibitors. Finally, glucocorticoids might stimulate the cellular inactivation and degradation of PA.

Harlow and coworkers (1987) reported that cortexolone, a glucocorticoid antagonist, significantly ameliorated the suppressive effect of glucocorticoids on PA secretion by rat granulosa cells. Furthermore, they observed that a time delay was required for glucocorticoids to take effect. Collectively, these data suggest that glucocorticoids bind to specific receptors and induce the synthesis of a protein with anti-protease activity (possibly a PAI). Coombs and Jenkins (1988) characterized a PAI induced by glucocorticoids in immature bovine Sertoli cells. This novel inhibitor has an apparent MW of 49 kD, and shares antigenic determinants with placental and macrophage PAI and fibroblast protease nexin.

Incubation of HTC rat hepatoma cells with the synthetic glucocorticoid dexamethasone induced a rapid 90% inhibition of PA activity and the concomitant appearance of PAI-1. Although immunologic and biochemical studies showed that dexamethasone treatment of HTC cells increased the amount of PAI 4- to 5-fold, this treatment did not decrease cellular levels of PA. These results reveal that glucocorticoid inhibition of PA activity in HTC cells is not due to a decrease in the amount of PA but is primarily due to the induction of PAI-1 (Cwikel et al., 1984; Gelehrter et al., 1987). Plasminogen activator inhibitor-1 mRNA has been shown to increase 4- to 5-fold when HTC cells were incubated with dexamethasone. Cycloheximide, a protein synthesis inhibitor, neither affected the accumulation of PAI-1 mRNA nor blocked its induction by dexamethasone; however, induction was blocked by actinomycin-D. Accordingly, the dexamethasone-induced increases in HTC cell PAI-1 activity are the result of a direct effect on the accumulation of PAI-1 mRNA (Heaton and Gelehrter, 1989). Therefore, in rat HTC hepatoma cells, glucocorticoid inhibition of PA activity is mediated at the molecular level by the induction of PAI-1 gene expression rather than by the suppression of PA gene expression.

Glucocorticoid inhibition of PA activity in rat hepatocytes, however, is different from HTC hepatoma cells. Heaton and coworkers (1989) studied the effect of dexamethasone and cAMP on PA activity in primary cultures of rat hepatocytes. Incubation of these cells with cAMP resulted in a 4-fold increase in PA activity, whereas dexamethasone caused more than a 90% decrease in PA

activity. Moreover, dexamethasone completely abolished the cAMP effect and significantly decreased PA activity. Both cAMP and dexamethasone induced the production of PAI-1. Hepatocyte PAI-1 mRNA levels were increased 4-fold by a combination of cAMP + dexamethasone. The authors concluded that cAMP induced PA activity directly by increasing PA mRNA accumulation. Dexamethasone, however, caused a decrease in PA activity by both decreasing PA mRNA and increasing PAI-1 mRNA. The primary effects of both agents are believed to be related to gene transcription or mRNA stability.

4. Phorbol esters.

Phorbol esters have been reported to enhance plasminogen activator production by a human melanoma cell line (Bowe) (Opdenakker et al., 1983), human bladder carcinoma cells T-24 (Rehemtulla and Hart, 1987) and human endothelial cells (Santell and Levin, 1988). In contrast, phorbol esters stimulated secretion of PAI in human rhabdomyosarcoma (RD) cell line (Meyer et al., 1988), mouse L-cells (Rehemtulla et al., 1987), and mouse resident peritoneal macrophages (Wohlwend et al., 1987). Phorbol esters were found to be without effect on PA activity in explants of involuting murine mammary gland and in primary cultures of rat pituitary cells (Dano et al., 1985).

The effect of phorbol esters on PA production is probably mediated through activation of the protein kinase C pathway, which has been identified as the primary cellular receptor for phorbol esters. The enhanced production of PA by Bowe melanoma cells after treatment with 12-O-tetradecanoylphorbol-13-acetate is accompanied by a concomitant increase in the cellular level of PA mRNA. This

suggests that phorbol esters participate, either directly or indirectly, in inducing PA gene transcription (Opdenakker et al., 1983). In addition to PA, endothelial cells release PAI-1, which may regulate PA activity in these cells. Santell and Levin (1988) investigated the effects of phorbol esters and cAMP on PA and PAI-1 secretion. Phorbol esters induced a time- and dose-dependent increase in PA release from endothelial cells, while cAMP had no significant effect. However, cAMP elevation increases phorbol ester-stimulated PA release 6-fold, and decreases secretion of PAI-1 by 30-40%. The inhibitory effect on PAI-1 secretion is directly attributed to the increased cAMP levels. They concluded that phorbol esters and cAMP acted coordinately to regulate and modify PA and PAI-1 secretion from endothelial cells.

The mechanisms by which phorbol esters enhanced the secretion of PAI-1 in some cells have been evaluated by several workers. Meyer and coworkers (1988) used an enzyme-linked immunosorbent assay (ELISA) to study the effect of phorbol 12-myristate 13-acetate on PA secretion in human RD cells. They found that PMA caused a 10-fold increase in PAI-1. The enhancement of PAI-1 secretion was accompanied by a 20-fold increase in the cellular level of PAI-1 mRNA and more than a 5-fold increase in PAI-1 gene transcription rate. Cycloheximide treatment did not suppress the PMA-stimulated increase of PAI-1 mRNA indicating that protein synthesis was not required for the PMA effect. Co-administration of cycloheximide with PMA resulted in a strong increase in PAI-1 mRNA levels, which indicated that a short-lived protein negatively regulates the level of PAI-1 mRNA either by suppressing PAI-1 gene

transcription or by destabilizing the mRNA. It is noteworthy to indicate that PMA in this study also stimulated PA production by RD cells, but with a smaller magnitude than the stimulation of PAI-1. The overall PA activity in RD cells was not affected by the PMA-stimulated increase in PAI-1. This paradoxical phenomenon might be explained by the difference in spatial ultrastructural localizations of PA and PAI-1. This explanation was confirmed by the finding that PA is localized mostly to cell-cell and focal contacts, whereas PAI-1 is uniformly deposited on the cell substratum. The distinct localization of the enzyme and its inhibitor saves the enzyme activity for the time when it is required (Pollanen et al., 1988).

5. Retinoic acid.

Retinoic acid (RA) has been reported to stimulate PA production in murine teratocarcinoma stem cells (Strickland and Mahdavi, 1978), chick embryo fibroblasts (Wilson and Reich, 1978), and a number of cultured human cell types derived from normal and neoplastic tissues (Dano et al., 1985).

Retinoids, derivatives of vitamin A, have been shown to influence cellular differentiation (Wolbach and Howe, 1925), induce proteolytic activity in cultured explants of the chick embryo limb bud (Fell et al., 1962), and promote the growth of experimental tumors (Polliack and Levi, 1969). The potential relationship between retinoid action and PA synthesis inspired some workers to study the regulatory mechanism of this enzyme in several cell types.

Wilson and Reich (1978) demonstrated that RA enhancement of PA production by chick embryo fibroblasts required protein and mRNA synthesis. In the teratocarcinoma cell line F9, RA increased the production of PA and a similar effect was observed in cAMP treated cells. Retinoic acid and cAMP, taken together, had an apparent synergistic effect on protease secretion; however, this effect was not immediate but required several days to be manifested. This delay may suggest that RA elevated cellular sensitivity to cAMP. Other morphological changes beside PA production indicate that RA induced the differentiation of embryonal carcinoma cells to endoderm (Strickland and Mahdavi, 1978).

6. Growth factors.

Basic fibroblast growth factor (bFGF) has been shown to increase PA production in cultured bovine capillary endothelial cells (Gross et al., 1982; Saksela et al., 1987), newborn rat cultured astroglial cells (Rogister et al., 1988), human endometrial adenocarcinoma cells (Presta et al., 1988), and cultured breast tumor cells (Mira-y-Lopez et al., 1986). Transforming growth factor (TGF-β) inhibited PA activity in capillary endothelial cells (Saksela et al., 1987), and stimulated production of PAI-1 in human endometrial adenocarcinoma cells (Presta et al., 1988) and human tumor cell line HT-1080 (Laiho et al., 1987).

Fibroblast growth factor (FGF) induces cell proliferation and protease production. Therefore, FGF may be involved in the regulation of the growth of both normal and tumor cells. In contrast, transforming growth factor-β (TGF-β) was shown to have

both stimulatory and inhibitory properties on cellular growth in vitro (Presta et al., 1988). Saksela and coworkers (1987) evaluated the opposing effects of bFGF and TGF-β on the regulation of PA activity in bovine capillary endothelial cells (BCEC). They found bFGF markedly increased PA production, while TGF-β caused a significant decrease in enzyme secretion. Administering bFGF and TGF-β simultaneously to BCEC resulted in the inhibition of the bFGF-simulation of PA activity. The stimulatory effect of bFGF on PA levels was due to the increased synthesis and secretion of PA. The suppression of PA activity by TGF-β may be a direct result of the decrease in the rate of synthesis of PA or indirectly by increasing synthesis of PAI-1 from endothelial cells. Prolonged culture of BCEC in bFGF and TGF-β diminished the TGF-β inhibitory effect; however, the bFGF stimulatory effect on PA activity was maintained. The bFGF stimulation of PA production by BCEC is important for the migration and proliferation of endothelial cells as well as the degradation of the subcellular matrix during angiogenesis. Transforming growth factor-β also stimulated synthesis and release of PAI-1 from human tumor cell line HT-1080, which was rapidly deposited in the cellular substratum. Pulse-phase experiments showed a relatively fast turnover of substratum-associated PAI-1. Transforming growth factor-β also inhibited the anchorage-independent growth of HT-1080 cells at the same concentrations necessary to induce PAI-1. These results indicate that TGF-β can modulate the extracellular proteolytic activity by stimulating the secretion and deposition of PAI-1 into ECM (Laiho et al., 1987).

Epidermal growth factor (EGF) has been reported to enhance PA activity in the HeLa cell line, cultured human foreskin fibroblasts, and rat kidney cell line NRK-536-3-1 (Dano et al., 1985). The stimulatory effect of EGF was observed on both intracellular and extracellular PA activity in HeLa cells. The induction of PA activity in these cells was blocked by actinomycin D and cycloheximide, suggesting that EGF induces de novo synthesis of PA in a manner dependent on the continued synthesis of PA mRNA (Dano et al., 1985).

7. Other biochemical factors.

Dimethyl sulfoxide (DMSO) greatly stimulates the production of cell-associated and secreted PA in auxotrophic mutants (cells that can not metabolize carbohydrates) of the Chinese hamster ovary cell line. Dimethyl sulfoxide stimulation of PA activity was completely blocked by actinomycin D and cycloheximide, indicating that new protein and mRNA synthesis were required for this process. Moreover, the stimulatory effect of DMSO was lost when the cells were hybridized with other complementary auxotrophic mutants, suggesting that the effect may be related to the auxotrophic mutation in this cell line (Carlsen, 1987).

Negatively charged phospholipids, contained within lipid vesicles, have the ability to activate the latent form of PAI-1. The presence of a net negative charge on the phospholipid head group is essential for the activation process. In the presence of negatively charged phosphatidylserine (PS), PAI-1 inhibited tPA 50-fold more effectively than in the absence of these phospholipids. Calcium ions interfered with the inhibitory activity of PS-

activated PAI-1, indicating that Ca^{2+} ions may be involved in regulating PAI-1 activity in the presence of negatively charged phospholipids. In conclusion, negatively charged phospholipids may play a critical role in controlling the fibrinolytic system by local activation of PAI-1 (Lambers et al., 1987).

Tumor necrosis factor (TNF) enhanced the production of PAI by cultured human endothelial cells from umbilical vein, 2-fold, and from foreskin microvessels, 4- to 5-fold. This increase in PAI production, accompanied by increased levels of PAI mRNA, indicated that TNF induced PAI synthesis. In vivo experiments with rats showed that TNF increased circulating PAI levels 5-fold. Tumor necrosis factor did not change PAI levels in primary monolayer cultures of rat and human hepatocytes. These results suggest that vascular endothelial cells are the primary targets for TNF. Furthermore, the induced PAI activity might decrease fibrinolysis within the blood vessels (Van Hinsbergh et al., 1988).

Sodium butyrate (NaB) significantly reduced PA activities in two human glioblastoma cell lines (SNB-19 and SNB-75). The lesser PA activities in these cells correlated with greatly reduced colonogenic properties, which is consistent with the suggested role of PA in promoting tumor proliferation. The inhibition of PA activity by NaB is most likely caused by a change in PA synthesis, since a direct inhibition assay revealed no changes in PAI levels. These results demonstrated that net cellular PA activity in glioma cells is regulated by a balance between PA production and protease-inhibitor levels. This balance can be altered by exogenous modulators such as NaB (Gross et al., 1988).

The stimulatory effect of dibutyryl cAMP (dbcAMP) on the production of PA in a wide variety of cells is well documented. However, data from one study suggested that the effect of dbcAMP on PA induction in cultured human endothelial cells may be attributed to the slow liberation of butyrate (Kooistra et al., 1987). They based their suggestion on the observation that free butyrate, but not 8-bromo-cAMP, resulted in the same increase in PA production as dbcAMP. The enhancement of PA activity by butyrate was accompanied by increased levels of PA mRNA. Butyrate, however, did not alter PAI-1 synthesis significantly indicating that this compound selectively induced PA production in endothelial cells.

Lipopolysaccharide (LPS), a mediator for the inflammatory response, has been found to stimulate the production of PAI-1 from human endothelial cells. This effect was accompanied by a considerable increase in PAI-1 mRNA. Lipopolysaccharide-mediated increases in PAI-1 mRNA is not dependent on protein synthesis, since co-treatment of endothelial cells with cycloheximide caused a superinduction of PAI-1 mRNA (Van Den Berg et al., 1988).

An extractable tPA cofactor from porcine coronary heart endothelium has been reported to enhance the rate of plasminogen activation by tPA. This novel cofactor increases V_{max} for plasminogen activation without affecting K_m. The mechanism of action of this cofactor differs from that of fibrin which stimulates plasminogen activation by lowering the K_m for plasminogen. The activity of this endothelial cofactor is high in coronary and aortic valve endothelium extracts, but not in fluids or tissues lacking endothelium (Machovich et al., 1988). Other

endogenous factor(s) have been shown to stimulate rat oocyte tPA activity during meiotic maturation (Bicsak and Hsueh, 1989).

Dermatan sulphate (DS), a component of the extracellular matrix, enhanced PA activity in the perfused rat hindquarters in a dose-dependent fashion. Dermatan sulphate has been shown to have a potent antithrombotic property. Therefore, it is reasonable to suggest that DS facilitates clot lysis by stimulating PA release from surrounding endothelial cells during vascular injury (Abbadini et al., 1987).

MATERIALS AND METHODS

Embryo Collection and Culture

Thirty-two crossbred beef cows from the Oregon State University beef herd were estrous-synchronized with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse, The Upjohn Co., Kalamazoo, MI) and superovulated with porcine follicle stimulating hormone (pFSH; Schering Corp., Kenilworth, NJ). Two 25-mg injections of $PGF_{2\alpha}$ were administered i.m. 14 days apart (Day 0 = first $PGF_{2\alpha}$ injection) to all cows. Porcine follicle stimulating hormone injections were administered twice daily i.m. at dosages of 5, 4, 2, and 1 mg on Days 12, 13, 14 and 15, respectively. Estrous detection was initiated 24 h after the second $PGF_{2\alpha}$ injection. Cows exhibiting estrus were either naturally mated at the onset of estrus and at 12-h intervals thereafter, or artificially inseminated with one straw of frozen semen at 0, 12, and 24 h after estrus onset.

Embryos were collected 8 days after mating either nonsurgically or from slaughtered cows. In the non-surgical collection, uteri were flushed with Dulbecco's phosphate buffered saline (PBS; Dulbecco and Vogt, 1954) containing .5% bovine serum albumin (BSA; Sigma) and 10 ml/l of an antibiotic-antimycotic solution (10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml in .9% sodium chloride; Sigma). Excised uteri recovered at slaughter were flushed within 30 minutes with Ham's F-12 containing 10 ml/l of the antibiotic-antimycotic solution (Sigma). Uterine flushings were examined under a dissecting microscope and embryos were recovered from the flushing by aspiration. Recovered embryos were washed at least three times in microdrops of Ham's F-12 with 1.5 mg/ml BSA and morphologically evaluated using an

inverted stage-phase contrast microscope. Only morphologically normal embryos were assigned to the various hormonal treatments. Embryos were individually cultured in 35 μl microdrops under paraffin oil (Fisher Scientific Co., Tustin, CA) in a humidified atmosphere of 5% CO_2 in air at 37°C for 5 days. At the initiation of culture and at subsequent 24 h intervals, embryonic cell stage was recorded and overall embryonic diameter (OD) was measured using an ocular micrometer. At 24 h intervals, embryos were transferred to fresh microdrops and 15 μl of the culture medium were recovered and frozen at -20°C until assayed for PA activity. The remaining medium was also recovered and pooled for every treatment and frozen at -20°C until zymography. Another 15 μl of medium from a microdrop without embryos was recovered for every treatment to correct for any spontaneous activation of plasminogen. At termination of culture, embryos were recovered and frozen at -20°C until zymography.

Effect of Hormones and Inducers of Intracellular Messengers on PA Production and OD by Cultured Bovine Embryos

Progesterone. Progesterone (Sigma) was initially dissolved in absolute ethanol and further diluted to final concentrations of 10^{-6}, 10^{-7}, and 10^{-8} g/ml with Ham's F-12 containing 15 mg/ml BSA. Two controls were included in this experiment: Ham's F-12 with 15 mg/ml BSA and this medium with 1% absolute ethanol. Twelve morphologically normal embryos were randomly assigned to each treatment.

Dexamethasone. Four levels (0, 10^{-8}, 10^{-7}, and 10^{-6} M) of dexamethasone (Sigma) were prepared by diluting a dexamethasone-ethanol stock solution with Ham's F-12 with 1.5 mg/ml BSA. The same levels of

absolute ethanol used to dissolve the drug was included in the control treatment (0 level). Eleven to 13 embryos were randomly assigned to each treatment.

Retinoic acid. Retinoic acid (Sigma) was dissolved in absolute ethanol and diluted to 0, 10^{-8}, 10^{-7}, and 10^{-6} M with Ham's F-12 containing 1.5 mg/ml BSA. Twelve to 13 embryos were assigned randomly to each treatment.

Estradiol-17β. Estradiol-17β (Sigma) was prepared with similar levels as progesterone (0, 10^{-8}, 10^{-7}, and 10^{-6} g/ml). Twelve to 14 embryos were randomly assigned to each treatment.

Phorbol myristate acetate and dibutyryl cyclic AMP.

Experiment 1. Phorbol-12-myristate 13-acetate (Sigma) was dissolved in dimethyl sulphoxide (DMSO; Sigma) and diluted with Ham's F-12 with 1.5 mg/ml BSA to a final concentration of 10 μg/ml. One percent of DMSO was added to Ham's F-12 with 1.5 mg/ml BSA as the control treatment. Dibutyryl cAMP (dbcAMP; Sigma) was diluted with Ham's F-12 containing 1.5 mg/ml BSA to 5 mM. A control treatment of Ham's F-12 with 1.5 mg/ml BSA was included in this experiment. Eight embryos were randomly assigned to each of these four treatments.

Experiment 2. Phorbol 12-myristate 13-acetate was further diluted with culture medium to 100 ng/ml, and dbcAMP was diluted to 0.5 mM. Two control treatments, one with 1% DMSO and the other with only culture medium (Ham's F-12 with 1.5 mg/ml BSA), were included

in this experiment. Seven to 10 embryos were randomly assigned to each treatment.

Experiment 3. Phorbol 12-myristate 13-acetate was diluted to 1 ng/ml with Ham's F-12 containing 1.5 mg/ml BSA, and 1% DMSO was added to the control treatment. Thirteen to 14 embryos were randomly assigned to each treatment.

Assay for PA Activity

Plasminogen activator activities were determined by a modified caseinolytic assay described by Menino and Williams (1987). The casein-agar solution was prepared by combining 2% agarose (Sigma) with an equal volume of warmed 2% nonfat milk (Carnation Co., Los Angeles, CA), dissolved in 0.038 M tris (hydroxymethyl)-aminomethane-0.1 M glycine buffer containing 0.195 g/l $CaCl_2 \cdot 2 H_2O$ and 0.325 g/l sodium azide. Fifteen milliliters of the warmed mixture was pipetted into 85 x 65 x 2 mm plastic diffusion plates and allowed to solidify at room temperature. Plasminogen activator activities were determined by combining 15 μl of 150 μg/ml human plasminogen (Sigma) with 15 μl of either conditioned medium or 0, .0009, .0045, .0091, .0455, and .0909 mU/ml urokinase (E.C. 3.4.21.7; Sigma) as the standard and incubated for 15 min at 37°C. Twenty-five microliters of the incubated mixture were aliquoted into 4 mm diameter wells cut in the casein-agar gel plate and incubated at room temperature for 24 h. Plates were fixed for 15 min with 3% acetic acid and rinsed in tap water; the resultant caseinolytic zones were measured with an electronic digital calipar. Concentrations of PA in the conditioned medium were determined from equation of the line calculations for caseinolytic ring diameter by log urokinase

concentration. The amount of the embryonic PA was determined by deducting the amount of PA in the medium without an embryo from the amount in the medium with an embryo for each 24-h interval. Plasminogen activator activity was expressed as milliunits PA x $10^4 \cdot ml^{-1} \cdot h^{-1} \cdot e^{-1}$.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Zymography

Molecular weights (MW) of PA were determined with the Protean Dual Slab Cell electrophoresis apparatus (Bio-Rad Laboratories, Richmond, CA) under non-reducing conditions using a modification of the SDS-PAGE and zymography techniques described by Granelli-Piperno and Reich (1978) and Vassalli and coworkers (1984). Media samples representing each 24-h interval were combined with equivalent volumes of sample buffer (5% SDS, 20% glycerol, 0.0025% bromophenol blue in 0.125 M Tris-HCl buffer). Embryos were thawed and homogenized by repeated aspiration with a micropipette before adding sample buffer. Urokinase standards at 1, .5 and .1 mU/ml were used as references and to indicate the sensitivity of the assay. A MW standard (Bio-Rad) with the following proteins: phosphorylase b (97.4 kD), bovine serum albumin (66.2 kD), ovalbumin (42.7 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and Lysozyme (14.4 kD) was included in each 160 x 135 x 1.5 mm electrophoretic slab gel. Urokinase and MW standards were diluted with sample buffer. One hundred microliters of samples and standards were pipetted into castellated wells in a 4% acrylamide stacking gel with a 12% separating gel. Electrophoresis was started at 15 mA through the stacking gel and 30 mA through the separating gel until the dye front reached the bottom edge of the separating gel. Following

electrophoresis, the lane containing the MW standards was cut and stained with .05% Commassie Brilliant Blue (Bio-Rad) in 65/25/10 water/isopropanol/acetic acid for 4-8 h and destained in 80/10/10 water/isopropanol/acetic acid overnight. The gel with the remaining lanes was gently agitated in 2.5% Triton X-100 (Sigma) for 30 min to remove the SDS, rinsed with distilled water 3 times, and incubated in PBS for 30 min. The gel was placed on plastic wrap and a casein-agar gel containing human plasminogen was carefully applied to the surface of the polyacrylamide gel. The casein-agar plates preparation was similar to those used in the caseinolytic assay with two exceptions; purified human plasminogen was added to the warmed mixture of 2% casein and 2% agarose to yield a final concentration of 25-30 μg/ml and 15 ml of this mixture was casted onto a warmed 13 x 10 cm glass plate. The gel sandwich (acrylamide gel + casein-agar gel) was incubated at room temperature for 40-48 h and observed for localized lysis periodically. Incubation was terminated by fixing the casein-agar gel with 3% acetic acid for ten min. Protease mobility was determined during incubation period and after fixing the gel by measuring the distance from the edge of the separating gel to the center of the lytic bands in each lane. Molecular weights of PA in the conditioned media, embryo homogenates and urokinase standards were determined from equation of the line calculations for relative mobility by log MW derived from the MW standards. Casein-agar gels were stained with 0.1% Amido Black (Bio-Rad) in 20/10/70 water/acetic acid/methanol for 3-4 min and destained in 70/10/20 methanol/acetic acid/water overnight.

Statistical Analyses

With the exception of the PMA treatments (10 μg/ml and 100 ng/ml), statistical analyses were performed on data obtained from embryos that successfully completed hatching. Statistical comparisons were made by two-way analyses of variance where hormonal treatment and time in culture were the main effects. Duncan's new multiple range test was used for multiple comparisons when appropriate. Molecular weights of PA were determined using correlation-regression analysis (Steel and Torrie, 1980).

RESULTS

Embryo Recovery

A total of 444 ova were collected from thirty-two cows with an average of 14 ova per cow. Thirty-five percent of these ova were comprised of unfertilized ova and degenerate or poor embryos. The remaining 65% were morphologically good embryos (287 embryos). The percentage of embryos that successfully completed in vitro hatching was 93.2% for all experiments.

Effects of Hormones and Inducers of Intracellular Messengers on PA Production and OD by Cultured Bovine Embryos

Progesterone. Fifty-nine morphologically normal embryos were assigned to the various treatments in this experiment and 54 (92%) embryos hatched. Ethanol vehicle (Figures 1 and 2), at levels used to dissolve progesterone, had no effect ($P>.1$) on PA production or OD when compared to the control of Ham's F-12 with 15 mg/ml BSA. Progesterone at levels of 10^{-6}, 10^{-7}, and 10^{-8} g/ml (Figures 1 and 2) neither affected PA production nor OD ($P>.1$); however, culture interval affected ($P<.05$) both PA production and OD. No interaction between time and hormonal level was observed ($P>.1$). Figure 1 shows the relationship between time and PA production. At the beginning of the culture (24 h to 48 h) PA production remained low with no change ($P>.05$). Plasminogen activator production, however, increased ($P<.05$) at 72 h of culture, peaked at 96 h, and plateaued thereafter. On the other hand, culture time increased ($P<.05$) OD where maximal OD was attained at 72 h then declined ($P<.05$) at the end of the culture period (Figure 2).

Dexamethasone. Forty-six morphologically good embryos were randomly assigned to four treatments (0,10^{-8}, 10^{-7}, 10^{-6} M) of dexamethasone. Forty-four embryos (96%) successfully completed hatching in culture. Plasminogen activator production and changes in OD for embryos hatched cultured in dexamethasone at levels indicated above are depicted in Figures 3 and 4. Plasminogen activator production and embryo OD were not affected ($P>.1$) by dexamethasone treatments. Time of culture, on the other hand, affected PA production and OD ($P<.05$) and no interaction was detected ($P>.05$) between the main effects. A progressive increase was observed in PA production (Figure 3) starting at 24 h which reached a maximum at 96 h, then decreased at 120 h of culture ($P<.05$). Similarly, OD increased ($P<.05$) during the first 48 h, peaked at 72 h then diminished at 96 and 120 h of culture (Figure 4).

Retinoic acid. Forty-four embryos were equally distributed over four RA levels (0, 10^{-8}, 10^{-7}, 10^{-6} M). Forty-one embryos (93%) successfully escaped from their zona pellucida. Retinoic acid did not affect ($P>.1$) PA production or OD (Figures 5 and 6). Time of culture had a great effect ($P<.01$) on PA production and OD and no interaction was observed between the RA treatments and time of culture ($P>.1$). Plasminogen activator production (Figure 5) remained low at the 24 and 48 h intervals ($P>.05$), increased at 72 h and persisted throughout the culture period ($P<.05$). Embryo OD (Figure 6) increased in a progressive fashion ($P<.05$) and at 72 h reached a maximal size which remained constant through 96 h then declined toward the end of culture ($P<.05$).

Estradiol-17β. Forty-eight normal embryos were equally distributed throughout 4 levels of estradiol 17β (0, 10^{-8}, 10^{-7}, 10^{-6} g/ml). Forty-five (94%) embryos hatched in vitro. No effect was observed in PA production and OD ($P>.1$) between the control treatment and treatments containing E_2 (Figures 7 and 8). In contrast, culture time greatly affected both PA production and OD ($P<.01$). Interaction between the treatment factor and time of culture was not observed ($P>.1$). Plasminogen activator production (Figure 7) remained unchanged at the first 48 h ($P>.05$), increased at 72 h ($P<.05$) and plateaued thereafter. Moreover, OD (Figure 8) increased during the first 48 h ($P<.05$) and reached the largest size at 72 h, remained constant to 96 h then declined at 120 h ($P<.05$).

Phorbol myristate acetate and dibutyryl cyclic AMP.

Experiment 1. Thirty-two embryos were randomly assigned to four treatments (10 μg/ml PMA, 5 mM dbcAMP, 1% DMSO, and Ham's F-12 with 1.5 mg/ml BSA as the control). Percentage of embryos hatching in PMA, dbcAMP, DMSO, and control were 0, 63, 88, and 100, respectively. Phorbol myristate acetate at 10 μg/ml caused embryos to degenerate within the first 24 h. Dibutyryl cAMP also reduced the number of embryos hatching in vitro and caused embryo degeneration toward the end of culture. Plasminogen activator production was not affected ($P>.1$) by dbcAMP when compared to the control treatment (Figure 9A). Production of this enzyme increased ($P<.01$) from 24 h to 48 h, plateaued during 72 and 96 h ($P>.05$) then decreased ($P<.05$) at the end of culture. No

interaction was observed between the two main effects ($P>.1$). A time-dependent decrease in OD ($P<.01$) was observed due to 5 mM dbcAMP; OD started to decrease after 48 h and remained low throughout the culture period (Figure 9B). The interaction between time of culture and treatment was apparent ($P<.05$). Dimethyl sulphoxide decreased PA production ($P<.01$) particularly during the first 72 h of culture. Levels of PA started to increase after 72 h reaching a comparable level as the control treatment (Figure 10A). Embryo OD was not affected ($P>.1$) by DMSO and remained unchanged during the first 48 h, increased to the largest size at 72 h ($P<.05$) and maintained the same size throughout the rest of culture (Figure 10B). Embryos cultured in 10 μg/ml PMA did not hatch and degenerated during the first 24 h of culture. Compared to embryos cultured in DMSO, PMA treatment and time of culture did not affect ($P>.1$) PA production (Figure 11A). Embryo OD was lower ($P<.01$) in PMA than DMSO and remained constant throughout the culture period (Figure 11B).

The second trial was designed to eliminate the side effect of the high levels of PMA and dbcAMP observed in the first trial. Thirty-three morphologically normal embryos were assigned to the following treatments: 100 ng/ml PMA, .5 mM dbcAMP, 1% DMSO, and control treatment (Ham's F-12 with 1.5 mg/ml BSA); hatching rates were 17, 90, 71, and 80 percent, respectively. There was no effect ($P>.1$) on PA production due to .5 mM dbcAMP (Figure 12A), but time of culture exerted a significant affect on the production of this enzyme (Figure 12A). Plasminogen activator increased ($P<.05$) at 48 h interval and remained unchanged during 72 and 96 h

intervals then declined toward 120 h. In contrast, OD was decreased ($P<.05$) in embryos cultured in .5 mM dbcAMP, however, time of culture and the interaction were not significant (Figure 12B). The results obtained for the DMSO effect on PA production were similar to those presented in experiment 1 with the exception of the effect on OD (Figure 13); an increase ($P<.01$) in OD was detected in embryos cultured in medium containing DMSO (Figure 13B). On the other hand, no significant effect on OD was observed with culture time. Phorbol myristate acetate at level of a 100 ng/ml had a similar effect on PA production and OD as in experiment 1 (Figure 14A and B), however, time of culture during this experiment significantly influenced PA production, which was low during the first 72 h,, increased ($P<.05$) at 96 h and 120 h (Figure 14A).

Experiment 3. Phorbol myristate acetate was diluted to a final concentration of 1 ng/ml. Twenty-six embryos were equally divided into two treatments, one containing 1 ng/ml PMA and the other containing 1% DMSO. Twenty-four embryos (92%) hatched in vitro. The toxic effect of PMA on embryos observed during the previous experiments was eliminated in this experiment. Phorbol myristate acetate compared to DMSO treatment did not ($P>.1$) affect PA activity (Figure 15A), yet OD were greater ($P<.05$) in the presence of PMA (Figure 15B). Time of culture did not affect PA production ($P>.05$) during the first 72 h, at 96 h production increased ($P>.05$) greatly and remained at the same level at 120 h (Figure 15A). Also, time of culture exerted a significant effect on OD which increased ($P<.05$) at 48 h, leveled at 72 and 96 h, then

decreased ($P<.05$) toward the end of culture (Figure 15B). No interactions between treatment and culture time were observed with regard to PA production and embryo OD.

SDS-PAGE and Zymography. Because of concentration differences due to interval of medium recovery, media from hormonal treatments were combined and compared to media obtained from control treatments. Molecular weights of urokinase standards, PA in embryo homogenates and culture media are displayed in Table 1. Urokinase standards showed two bands at 36.8 ± 0.8 and 52.4 ± 1.2 kD for the low and high MW forms, respectively. The MW of PA in embryo homogenates and media at 24, 48, 72, 96 and 120-h intervals were 47.2 ± 0.6, 47.1 ± 0.9, 47.1 ± 0.9, 46.3 ± 1.5, 46.7 ± 0.9, and 47.0 ± 0.6 kD, respectively. The pattern of the MW of PA observed in both the embryo homogenate and the culture media indicates that bovine embryos secrete a urokinase-type plasminogen activator (Figure 16).

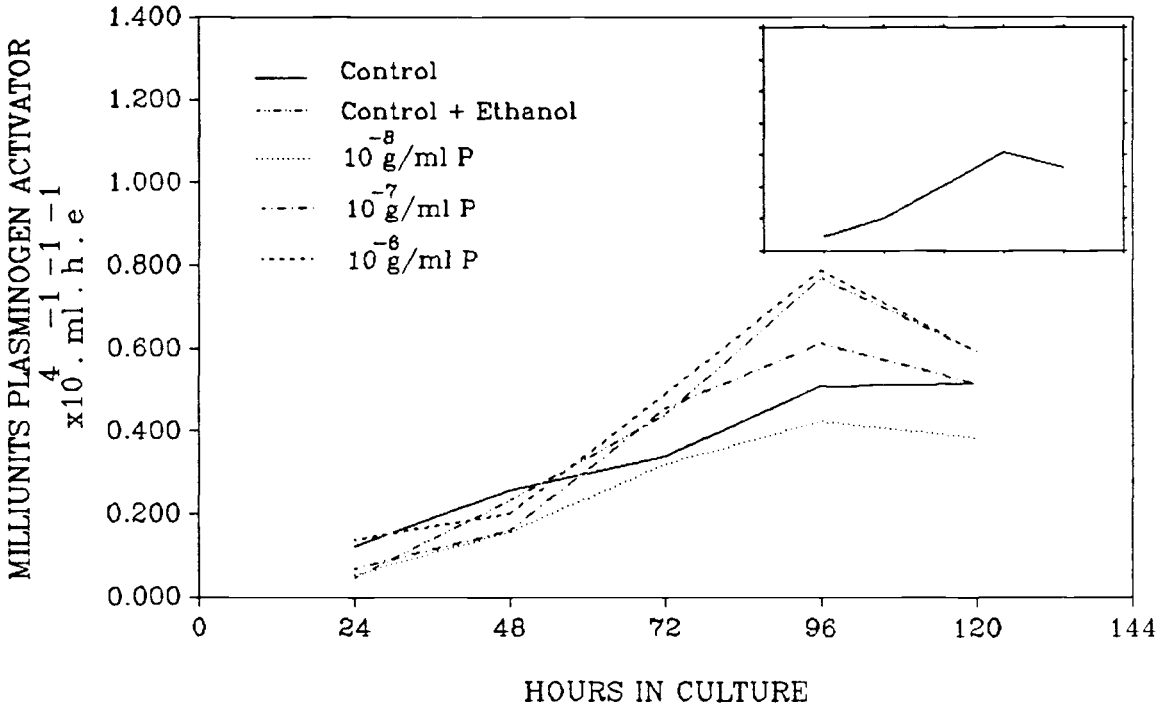

Figure 1. Plasminogen activator production by bovine embryos cultured in Ham's F-12 with 15 mg/ml BSA containing 0 (——), 0 + ethanol (-···-), 10^{-8} (····), 10^{-7} (·-·-·) or 10^{-6} (----) g/ml progesterone (P). Insert depicts overall plasminogen activator production for embryos cultured in the five treatments. Error mean square is 0.222.

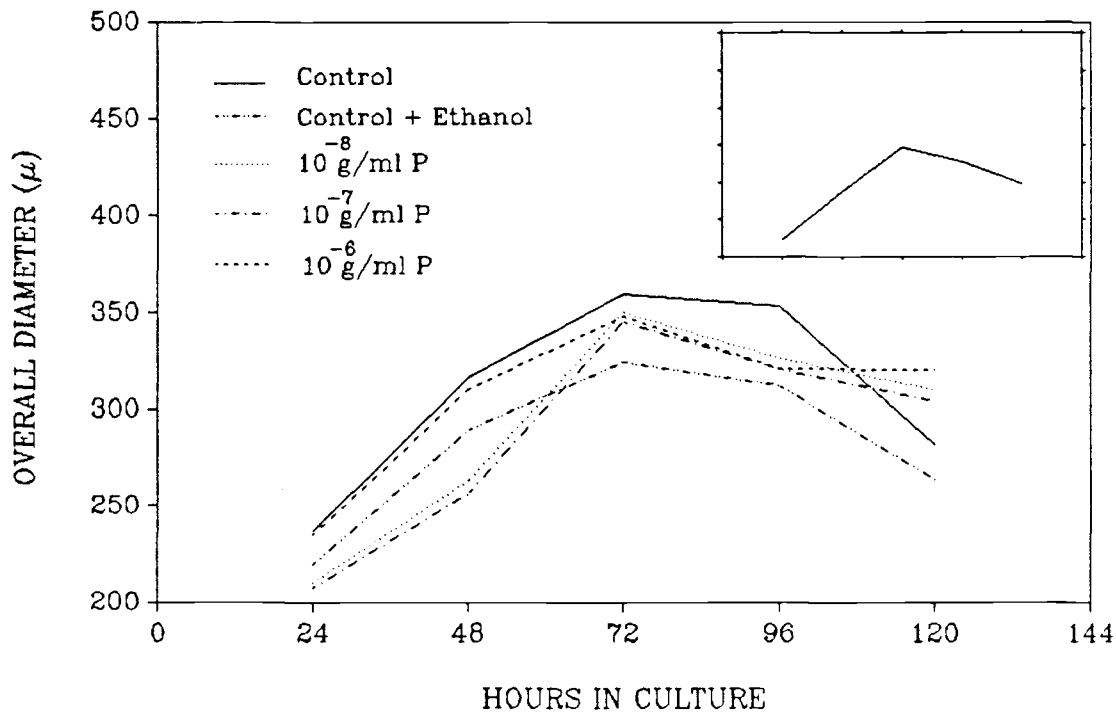

Figure 2. Embryonic overall diameter of bovine embryos cultured in Ham's F-12 with 15 mg/ml BSA containing 0 (——), 0 + ethanol (··-··-), 10^{-8} (····), 10^{-7} (·-·-·) or 10^{-6} (----) g/ml progesterone (P). Insert depicts average embryonic overall diameter for embryos cultured in the five treatments. Error mean square is 8587.

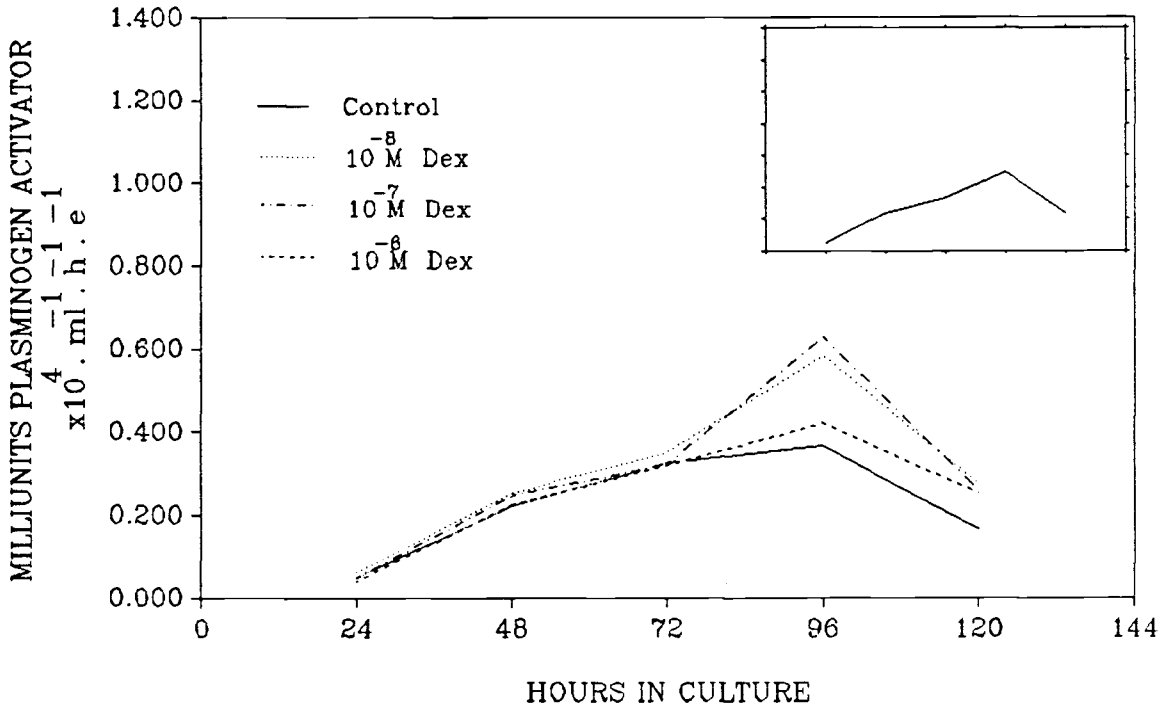

Figure 3. Plasminogen activator production by bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——), 10^{-8} (····), 10^{-7} (·-·-·) or 10^{-6} (----) M dexamethasone (Dex). Insert represents overall plasminogen activator production for embryos cultured in the four treatments. Error mean square is 0.046.

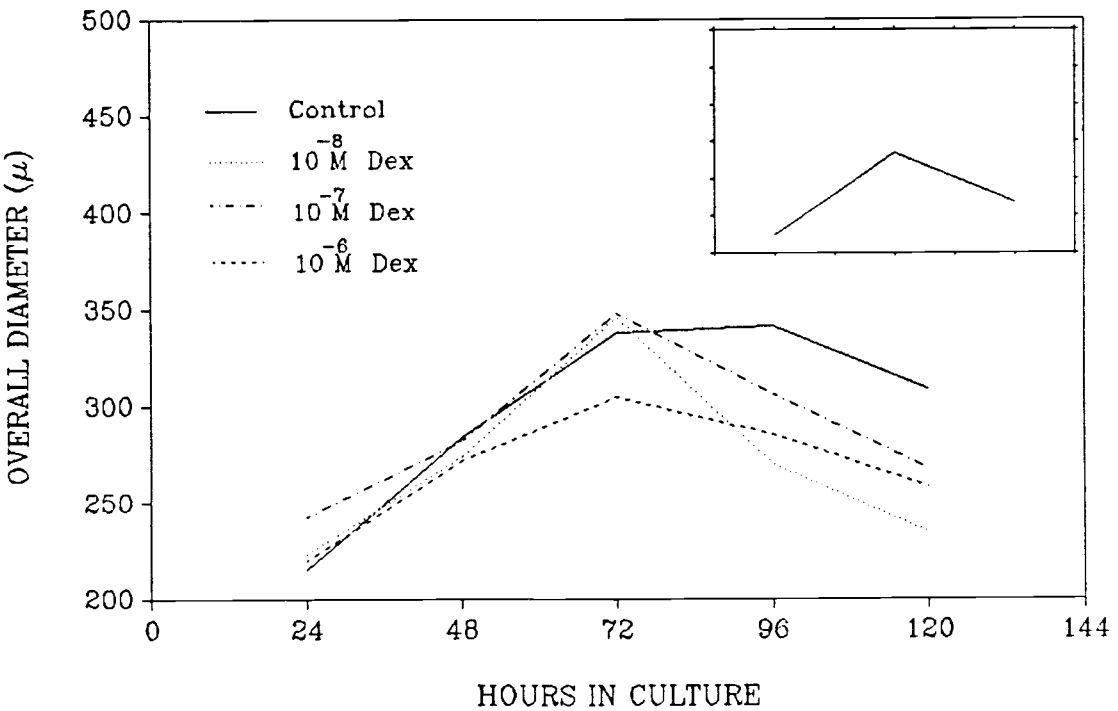

Figure 4. Embryonic overall diameter of bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——), 10^{-8} (····), 10^{-7} (·-·-·) or 10^{-6} (----) M dexamethasone (Dex). Insert depicts average embryonic overall diameter for embryos cultured in the four treatments. Error mean square is 6166.

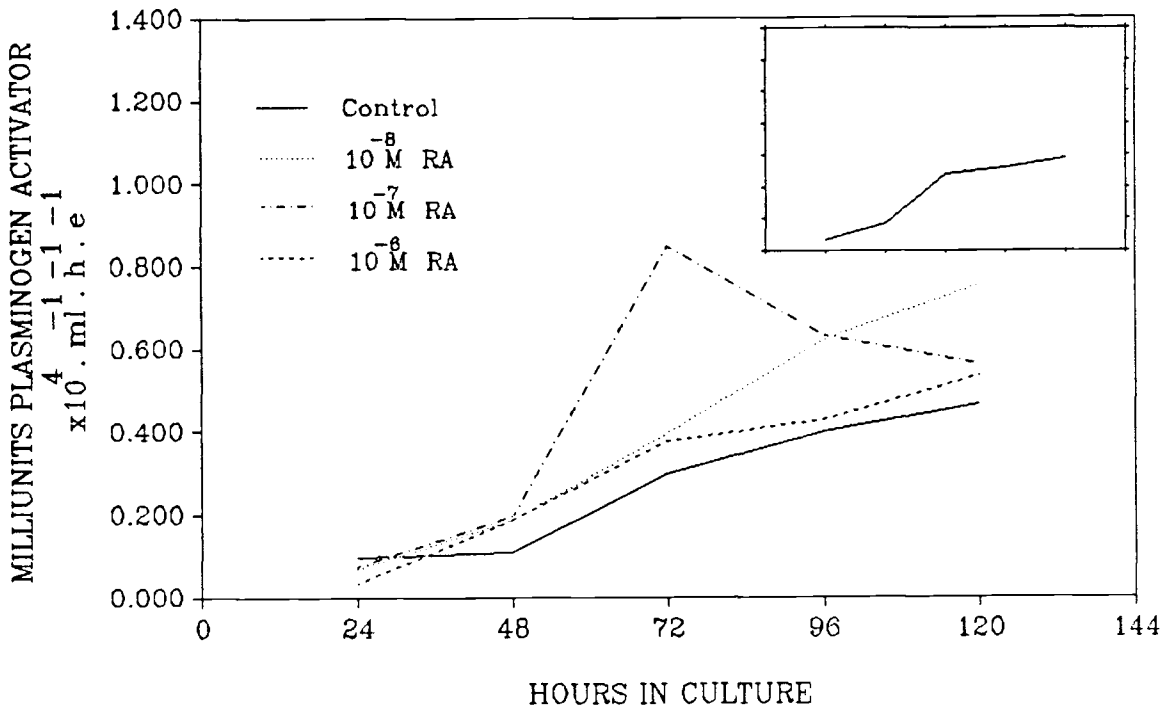

Figure 5. Plasminogen activator production by bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——), 10^{-8} (····), 10^{-7} (·-··-·) or 10^{-6} (----) M retinoic acid (RA). Insert represents overall plasminogen activator production for embryos cultured in the four treatments. Error mean square is 0.292.

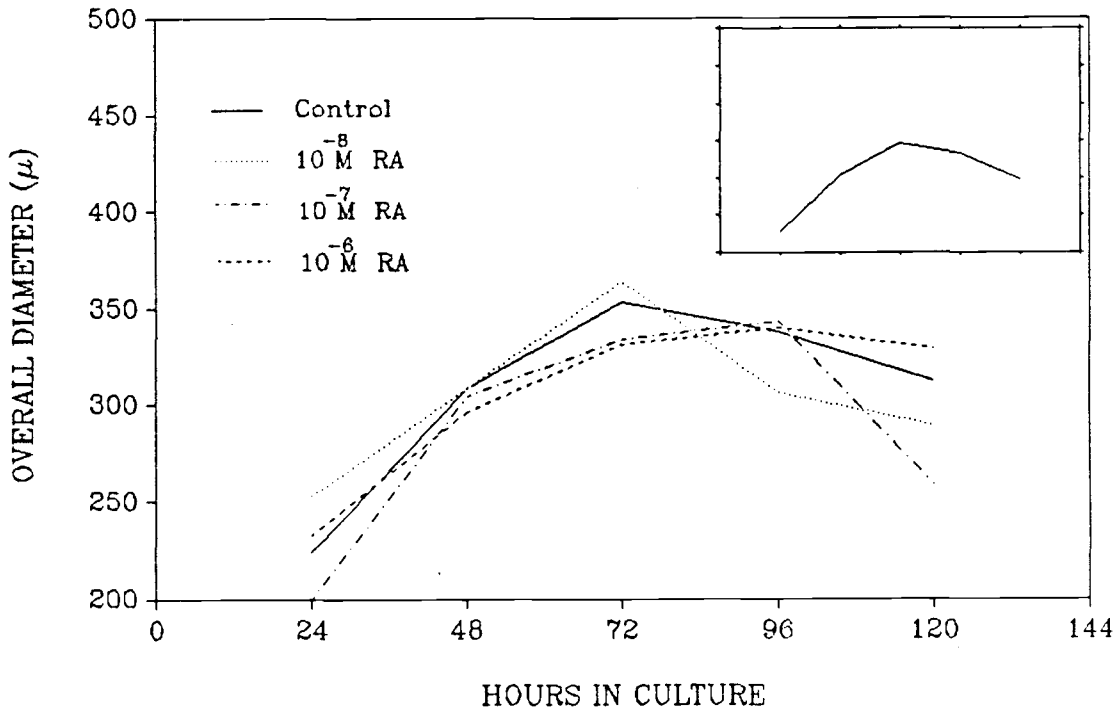

Figure 6. Embryonic overall diameter of bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——), 10^{-8} (····), 10^{-7} (·-·-·) or 10^{-6} (----) M retinoic acid (RA). Insert depicts average embryonic overall diameter for embryos cultured in the four treatments. Error mean square is 6377.

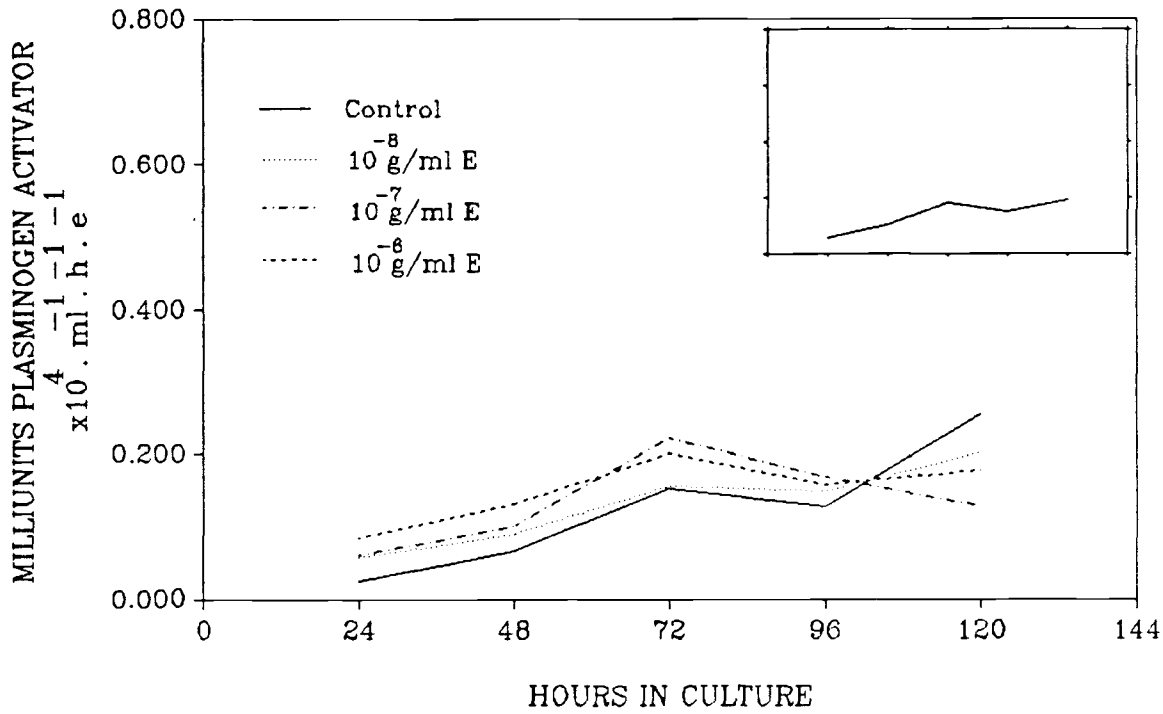

Figure 7. Plasminogen activator production by bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——), 10^{-8} (····), 10^{-7} (·-·-·) or 10^{-6} (----) g/ml estradiol-17β (E). Insert depicts overall plasminogen activator production for embryos cultured in the four treatments. Error mean square is 0.015.

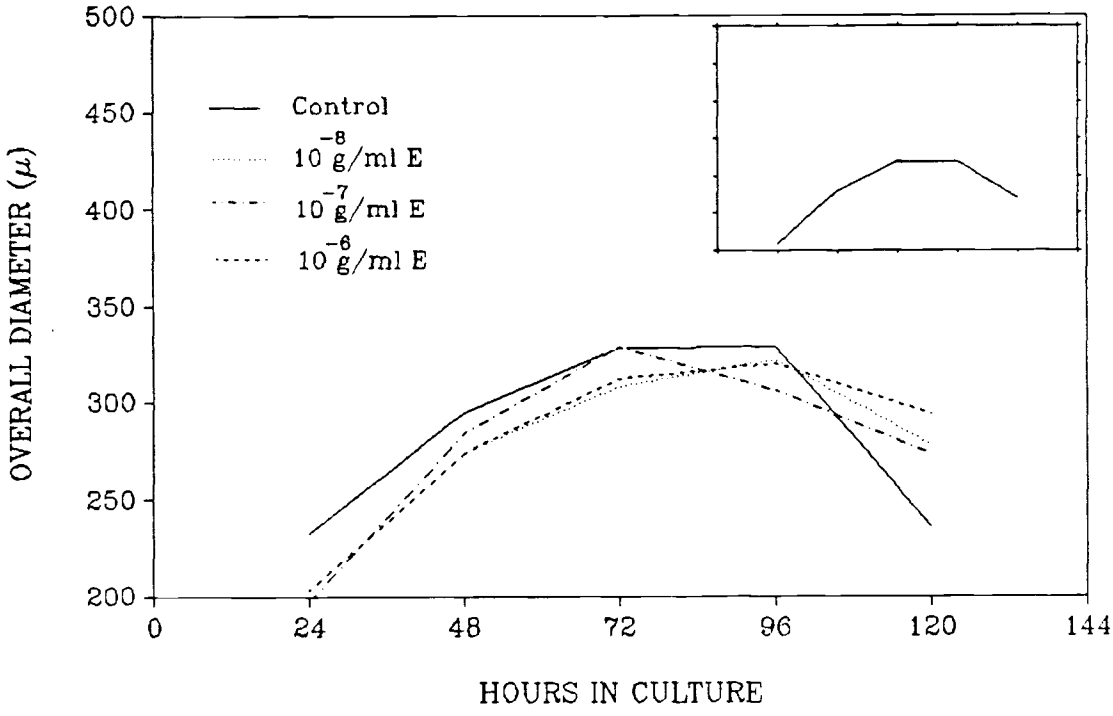

Figure 8. Embryonic overall diameter of bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——), 10^{-8} (····), 10^{-7} (·-·-·) or 10^{-6} (----) g/ml estradiol-17β (E). Insert depicts average embryonic overall diameter for embryos cultured in the four treatments. Error mean square is 6377.

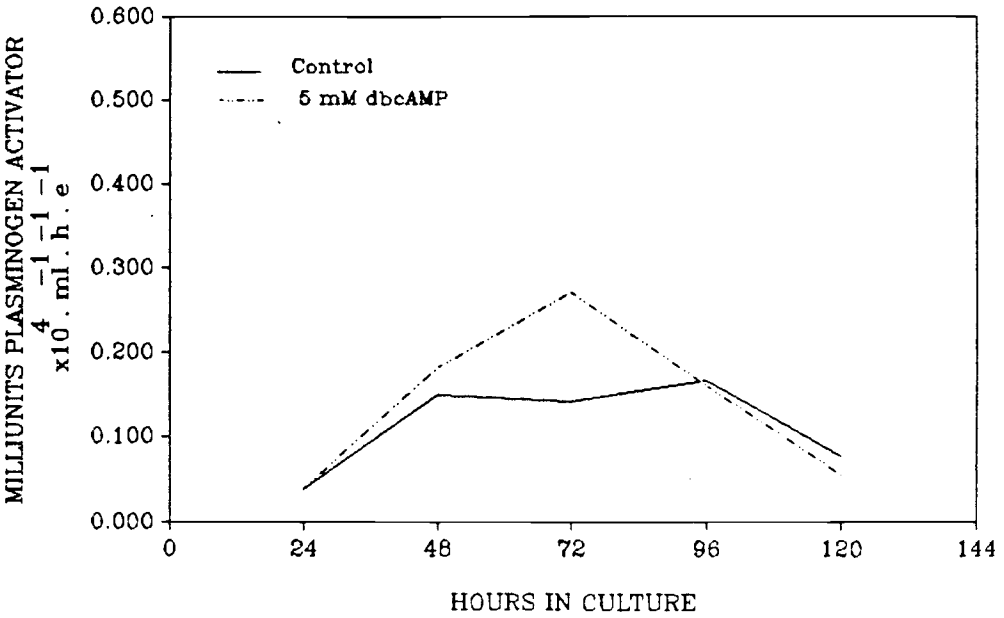

Figure 9A. Plasminogen activator production by bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——), or 5 (-···-···) mM dibutyryl cAMP (dbcAMP). Error mean square is 0.008.

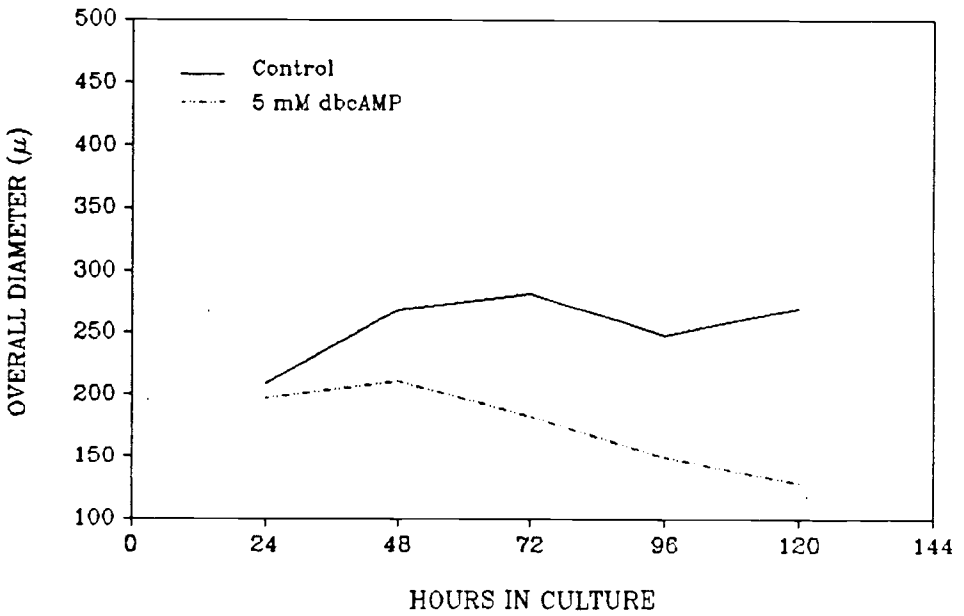

Figure 9B. Embryonic overall diameter of bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——), or 5 (-···-···) mM dibutyryl cAMP (dbcAMP). Error mean square is 2583.

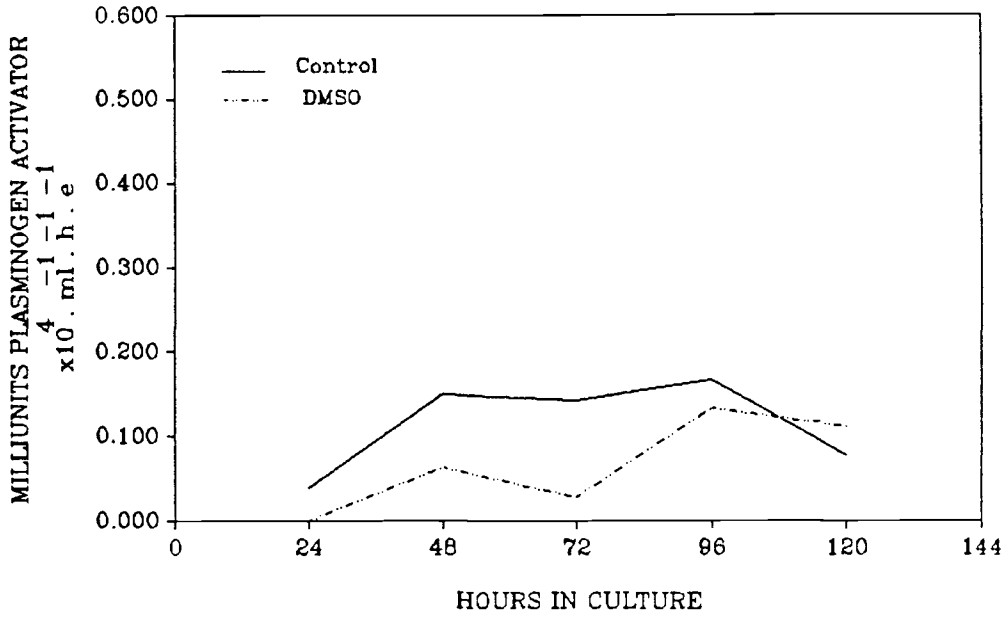

Figure 10A. Plasminogen activator production by bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——), or 1 (-···-··) percent dimethyl sulfoxide (DMSO). Error mean square is 0.004.

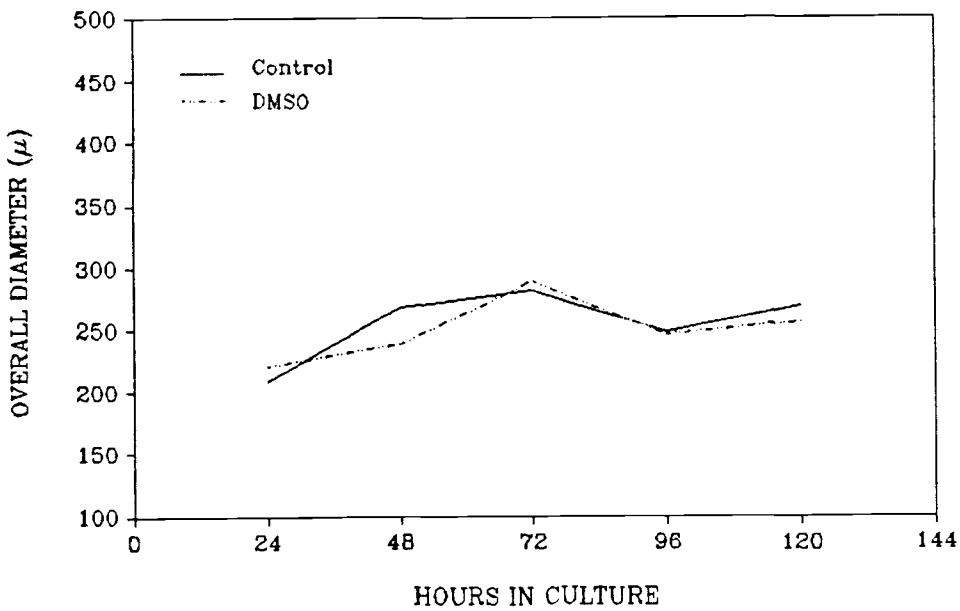

Figure 10B. Embryonic overall diameter of bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——), or 1 (-···-··) percent dimethyl sulfoxide (DMSO). Error mean square is 3180.

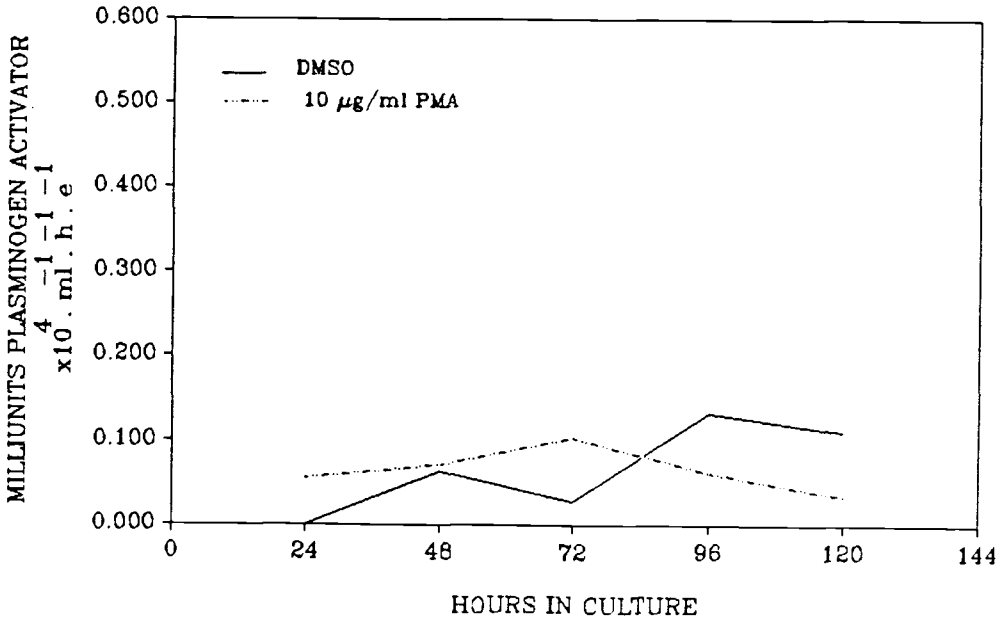

Figure 11A. Plasminogen activator production by bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 1 (——) percent dimethyl sulfoxide (DMSO) or 10 (-···-··) μg/ml phorbol-12-myristate 13-acetate (PMA). Error mean square is 0.005

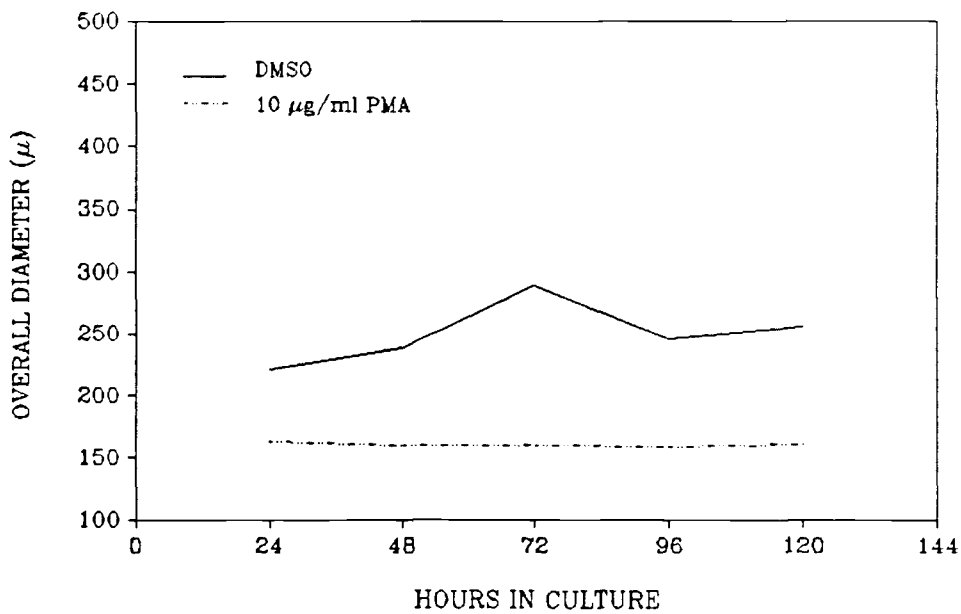

Figure 11B. Embryonic overall diameter of bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 1 (——) percent dimethyl sulfoxide (DMSO), or 10 (-···-··) μg/ml phorbol 12-myristate 13-acetate (PMA). Error mean square is 1430.

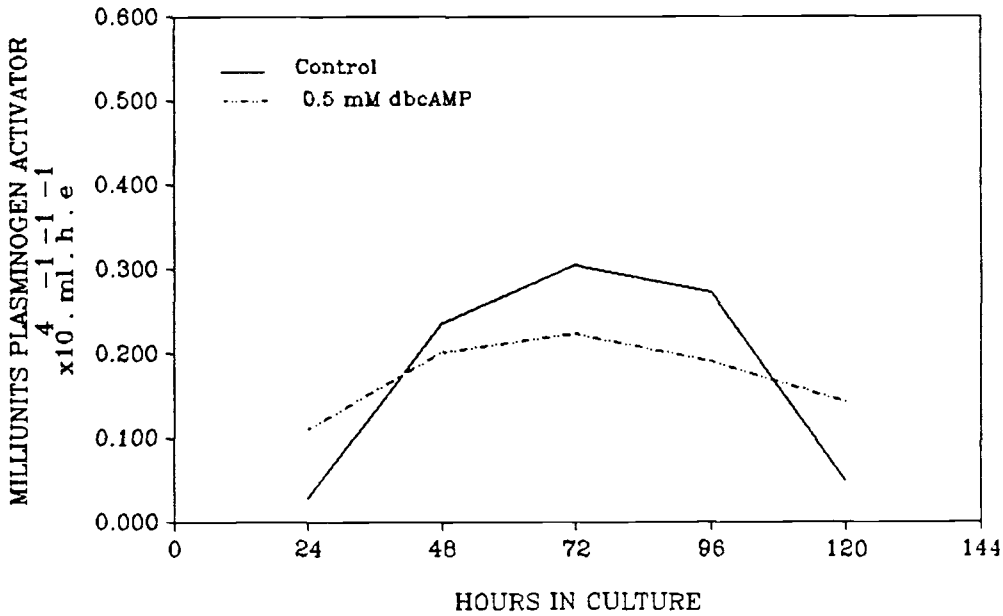

Figure 12A. Plasminogen activator production by bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——) or 0.5 (-···-··) mM dibutyryl cAMP (dbcAMP). Error mean square is 0.027.

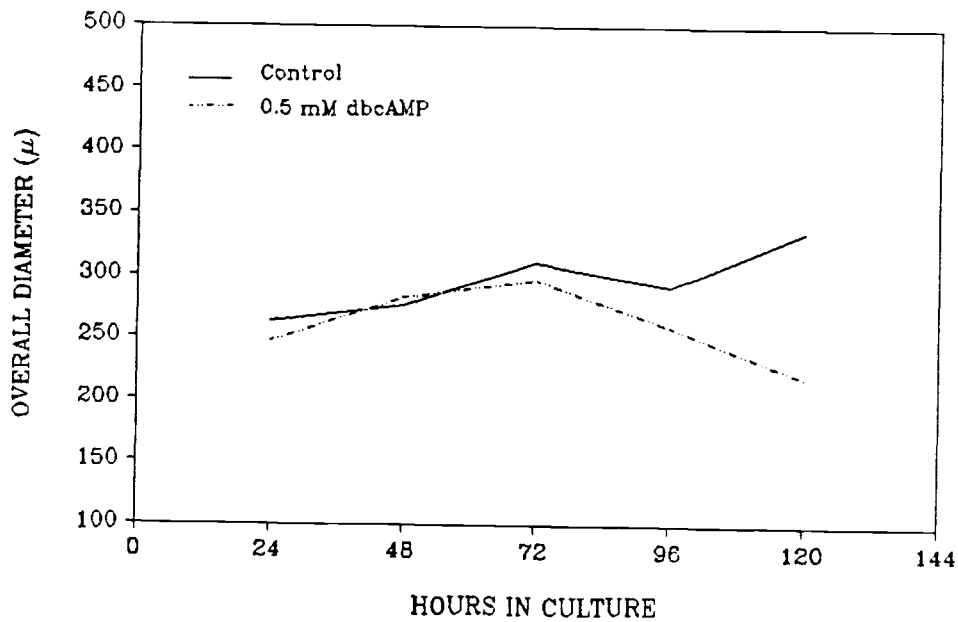

Figure 12B. Embryonic overall diameter of bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——) or 0.5 (-···-··) dibutyryl cAMP (dbcAMP). Error mean square is 5802.

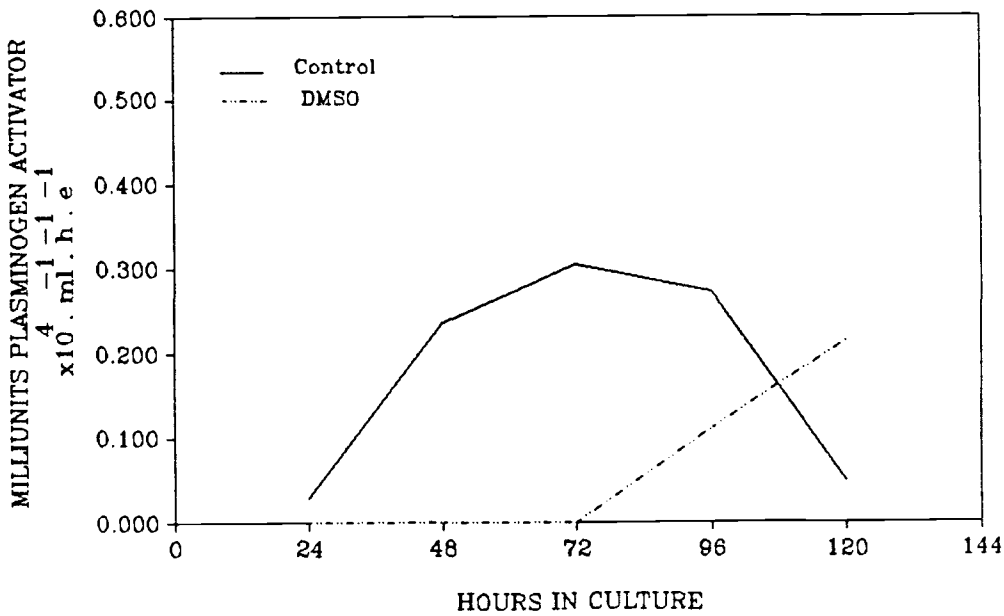

Figure 13A. Plasminogen activator production by bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——) or 1 (-···-···) percent dimethyl sulfoxide (DMSO). Error mean square is 0.032.

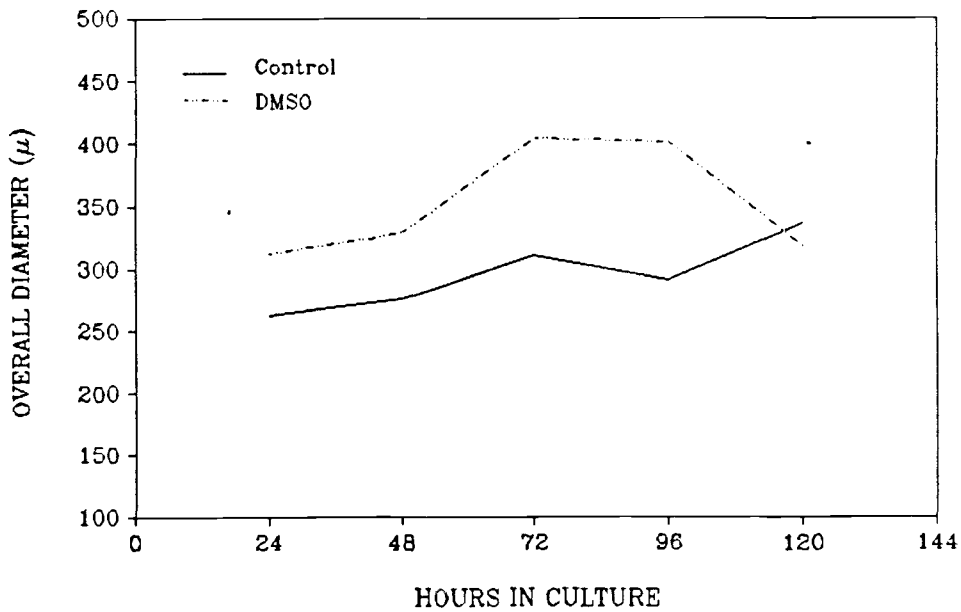

Figure 13B. Embryonic overall diameter of bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 0 (——) or 1 (-···-···) percent dimethyl sulfoxide (DMSO). Error mean square is 5889.

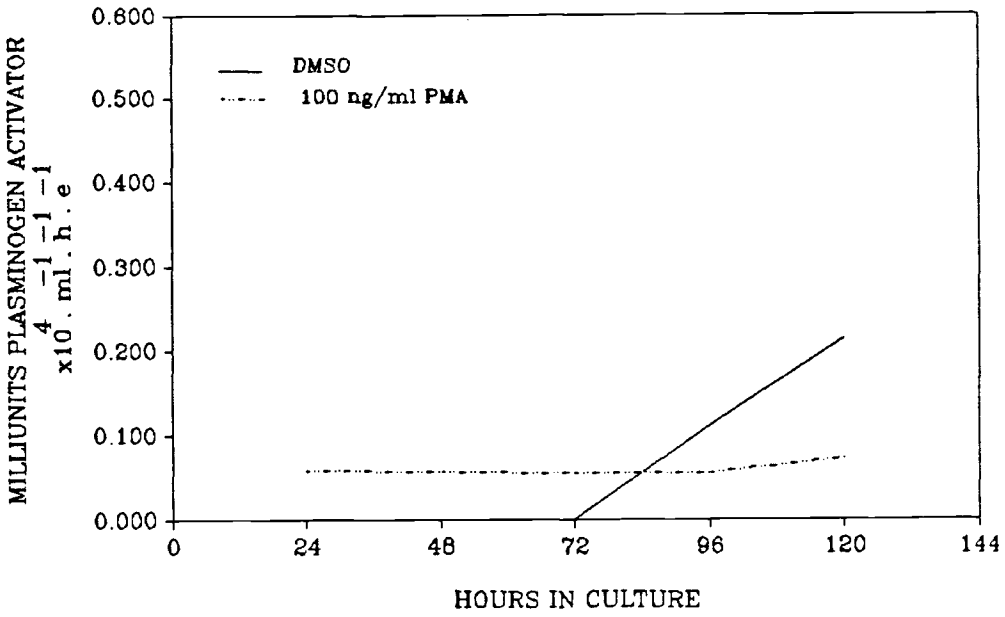

Figure 14A. Plasminogen activator production by bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 1 (——) percent dimethyl sulfoxide (DMSO) or 100 (-···-··) ng/ml phorbol 12-myristate 13-acetate (PMA). Error mean square is 0.007.

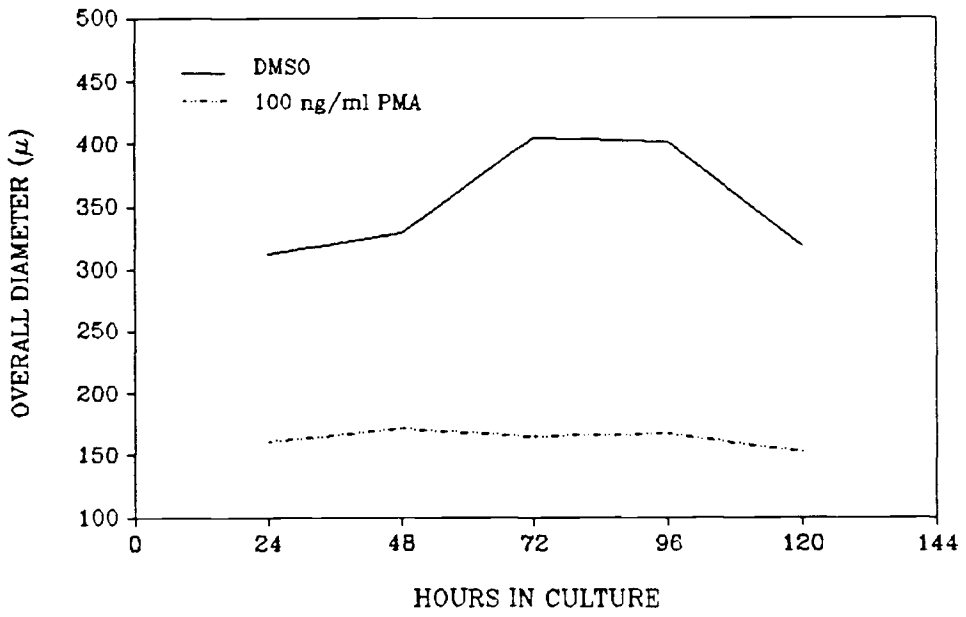

Figure 14B. Embryonic overall diameter of bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 1 (——) percent dimethyl sulfoxide (DMSO) or 100 (-···-··) ng/ml phorbol 12-myristate 13-acetate (PMA). Error mean square is 2949.

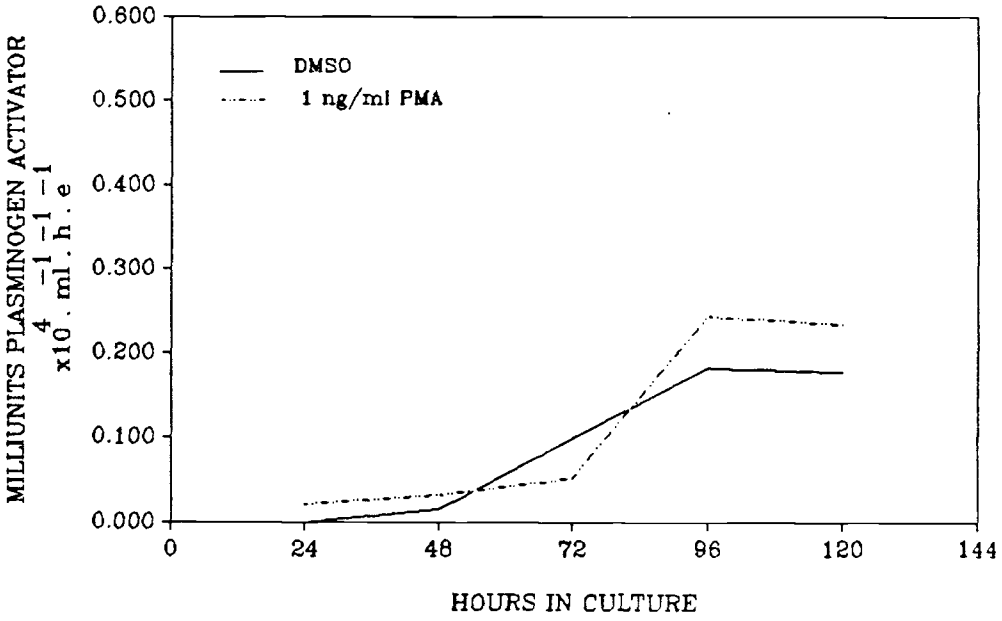

Figure 15A. Plasminogen activator production by bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 1 (——) percent dimethyl sulfoxide (DMSO) or 1 (-···-··) ng/ml phorbol 12-myristate 13-acetate (PMA). Error mean square is 0.013.

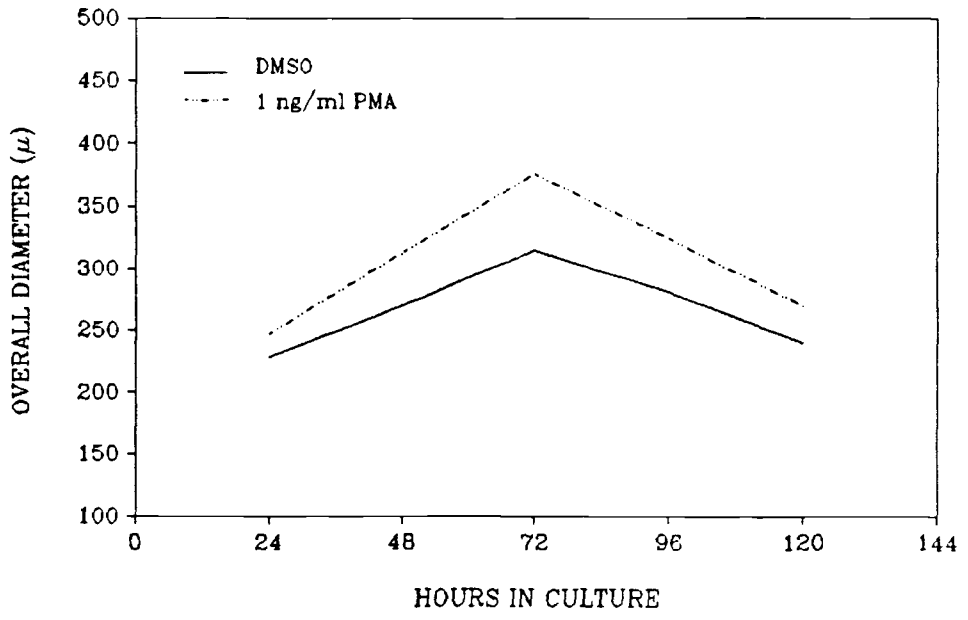

Figure 15B. Embryonic overall diameter of bovine embryos cultured in Ham's F-12 with 1.5 mg/ml BSA containing 1 (——) percent dimethyl sulfoxide (DMSO) or 1 (-···-··) ng/ml phorbol 12-myristate 13-acetate (PMA). Error mean square is 5945.

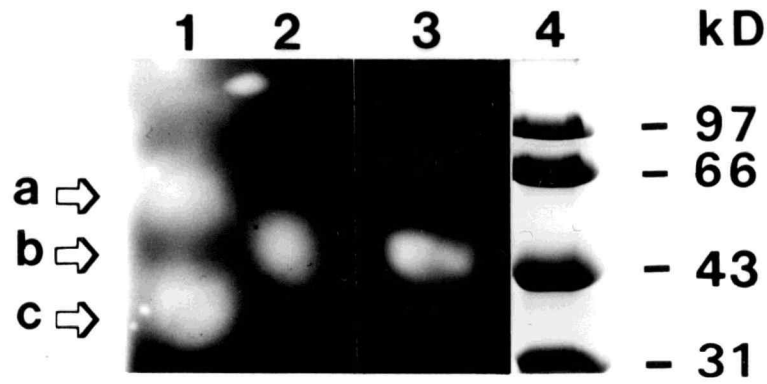

Figure 16. A representative zymograph of urokinase standards and embryonic plasminogen activators. Lane 1, urokinase standards; Lane 2, embryo homogenate; Lane 3, conditioned medium; Lane 4, stained polyacrylamide gel of molecular weight standards. Arrow heads A and C, high MW and low MW form of urokinase; arrow head B, embryonic plasminogen activator.

Table 1. Molecular weights (KD) of urokinase standards and plasminogen activators produced by bovine embryos.

Source	n	$\bar{X} \pm SE$[a]
Urokinase		
Low MW form	19	36.3 ± 0.8
High MW form	19	52.4 ± 1.2
Embryo homogenates	6	47.2 ± 0.6
Medium		
24 h	2	47.1 ± 0.9
48 h	2	47.1 ± 0.9
72 h	3	46.3 ± 1.5
96 h	3	46.7 ± 0.9
120 h	3	47.0 ± 0.6

[a] Mean ± standard error of the mean.

DISCUSSION

Endocrine regulation of plasminogen activator (PA) has been studied in various reproductive processes. The increase in PA activity during the ovulatory process is highly correlated to the preovulatory surge of gonadotropins (Beers et al., 1975; Beers et al., 1975; Strickland and Beers, 1976; Reich et al., 1985; Canipari and Strickland, 1986; Harlow et al., 1987; Liu et al., 1987; Bicsak et al., 1989). Follicle stimulating hormone stimulates PA production from Sertoli cells and segments of seminiferous tubule during spermatogenesis (Lacroix et al., 1981; Marzowski et al., 1985; Hettle et al., 1986; Coombs et al., 1987). These studies clearly establish the close relationship between PA activity and the hormonal profile during ovulation and spermatogenesis. Moreover, much evidence has been presented indicating an important physiological role for PA in these processes. Therefore, examining the effect of hormones on embryonic PA may provide some insight on the significance of this protease during early bovine embryogenesis.

In this study, several known modulators of PA have been evaluated in cultured Day 8 bovine embryos. Progesterone, estradiol-17β, dexamethasone, and retinoic acid did not affect the production of PA by cultured bovine embryos. Furthermore, these modulators neither affected embryo overall diameter nor hatching rate. Estradiol-17β increases PA activity in human breast carcinoma cell lines MCF-7 (Dickerman et al., 1989) and stimulates uptake of plasminogen by the mouse uterus (Finlay et al., 1983). Retinoic acid stimulates PA production in chick embryo fibroblasts and muscle cells (Wilson and Reich, 1978; Miskin et al.,

1978). Estradiol-17β and retinoic acid stimulation of PA production in these systems may be due to direct activation of mRNA synthesis. On the other hand, progesterone and dexamethasone have been shown to stimulate the production of an inhibitor that decreases plasminogen activation in several tissues including porcine endometrium (Fazleabas et al., 1982), rat granulosa cells (Harlow et al., 1987) and bovine Sertoli cells (Coombs and Jenkins, 1988). However, dexamethasone has been shown to have no effect on PA production by human melanoma cells (Roblin and Young, 1980) and rat ovarian granulosa cells (Strickland and Beers, 1976). Ovarian steroids also did not stimulate PA production in rat granulosa cells (Strickland and Beers, 1976; Reich et al., 1985).

The effect of ovarian steroids on early embryo development has been studied extensively in rodents and pigs. These hormones could act directly on the embryo, which would be a target tissue, or indirectly through the genital tract, which would provide the proper microenvironment required for embryos during the preimplantation period. Transport of mouse embryos through the oviduct, cleavage rate and transformation of morulae to blastocysts were delayed in females ovariectomized on Day 2 of pregnancy. Estradiol-17β and progesterone injected for 3 days after ovariectomy restored embryo transport rate, cleavage rate, and transformation rate to normal levels (Roblero and Garavagno, 1979). Estrogen also induced uptake of nucleic acid precursors in cultured mouse blastocysts (Harrer and Lee, 1973) and is required for implantation in rodents (Smith, 1968; Smith and Biggers, 1968). In pigs, estradiol-17β is important for the transformation of the compacted morulae to the cavitated blastocysts (Niemann and Elsaesser, 1986). Furthermore, embryonic synthesis of estrogens

increases dramatically at Day 12 of pregnancy which appears to be important in maternal recognition of pregnancy (Heap et al., 1979). Ovarian steroids, dexamethasone, and retinoic acid in the present study did not affect the viability of the bovine embryo in vitro. There are three possible explanations for the lack of effects of these hormones on preimplantation bovine embryos. The first possibility is that ovarian steroids may affect embryos indirectly through the stimulation of genital tract motility and secretions. Wilmut and coworkers (1985) found that asynchrony between embryo donor and recipient increases embryo loss, indicating the importance of the close relationship between the maternal hormone profile and embryo stage. The second possibility is concerned with the capability of the embryo to synthesize its own hormones, which can act in paracrine fashion to regulate early events of embryo development. Measurable concentrations of estrogen, progesterone and steroidogenic enzymes have been detected in the embryos of the mouse (Sengupta et al., 1982; Wu, 1987), rabbit (Dickmann et al., 1975; Borland et al., 1977), pig (Perry et al., 1973; Niemann and Elsaesser, 1987), horse (Paulo and Tischner, 1985) and cow (Shemesh et al., 1979). Finally, the ability of the embryo to achieve normal development to the blastocyst stage in vitro or in the oviduct of other species regardless of their hormonal status, signifies a considerable autonomy in early embryonic development (Brinster, 1963; Betteridge and Flechon, 1988). The normal growth of cultured bovine embryos observed in this study supports the existence of an endogenous control mechanism which contributes to the independence of the embryo from maternal hormones during in vitro development.

Although PA production by cultured bovine embryos was not effected by the hormonal treatments, a substantial increase in this enzyme was observed at particular intervals during culture. The temporal pattern of PA production was consistent in all experiments. Plasminogen activator production was relatively low during the first 48 h of culture, increased at 72 h, peaked at 96 h, then plateaued thereafter. Some slight deviations from this pattern were noted with dexamethasone, where PA production decreased toward the end of culture, and estradiol-17β, where peak PA production was attained at 72 h then plateaued thereafter. Moreover, PA production by embryos cultured in the presence and absence of estradiol-17β was lower than the levels observed in the other experiments. Kaaekuahiwi and Menino (1989) observed that cow source significantly affected embryo overall diameter and PA production by bovine embryos developing in vitro. Different cows were used as embryo donors for every experiment, therefore it is conceivable that variations in PA production between experiments may be due to differences in embryo donors.

The majority of embryos completed hatching before 72 h. Peak production of PA coincided to the period where the greatest embryonic diameter was attained, which occurred at 24 to 48 h after hatching. This is consistent with the finding of Kaaekuahiwi and Menino (1989), but different from the earlier observation of Menino and Williams (1987). The latter observed that peak PA production was associated with embryos initiating the hatching process; however, those investigators used day 5 to day 6 embryos and cultured them for 12 days. In spite of these differences the patterns of PA production by bovine embryos developing in vitro were similar. Other investigators working with

different species found similar results. A biphasic pattern of PA production has been reported for the mouse, where the first phase corresponds to trophoblast invasiveness and the second to acquisition of the migratory capability by differentiated embryonic cells (Sherman et al., 1976; Strickland et al., 1976). Porcine embryos developing in vitro secrete PA in a biphasic pattern, where the initial phase coincides with blastocyst elongation, and the second corresponds to the period of increased embryonic DNA content (Fazleabas et al., 1983).

Dibutyryl cAMP, a known inducer of cAMP-dependent protein kinase A, has been found to stimulate the production of PA in various types of cells (Beers et al., 1975; Hettle et al., 1986; Queenan et al., 1987) by promoting the transcriptional activity of the PA gene (Nagamine et al., 1983; Roesler et al., 1988). In this study, although dbcAMP did not affect PA production, a progressive decrease in OD was observed. This inhibitory effect persisted, but to a lesser extent, when the dbcAMP concentration was reduced tenfold. This is not surprising, since cAMP has been hypothesized to diminish during preimplantation stages of development (Robison, 1972). Moreover, Fisher and Gunaga (1975) have reported that mouse blastocyst development was retarded when cAMP accumulation was induced by theophylline, an inhibitor of intracellular breakdown of cAMP. High levels of cAMP are generally associated with inhibition of cellular proliferation (Bombik and Burger, 1973), presumably through inhibition of the rate of replication (Thomas et al., 1973). In contrast, Manejawala and coworkers (1986, 1989) demonstrated that cAMP analogs, which activate the cAMP-dependent protein kinase, stimulate the rate of blastocoel expansion and Na^+ uptake by mouse preimplantation embryos, suggesting a possible function for cAMP in

blastocoel formation. The apparent paradox between the observations of Manejawala et al. (1986, 1989) and our results could be explained by differences in the duration of culture. While embryos in this study were cultured for 5 days, Manejawala and his colleagues incubated embryos for only 8 h. Furthermore, our results with bovine embryos demonstrated that dbcAMP was without any effect on OD during the first 24 h. The inhibitory effect of dbcAMP was exerted at later intervals of culture, suggesting that prolonged exposure to this compound is required.

Phorbol 12-myristate 13-acetate (PMA), a potent tumor promotor, has been found to enhance PA activity in many cell types (Wigler et al., 1970; Vassalli et al., 1977; Quigley, 1979) through the activation of phospholipid-dependent protein kinase C (Castagna et al., 1982). Therefore, the possibility that the embryo utilizes protein kinase C in regulating PA production was investigated. Although PMA inhibited PA production by bovine embryos during the first 72 h of culture, this inhibitory effect was attributed to DMSO which was used to dissolve the PMA in the culture medium. Results from the first and second trials comparing the effect of DMSO to the control treatment suggests that this polar solvent inhibited PA production, particularly during the first 72 h of culture. Phorbol myristate acetate neither potentiated nor decreased PA production when compared to DMSO. Accordingly, the inhibitory effect of PMA on PA activity was largely due to DMSO. Carlsen (1987), however, found that DMSO concentrations up to 2.5% greatly stimulated the production of PA in Chinese hamster ovary cells, and new protein and RNA synthesis were required for this process. The inhibitory effect of DMSO on PA production observed in this study was

not accompanied by any changes in embryo viability; in contrast, a stimulatory effect was observed on OD during the second trial. The reason for this inhibitory effect of DMSO is not clear, hence, further investigation is required to elucidate the mechanism by which DMSO inhibited PA production.

Embryos cultured in the presence of high levels of PMA failed to hatch, showed signs of degeneration within 24 h, and completely degenerated by 72 h of culture, whereas PMA at low concentrations significantly stimulated embryonic diameter. Phorbol esters have been shown to stimulate RNA synthesis (Sivak and Van Duuren, 1970), increase cell division (Driedger and Blumberg, 1977), enhance protein synthesis (Hiwasa et al., 1982), and increase the specific activity of cell membrane associated Na^+/K^+-ATPase (Sivak et al., 1972). Thus, the effect of PMA on bovine embryo development reported herein may be due to stimulation of cell division and/or an increase in blastocoel expansion due to enhancement in Na^+/K^+-ATPase activity. Ultimately, these processes would lead to an increase in embryonic diameter. It is interesting that PMA, which promotes tumor growth, exerted such an effect on the embryo, supporting the relevance of embryos in cancer research as suggested by Betteridge and Flechon (1988). Embryos cultured in the presence of high levels of PMA degenerated, yet liberated measurable amounts of PA. Degeneration of the embryo is accompanied by progressive cell lysis, releasing cell-associated PA into the medium which explains the persistence of enzyme activity in degenerating embryos.

Zymographic analysis indicated that bovine embryos produce uPA. Analysis of embryonic tissues and media collected during the culture

period revealed great similarities in MW between the released- and cell-associated forms of PA. The MW of bovine embryonic PA is similar to mouse uPA reported by Marotti and coworkers (1982); however, in addition to uPA, mouse embryos produce tPA which was not detected in the present study. It is possible that tPA is produced during advanced stages of embryogenesis.

The exact physiologic role of PA in early embryogenesis is not clear. However, the ability of embryos of different species to produce this enzyme during early stages of development prompted several workers to implicate PA in various processes such as embryo hatching (Menino and Williams, 1987), tissue remodeling and proliferation (Fazleabas et al., 1983), migration of parietal endoderm (Strickland et al., 1976) and implantation (Strickland et al., 1976; Sherman, 1980; Axelrod, 1985). The fact that DMSO in this study inhibited PA production during the first 72 h of culture without affecting embryo hatching rate suggests that this protease is not involved directly in the hatching process. Menino and Williams (1987) concluded that plasminogen activation accelerated the hatching process without increasing the rate of hatching, and they suggested the involvement of other factors beside PA in this process (Menino et al., 1989). Furthermore, Sherman (1980) has suggested that PA is not directly involved in hatching. In the present study, maximum concentrations of PA occurred at about 48 h after hatching which coincided with blastocyst expansion and cellular proliferation and migration. Endodermal cells of the bovine embryo begin to migrate at Day 8 and their migration involves the interaction with an extracellular matrix (Betteridge and Flechon, 1988). Plasminogen activator, particularly uPA, has been implicated in the

migration of cultured cells (Ossowski et al., 1975) as well as parietal endoderm in mouse embryos (Strickland et al., 1976). Accordingly, PA released by bovine embryos may play a role in facilitating cellular migration during the preimplantation period.

In conclusion, bovine preimplantation embryos developing in vitro release an appreciable amount of uPA, which is neither dependent upon nor influenced by hormonal regulation. With the exception of PMA and dbcAMP, the hormones tested herein showed no effect on embryonic diameter. Although this study did not provide a conclusive role for PA in early embryo development, this enzyme may be important for cellular movement during bovine embryogenesis. Further research is required to elucidate the function of PA during the early stages of bovine embryo development.

BIBLIOGRAPHY

Abbadini M, Zhu GJ, Maggi A, Pangrazzi J, Donati MB, Mussoni L, 1987. Dermatan sulphate induces plasminogen activator release in the perfused rat hindquarters. Blood 70:1858-60.

Amin W, Karlan BY, Littlefield BA, 1987. Glucocorticoid sensitivity of OVCA 433 human ovarian carcinoma cells: inhibition of plasminogen activators, cell growth, and morphological alterations. Cancer Res. 47:6040-45.

Andreasen PA, Christensen TH, Hwang J-Y, Nielsen LS, Wilson EL, Dano K, 1986. Hormonal regulation of extracellular plasminogen activators and Mr ~ 54000 plasminogen activator inhibitor in human neoplastic cell lines; studies with monoclonal antibodies. Mol Cel Endocr 45:137-47.

Andreasen PA, Pyke C, Riccio A, Kristensen P, Nielsen LS, Lund LR, Blasi F, Dano K, 1987. Plasminogen activator inhibitor type 1 biosynthesis and mRNA level are increased by dexamethasone in human fibrosacroma cells. Mol Cell Biol 7:3021-25.

Astrup T, 1978. Fibrinolysis, an overview. In: Davidson JF, Rowan RM, Saramaria MM, Desnoyers PC (eds.), Progress in Chemical Fibrinolysis and Thrombolysis. Vol. 3. New York: Raven Press, pp. 1-97.

Axelrod HR, 1985. Altered trophoblast functions in implantation-defective mouse embryo. Devel Biol 108:185-90.

Baron-Van Evercooren A, Leprince P, Rogister B, Lefebvre PP, Delree P, Selak I, Moonen G, 1987. Plasminogen activators in developing peripheral nervous system, cellular origin and mitogenic effect. Devel. Brain Res. 36:101-08.

Bayliss WM, Starling EH, 1902. The mechanism of pancreatic secretion. J Physiol 28:325-53.

Beers WH, 1975. Follicular plasminogen and plasminogen activator and the effect of plasmin on ovarian follicle wall. Cell 6:379-86.

Beers WH, Strickland S, Reich E, 1975. Ovarian plasminogen activator: relationship to ovulation and hormonal regulation. Cell 6:387-94.

Bergh A, Damber JE, Jacobesson H, Nilsson TK, 1987. Production of lactate and tissue plasminogen activator in vitro by seminiferous tubules obtained from adult unilaterally cryptorchid rats. Arch Androl 19:177-82.

Betteridge KJ, Flechon JE, 1988. The anatomy and physiology of pre-attachment bovine embryos. Theriogenology 29:154-87.

Bicsak TA, Cajander SB, Peng X-R, Ny T, LaPlot PS, Lu JKH, Kristensen P, Tsafriri A, Hsueh AJW, 1989. Tissue type plasminogen activator in rat oocytes: expression during the periovulatory period, after fertilization, and during follicular atresia. Endocrinology 124:187-94.

Bicsak TA, Hsueh AJ, 1989. Rat oocyte tissue plasminogen activator is a catalytically efficient enzyme in the absence of fibrin. J Biol Chem 264:630-34.

Blasi F, Vassalli J-D, Dano K, 1987. Urokinase-type plasminogen activator: Proenzyme, receptor and inhibitors. J Cell Biol 104:801-04.

Bode VC, Dziadek MA, 1979. Plasminogen activator secretion during mouse embryogenesis. Devel Biol 73:272-89.

Bombik BM, Burger MM, 1973. c-AMP and the cell cycle: inhibition of growth stimulation. Exp Cell Res 20:88-94.

Borland RM, Erickson GF, Ducibella T, 1977. Accumulation of steroids in rabbit preimplantation blastocysts. J Reprod Fert 49:219-24.

Brinster RL, 1963. A method for in vitro cultivation of mouse ova from two-cell to blastocyst. Expl Cell Res 32:205-08.

Browne MJ, Carey JE, Chapman CG, Tyrrell AWR, Entwisle C, Lawrence GMP, Revy B, Dodd I, Esmail A, Robinson JH, 1988. A tissue-type plasminogen activator mutant with prolonged clearance in vitro. J Biol Chem. 263:1955-02.

Burtin P, Fondaneche M-C, 1988. Receptor for plasmin on human carinoma cells. J Natl Cancer Inst 80:762-65.

Canipari R, O'Connell ML, Meyer G, Strickland S, 1987. Mouse ovarian granulosa cells produce urokinase-type plasminogen activator, whereas the corresponding rat cells produce tissue-type plasminogen activator. J Cell Biol 105:977-81.

Canipari R, Strickland S, 1986. Studies on the hormonal regulation of plasminogen activator production in the rat ovary. Endocrinology 118:1652-59.

Carlsen SA, 1987. Stimulation of plasminogen activator production by dimethyl sulfoxide in Chinese hamster ovary cells. Biochem Cell Biol 65:710-16.

Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y, 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem 257:7847-51.

Chow G, Woronick A, Kinkade P, DeLeon R, Farina PR, Malteo MR, 1987. Pharmacological modulation of plasminogen activator secretion by F388D1 cell line. Agents & Actions 21:387-89.

Christman JK, Acs G, Silagi S, Silverstein SC, 1975. Plasminogen activator: biochemical characterization and correlation with tumorigenicity. In: Reich E,Rifkin DB, Shaw E (eds.), Proteases and Biological Control. New York: Cold Spring Harbon Laboratory, pp. 827-39.

Christman JK, Silverstein SC, and Acs G, 1977. Plasminogen activators. In: Barrett AJ (ed.), Proteinases in Mammalian Cells and Tissues. Amsterdam: Elsevier/North-Holland Biomedical Press, pp. 91-149.

Clermont Y, 1972. Kinetics of spermatogenesis in mammals, seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev 52:198-236.

Coombs RJ, Ellison J, Woods A, Jenkin N, 1988. Only tissue-type plasminogen activator is screted by immature bovine Sertoli cell-enriched cultures. J Endocr. 117:63-67.

Coombs RJ, Jenkins N, 1988. Characterization of a plasminogen activator inhibitor induced by glucocorticoids in immature bovine Sertoli cell enriched cultures. J. Endocr 117:69-74.

Cwikel BJ, Barouski-Miller PA, Coleman PL, Gelehrter TD, 1984. Dexamethasone induction of an inhibitor of plasminogen activator in HTC rat hepatoma cells. J Biol Chem 257:4260-64.

Dano K, Andreasen A, Grondahl-Hansen J, Kristensen P, Nielsen LS, Skriver L, 1985. Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 44:139-266.

Declerck PJ, DeMol M, Aless M-C, Baudner S, Pagues E-P, Preissner KT, Berghaus G-M, Collen D, 1988. Purification and characterization of a plasminogen activator inhibitor 1 binding protein from human plasma. J Biol Chem 263:15454-61.

Degen SJF, Heckel JL, Reich E, Degen JL, 1987. The murine urokinase-type plasminogen activator gene. Biochemistry 26:8270-79.

Degen SFJ, Rajput B, Reich E, 1986. The human tissue plasminogen activator gene. J Biol Chem 261:6972-85.

Deguchi K, Shirakawa S, 1988. Plasminogen activation by tissue plasminogen activator in the presence of platelets. Tromb. Res. Supp. VIII:65-72.

Dickerman HW, Martinez HL, Seeger JI, Kumar SA, 1989. Estrogen regulation of human breast cancer cell line MCF-7 tissue plasminogen activator. Endrocrinology 125:492-500.

Dickmann Z, Dey SK, Gupta JE, 1975. Steroidogenesis in rabbit preimplantation embryos. Proc Nat Acad Sci USA 72:298-300.

Driedger PE, Blumberg PM, 1977. The effect of phorbol diesters on chick embryo fibroblasts. Cancer Res 37:3257-65.

Dulbecco R, Vogt M, 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J Exp Med 99:167-82.

Espey LL, 1975. Evaluation of proteolytic activity in mammalian ovulation. In: Reich E, Rifkin DB, Shaw E (eds.), Proteases and Biological Control. New York: Cold Spring Harbor Laboratory, pp. 767-76.

Espey L, Shimada H, Okamura H, Mori T, 1985. Effect of various agents on ovarian plasminogen activator activity during ovulation in pregnant mare's serum gonadotropin-primed immature rats. Biol Reprod 32:1087-94.

Fazleabas AT, Bazer FW, Roberts RM, 1982. Purification and properties of a progesterone-induced plasmin/trypsin inhibitor from uterine secretion of pigs and its immunocytochemical localization in the pregnant uterus. J Biol Chem 257:6886-97.

Fazleabas AT, Geisert RD, Baser FW, Roberts RM, 1983. Relationship between release of plasminogen activator and estrogen by blastocysts and secretion of plasmin inhibitor by uterine endometrium in the pregnant pig.Biol Reprod 29:225-38.

Fears R, 1988. Kinetic studies on the effect of heparin and fibrin on plasminogen activators. Biochem J 249:77-81.

Fell HB, Dingle JT, Webb CT, 1962. Studies on the mode of action of excess Vitamin A. The specificity of the effect an embryonic chick-limb catilage in culture and on isolated rat liver lysosomes. Biochem J 83:63-69.

Finlay TH, Katz J. Levitz M, Nathoo SA, Seiler S, 1983. Estrogen-stimulated uptake of plasminogen by the mouse uterus. Endocrinology 112:856-61.

Fisher DL, Gunaga KP, 1976. Theophylline induced variations in cyclic AMP content of the superovulated preimplantation mouse embryo. Biol Reprod 12:471-76.

Fritz I, Rommerts FFG, Louis BG, Dorrington JH, 1976. Regulation of follicle stimulating hormone and dibutyryl cyclic AMP on androgen binding protein formation by Sertoli cell enriched cultures. J. Reprod Fertil 46:17-24.

Fulton RJ, Hart DA, 1987. Expression of plasminogen activator activity by rate thymocytes is restricted to a subpopulation of cells. Med Sci Res 15:1089-90.

Gelehrter TD, Sznycer-Laszuk R, Zeheb R, Cwikel BJ, 1987. Dexamethasone inhibition of tissue-type plasminogen activator (tPA) activity: paradoxical induction of both antigen and plasminogen activator inhibitor. Mol Endocr. 1:97-101.

Gerard R, Chien K, Meidell R, 1986. Molecular biology of tissue pasminogen activator and endogenous inhibitors. Mol Biol Med 3:449-57.

Gheysen D, Lijnen HR, Pierard L, deForesta F, Demarsin E, Jacobs P, DeWild M, Bollen A, Collen D, 1987. Characterization of a recombinant fusion protein of the finger domain of tissue-type plasminogen activator with a truncated single chain urokinase-typeplasminogen activator. J Biol Chem 262:11779-84.

Gierthy JF, Lincoln DW, Gillespie MB, Seeger JI, Martinez HL, Dickerman HW, Kumar SA, 1987. Suppression of estrogen-regulated extracellular tissue plasminogen activator activity of MCF-7 cells by 2, 3, 7, 8 tetrachlorodibenzo-p-dioxin. Cancer Res. 47:6198-203.

Granelli-Piperno A, Reich E, 1978. A study of proteases and protease-inhibitor complexes in biological fluids. J Exp Med 148:223-34.

Gross JL, Behrens DL, Mullins DE, Kornblith PL, Dexter DL, 1988. Plasminogen activator and inhibitor activity in human glioma cells and modulation by sodium butyrate. Cancer Res 48:291-96.

Gross JL, Moscatelli D, Jaffe EA, Rifkin DB, 1982. Plasminogen activator and collagenase production by cultured capillary endothelial cells. J Cell Biol 95:974-81.

Grossbard EB, 1987. Recombinant tissue plasminogen activator: A brief review. Pharmacut. Res. 4:375-78.

Gudewicz PW, Gilboa N, 1987. Human urokinase-type plasminogen activator stimulates chemotaxis of human neutrophils. Biochem Biophysic Res Comm. 147:1176-81.

Guillou F, Martinant N, Combarnous Y, 1986. Study of the superactivity of equine follicle-stimulating hormone in in-vitro stimulation of rat Sertoli cells. Biochem Biophysic Acta 887:196-203.

Hansen L, Blue Y, Barone K, Collen D, Larsen GR, 1988. Functional effects of asparagine-linked oligosaccharide on natural and variant human tissue-type plasminogen activator. J Biol Chem 263:15713-19.

Hantai D, Rao JS, Kahler C, Festoff BW, 1989. Decrease in plasminogen activator correlates with synapse elimination during neonatal development of mouse skeletal muscle. Proc Natl Acad Sci USA 86:362-66.

Harlow CR, Coombs RJ, Hodges JK, Jenkins N, 1987. Modulation of plasminogen activation by glucocorticoid hormones in rat granulosa cell. J Endocr 114:207-12.

Harpel P, Bang NU, Hamburger F, Treger A, 1966. Plasminogen activator in mouse uterine fluid: Its suppression by estradiol and progesterone. Proc Soc Exp Biol Med 122:1192-95.

Harpel PC, Homburger F, Treger A, 1967. Mouse uterine fluid plasminogen activator, acid phosphatase, and contraceptive hormones. Am J Physiol 215:928-31.

Harrer JA, Lee HH, 1973. Differential effects of oestrogen on the uptake of nucleic acid precursors by mouse blastocysts in vitro. J Reprod Fertil 33:327-30.

Hart DA, Rehemitulla A, 1988. Plasminogen activators and their inhibitors: Regulators of extracellular proteolysis and cell function. Comp Biochem Physiol 90B:691-708.

Heap RB, Flint APF, Gadsby JE, Rice C, 1979. Hormones, the early embryo and the uterine environment. J Reprod Fertil 55:267-75.

Heaton JH, Gelehrter TD, 1989. Glucocorticoid induction of plasminogen activator-inhibitor messenger RNA in rat hepatoma cells. Mol Endocr 3:349-55.

Heaton JH, Nebes VL, O'Dell LG, Morris SM, Gelehrter TD, 1989. Glucocorticoid and cyclic nucleotide regulation of plasminogen activator-inhibitor gene expression in primary cultures of rat hepatocytes. Mol Endocr 3:185-92.

Hettle, JA, Balekjian E, Tung PS, Fritz IB, 1988. Rat testicular peritubular cells in culture secrete an inhibitor of plasminogen activator activity. Biol Reprod 38:359-71.

Hettle JA, Waller EK, Fritz IB, 1986. Hormonal regulation alters the type of plasminogen activator produced by Sertoli cells. Biol Reprod 34:895-904.

Hiwasa T, Fujimura S, Sakiyama S, 1982. Tumor promotors increase the synthesis of a 32,000-dalton protein in BALB/c 3T3 cells. Proc Natl Acad Sci USA 79:1800-804.

Hortin GL, Gibson BL, Fok KF, 1988. a_2-antiplasmin's carboxy-terminal lysine residue is a major site of interaction with plasmin. Bioch Biophysi Res Comm 155:591-96.

Hoylaerts M, Rijken DC, Lijnen HR, Collen D, 1982. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of Fibrin. J Biol Chem 257:2912-19.

Huarte J, Belin D, Vassalli J-D, 1985. Plasminogen activator in mouse and rat oocytes: Induction during meiotic maturation. Cell 43:551-58.

Huarte J, Belin D, Vassalli A, Strickland S, Vassalli J-D, 1987. Meiotic maturation of mouse oocytes triggers the translation and polyadenylation of dormant tissue-type plasminogen activator mRNA. Genes Devel 1:1201-11.

Huff, K, Lipmann ME, 1984. Hormonal control of plasminogen activator secretion in ZR-75-1 human breast cancer cell line in culture. Endocrinology 114:1702-10.

Jans DA, Dierks-Ventling C, Hemmings BA, 1987. Pathway of urokinase-type plasminogen activator induction in T47D and LLC-PK_1 cell lines. Exp Cell Res 172:76-83.

Kaaekuahiwi MA, Menino AR, 1989. The relationship between plasminogen activator production and various aspects of bovine embryo development in vitro. Proc West Sec Am Soc Anim Sci 40:302-05.

Keski-Oja J, Vaheri A, 1982. The cellular target for the plasminogen activator, Urokinase, in human fibroblasts - 66,000 dalton protein. Biochemica Biophyisca Acta 720:141-146.

Kooistra T, Van Den Berg J, Tons A, Platenburg G, Rijken DC, Van Den Berg E, 1987. Butyrate stimulates tissue-type plasminogen activator synthesis in cultured human endothelial cells. Biochem J 247:605-12.

Kruithof EKO, 1988. Plasminogen activator inhibitors - a review. Enzyme 40:113-21.

Kuiper J, Otter M, Rijken DC, Van Berkel TJC, 1988. Characterization of the interaction in vivo of tissue-type plasminogen activator with liver cells. J Biol Chem 263:18220-24.

Krystosek A, Verrall S, Seeds NW, 1988. Plasminogen activator secretion in relation to Schwann cell activities. Int J Neuroscience. 6:483-93.

Lacroix M, Parvienen M, Fritz I, 1981. Localization of testicular plasminogen activator in discrete positions (stages VII and VIII) of the seminiferous tubule. Biol Reprod 25:143-46.

Laiho M, Saksela O, Keski-Oja J, 1987. Transforming growth factor-ß induction of type-1 plasminogen activator inhibitor. J Biol Chem 262:17467-74.

Lambers JWJ, Cammenga M, Konig BW, Mertens K, Pannekoek H, Van Mourik JA, 1987. Activation of human endothelialcell-type plasminogen activator inhibitor (PAI-1) by negatively charged phospholipids. J Biol Chem 262:17492-96.

Levin EG, Santell L, 1987. Conversion of the active to latent plasminogen activator inhibitor from human endothelial cells. Blood. 70:1090-98.

Liedholm P, Astedt B, 1975. Fibrinolytic activity of the rat ovum, appearance during tubual passage and disappearance at implantation. Int J Fertil 20:24-26.

Lijnen HR, Collen D, 1988. Mechanisms of plasminogen activation by mammalian plasminogen activators. Enzyme 40:90-96.

Lijnen HR, Nelles L, Van Hoff B, Demarsin E, Collen D., 1988. Structural and functional characterization of mutants of recombinant single-chain urokinase-type plasminogen activator obtained by site-specific mutagenesis of Lys^{158}, Ile^{159} and Ile^{160}. Eur. J Biochem 177:575-82.

Lijnen HR, Van Hoff B, Collen D, 1987. Activation with plasmin of two-chain urokinase-plasminogen activator derived from single-chain urokinase-type plasminogen activator by treatment with thrombin. Eur J Brochem 169:359-64.

Lijnen HR, Zamarron C, Blabler, Winkerler ME, Collen D, 1986. Activation of plasminogen by pro-urokinase; I. Mechanism. J Biol Chem 261:1253-58.

Liu Y-X, Cajander SB, Ny T, Kristensen P, Hsueh AJW, 1987. Gonadotropin regulation of tissue-type plasminogen activators in rat granulosa and theca-interstitial cells during periovulatory period. Mol Cell Endocr 54:221-29.

Liu Y-X, Hsueh AJW, 1987. Plasminogen activator activity in cumulus-oocyte complexes of gonadotropin-treated rats during the periovulatory period. Biol Reprod 36:1055-62.

Loscalzo J, Vaughan DE, 1987. Tissue plasminogen activator promotes platelet disaggregation in plasma. J Clin Invest 79:1749-55.

Machovich R, Owen WG, 1988. A factor from endothelium facilitates activation of plasminogen by tissue plasminogen activator. Enzyme 40:109-12.

Manejawala F, Kajie, Schultz RM, 1986. Development of activatable adenylate cyclase in preimplantation mouse embryos and a role of cyclic AMP in blastocoel formation. Cell 46:95-103.

Manejawala FM, Schultz RM, 1989. Blastocoel expansion in the preimplantation mouse embryo: stimulation of sodium uptake by cAMP and possible involvement of cAMP-dependent protein kinase. Devel Biol 136:560- 63.

Markus G, Takita H, Camiolo SM, Corasanti JG, Evers JL, Hobika GH, 1980. Content and characterization of plasminogen activators in human-lung tumors and normal-lung tissues. Cancer Res 40:841-48.

Marotti KR, Belin D, Strickland S, 1982. The production of distinct forms of plasminogen activator by mouse embryonic cells. Devel Biol 90:154-59.

Marzowski J, Sylvestern SR, Gilmont RR, Griswold MD, 1985. Isolation and characterization of Sertoli cell plasma membranes and associated plasminogen activator acti vity. Biol Reprod 32:1237-45.

Masson C, Angles-Cano E, 1988. Kinetic analysis of the interaction between plasminogen activator inhibitor-1 and tissue plasminogen activator. Biochem J 256:237- 44.

Mayer M, Lund LR, Riccio A, Jan Skouv LSN, Stacy SN, 1988. Plasminogen activator inhibitor type-1 protein, m-RNA and gene transcription are increased by phorbol esters in human Rhabdomyosarcoma cells. J Biol Chem 263:15688-93.

Menino AR, Dyk AR, Gardiner CS, Grobner MA, Kaaekuahiwi MA, Williams JS, 1989. The effects of plasminogen on in vitro ovine embryo development. Biol Reprod 41:899-905.

Menino AR, O'Claray JL, 1986. Enhancement of hatching and trophoblastic outgrowth by mouse embryos cultured in Whitten's medium containing plasmin and plasminogen. J Reprod Fertil 77:159-67.

Menino AR, Williams JS, 1987. Activation of plasminogen by the early bovine embryo. Biol Reprod 36:1289-95.

Mimuro J, Loskutoff DJ, 1989a. Purification of a protein from bovine plasma that binds to type 1 plasminogen activator inhibitor and prevents its interaction with extracellular matrix. J Biol Chem 264:936-39.

Mimuro J, Loskutoff DJ, 1989b. Binding of type 1 plasmi nogen activator inhibitor to the extracellular matrix of cultured bovine endothelial cells. J Biol Chem 264:5058-63.

Mimuro J, Schleef RR, Loskutoff DJ, 1987. Extracellular matrix of cultured bovine aortic endothelial cells contains functionally active type-1 plasminogen activator inhibitor. Blood 70:721-28.

Mira-y-lopez R, Joseph-Silverstein J, Rifkin DB, Ossowski L, 1986. Identification of a pituitary factor responsible for enhancement of plasminogen activator activity in breast tumor cells. Proc Natl Acad Sci USA 83:7780-84.

Miskin R, Easton TG, Reich E, 1978. Plasminogen activator in chick embryo muscle cells: induction of enzyme by RSV, PMA and retinoic acid. Cell 15:1301-12.

Mullins DE, Bazer FW, Roberts RM, 1980. Secretion of a progesterone-induced inhibitor of plasminogen activa tor by the porcine uterus. Cell 20:865-72.

Nagamine Y, Sudol M, Reich E, 1983. Hormonal regulation of plasminogen activator mRNA production in porcine kidney cells. Cell 32:1181-90.

Nelles L, Lijnen HR, Collen D, Holmes WE, 1987. Charac terization of a fusion protein consisting of amino acids 1 to 263 of tissue-type plasminogen activator and amino acids 144 to 411 urokinase-type plasminogen activator. J Biol Chem 262:10855-62.

Niemann H, Elsaesser F, 1986. Evidence for estrogen- dependent blastocyst formation in the pig. Biol Reprod. 35:10-16.

Niemann H, Elsaesser F, 1987. Accumulation of oestrone by pig blastocysts and its potential physiologic signi ficance for blastocyst development in vitro. J Reprod Fertil 80:221-27.

Nigg EA, Eppenberger HM, Jans DA, Hemmings BA, Hilz H, 1988. Evidence for transcriptional activation of the plasminogen activator gene by the catalytic subunit of cAMP-dependent protein kinase. In: Mechanism of control of gene expression. Alan R Liss Inc., pp. 169-77.

Ny T, Liu Y-X, Ohlsson M, Jones PBC, Hsueh AJW, 1987. Regulation of tissue-type plasminogen activator activity and messenger RNA levels by gonadotropin- releasing hormone in cultured rat granulosa cells and cumulus-oocyte complexes. J Biol Chem 262:11790-93.

Opdenakker G, Ashino-Fuse H, VanDamme J, Billiau A, DeSomer P, 1983. Effect of 12-O-tetradecanoylphorbol-13- acetate on the production of mRNAs for human tissue- type plasminogen activator. Eur J Biochem 131:481- 87.

Opdenakker G, Bosman F, Decock B, Gabeza-Arvelaiz Y, Damme JV, Billiau A, 1988. Heterogeneity of human tissue- type plasminogen activator. FEB 238:129-34.

Ossowski L, Quigley JP, Kellerman GM, Reich E, 1973. Fibrinolysis associated with oncogenic transforma tion. Requirement of plasminogen for correlated changes in cellular morphology, colony formation in agar, and cell migration. J Exp Med 138:1056-64.

Ossowski L, Quigley JP, Reich E, 1974. Fibrinolysis asso ciated with oncogenic transformation. J Biol Chem 249:4312-20.

Ossowski L, Quigley JP, Reich E, 1975. Plasminogen, a necessary factor for cell migration in vitro. In: Reich E, Rifkin DB, Shaw E (eds.), Proteases and Biological Control. New York: Cold Spring Harbor Laboratory, pp. 901-13.

Ossowski L, Unkeless JC, Tobia A, Quigley JP, Rifkin DB, Reich E, 1973. An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. II. Mammalian fibroblast cultures transformed by DNA and RNA tumor viruses. J Exp Med 137:112-126.

Paulo E, Tischner M, 1985. Activity of $\Delta^5$3β-hydroxy- steroid dehydrogenase and steroid hormones content in early preimplantation horse embryos. Folia Histochemica et Cytobiologica 23:81-84.

Perry JS, Heap RB, Amoroso EC, 1973. Steroid hormone production by pig blastocysts. Nature (Lond) 245:45-47.

Petersen LC, Lund LR, Nielsen LS, Dano K, Skriver L, 1988. One-chain urokinase-type plasminogen activator from human sacroma cells is a proenzyme with little or no intrinsic activity. J Biol Chem 263:11189-95.

Pittman RN, 1985. Release of plasminogen activator and a calcium-depedent metalloprotease from cultured sympathetic and sensory neurons. Devel Biol 110:91-101.

Pollanen J, Hedman K, Nielesen LS, Dano K, Vaheri A, 1988. Ultrastructural localization of plasma membrane-associated urokinase-type plasminogen activator to focal contacts. J cell Biol 106:87-95.

Polliack A, Levij IS, 1969. The effect of tropical vitamin A on papillomas and intraepithelial carcinomas induced in hamster cheek pouches with 9, 10-dimethyl-1,2-benzathracene. Cancer Res. 29:327-32.

Presta M, Maier JAM, Moscatelli D, Ragnotti G, 1988. Modulation of plasminogen activator activity in human endometrial adenocarcinoma cells by basic fibroblast growth factor and transforming growth factor β. Cancer Res 48:6384-89.

Queenan, Jr JT, Kao L-C, Arboleda CE, Ulloa-Aquirre A, Golos TG, Cines DB, Strauss III JF, 1987. Regulation of urokinase-type plasminogen activator production by cultured human cytotrophoblasts. J Bio Chem 262:10903-06.

Quigley JP, 1979. Phorobol ester-induced morphological changes in transformed chick fibroblasts: evidence for direct catalytic involvement of plasminogen activator. Cell 17:131-41.

Rajput B, Degen S, Reich E, Walker E, Alexrod J, Eddy R, Shows T, 1985. Chromosome locations of human tissue plasminogen activator and urokinase gene. Science 230:672-74.

Rajput B, Marshall A, Killary AM, Lalley PL, Naylor SL, Belin D, Rickles RJ, Strickland S, 1987. Chromosomal assignments of genes for tissue plasminogen activator and urokinase in mouse. Somatic Cell Mol Genetics 13:581-86.

Ranby M, Brandstrom A, 1988. Biological control of tissue plasminogen activator-mediated fibrinolysis. Enzyme 40:130-43.

Rehemtulla A, Gates J, Hart DA, 1987. Treatment of mouse L-cells with phorbol myristate acetate induces the secretion of a plasminogen activator inhibitor which binds to human and mouse urokinase and human tissue plasminogen activator. Comp Biochem Physiol 88B:227-83.

Rehemtulla A, Smith R, Hart DA, 1987. Regulation of plasminogen activator and plasminogen activator-inhibitor production by tissue culture cells: evidence for independent induction and regulation. Fibrinolysis 1:109-16.

Reich R, Misken R, Tsafriri A, 1985. Follicular plas minogen activator: involvement in ovulation. Endocrinology 116:516-21.

Reich R, Tsafriri A, Mechanic GL, 1985. The involvement of collagenolysis in ovulation in the rat. Endocrinology 116:522-27.

Rijken DC, Groeneveld E, Kluft C, Nieuwenhuis HK, 1988. a_2-Antiplasmin enschede is not an inhibitor, but a substrate of plasmin. Biochem J 255:609-15.

Roblero LS, Garavagno AC, 1979. Effect of oestradiol-17β and progesterone on oviductal transport and early development of mouse embryos. J Reprod Fertil 57:91-95.

Roblin R, Young PL, 1980. Dexamethasone regulation of plasminogen activator in embryonic and tumor-derived human cells. Cancer Res 40:2706-13.

Roesler WJ, Vandenbark GR, Hanson RW, 1988. Cyclic AMP and the induction of eukaryotic gene transcription. J Biol Chem 263:9063-66.

Rogister B, Leprince P, Pettmann B, Labouradette G, Sensebrenner M, Moonen G, 1988. Brain basic fibroblast growth factor stimulates the release of plasminogen activators by newborn rat cultured astroglial cells. Neurosci LETT 91:321-26.

Ryan TJ, 1987. Photoaffinity labeling of human plasmin and plasminogen. Pharmac. Ther. 34:335-48.

Saksela O, Moscatelli D, Rifkin DB, 1987. The opposing effect of basic fibroblast growth factor and transforming growth factor beta on the regulation of plasminogen activator activity in capillary endothelial cells. J Cell Biol 105:957-63.

Samuel CA, 1971. The development of the pig trophoblast in ectopic sites. J Reprod Fertil 27:494-95.

Santell L, Levin EG, 1988. Cyclic AMP potentiates phorbol ester stimulation of tissue plasminogen activator release and inhibits secretion of plasminogen activator inhibitor-1 from human endothelial cells. J Biol Chem 263:16802-08.

Sauer MJ, 1979. Hormonal involvement in the establishment of pregnancy. J Reprod Fertil 56:725-43.

Sawaya R, Highsmith R, 1988. Plasminogen activator and molecular weight patterns in human brain tumors. J Neurosurg 68:73-79.

Schafer AI, Adelman B, 1985. Plasmin inhibition of platelet function and arachidonic acid metabolism. J Clin Invest 75:456-61.

Schaller J, Moser PW, Dannegger-Muller GAK, Rosselet SJ, Kampfer U, 1985. Complete amino acid sequence of bovine plasminogen. Comparison with human plasminogen. Eur J Biochem 149:267-78.

Schaller J, Rickli EE, 1988. Structural aspects of plasminogen of various species. Enzyme 40:63-69.

Sengupta J, Roy SK, Manchanda, 1982. Effect of an oestrogen synthesis inhibitor, 1,4,6-androstratriene-3,17-dione on mouse embryo development in vitro. J Reprod Fertil 66:63-66.

Shemesh M, Milaguir F, Ayalon N, Hansel W, 1979. Steroidogenesis and prostaglandin synthesis by cultured bovine blastocysts. J Reprod Fertil 56:181-85.

Sherman, MI, 1980. Studies on the temporal correlation between secretion of plasminogen activator and stages of early mouse embryogenesis. Onco Biol Med 1:7-17.

Sherman, MI, Strickland S, Reich E, 1976. Differentiation of early mouse embryonic and teratocarcinoma cells in vitro: plasminogen activator production. Cancer Res 36:4208-16.

Sivak A, Mossman BT, Van Durren BL, 1972. Activation of cell membrane enzymes in the stimulation of cell division. Biochem Biophys Res Commun 46:606-09.

Sivak A, Van Duuren BL, 1970. RNA synthesis induction by a tumor promoter. Cancer Res 30:1203-05.

Smith, DM, 1968. The effect on implantation of treating cultured mouse blastocysts with oestrogen in vitro and the uptake of oestrogen by blastocysts. J Endocr 41:17-29.

Smith DM, Biggers JD, 1968. The oestrogen requirement for implantation and the effect of its dose on the implantation response in the mouse. J Endocr 41:1-9.

Smokovitis A, Kokolis N, Alexaki-Tzivandidou E, 1988. The plasminogen activator activity is markedly increased mainly at the area of the rupture of the follicular wall at the time of ovulation. Animal Reprod Sci. 16:285-94.

Steel RDG, Torrie JH, 1980. Principles and procedures of statistics. New York: McGraw Hill Book Company.

Strickland S, Beers WH, 1976. Studies on the role of plasminogen activator in ovulation. In vitro response of granulosa cells to gonadotropins, cyclic nucleotides, and prostaglandins. J Biol Chem 251:5694-702.

Strickland S, Mahdavi V, 1978. The induction of differen tiation in teratocarcinoma stem cells by retinoic acid. Cell 15:393-403.

Strickland S, Reich E, Sherman MI, 1976. Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm. Cell 9:231-40.

Stump DC, Thienpont M, Collen D, 1986. Purification and characterization of a novel inhibitor of urokinase from human urine. J Biol Chem 261:12759-66.

Summaria L, Arzadon L, Bernabe P, Robbins K, 1973. Iso lation, characterization, and comparison of the S-carboxy-methyl Heavy (A) and Light (B) chain derivatives of cat, dog, rabbit, and bovine plasmins. J Biol Chem 248:6522-27.

Thewes T, Ramesh V, Simplacenu FL, Llinas M, 1988. Analysis of the aromatic ^{1}H-NMR spectrum of the Kringle 5 domain from human plasminogen. Evidence for a conserved Kringle fold. Eur J Biochem 175:237-49.

Thomas DB, Medley G, Lingwood CA, 1973. Growth inhibition of murine tumor cells, in vitro, by puromycin, (^{6}N) O^{2} -dibutyrul 3′,5′-adenosine monophosphate or adenosine. J Cell Biol 57:397-405.

Tsafriri A, Bicsak TA, Cajander SB, Ny T, Hsueh AJW, 1989. Suppression of ovulation rate by antibodies to tissue-plasminogen activator and a_2-antiplasmin. Endocrinology 124:415-21.

Unkeless JFC, Gordon S, Reich E, 1974. Secretion of plas minogen activator by stimulated macrophages. J Exp Med 139:834-50.

Van Den Berg EA, Sprengers ED, Jaye M, Burgess W, Maciag T, Van Hinsbergh VWM, 1988. Regulation of plasmino gen activator inhibitor-1 mRNA in human endothelial cells. Thromb Haemost 60:63-67.

Van Hinsbergh VWM, Kooistra T, Van Den Berg E, Princen HMG, Fiers W, Emeis JJ, 1988. Tumor necrosis factor increases the production of plasminogen activator inhibitor in human endothelial cells in vitro and in rats in vivo. Blood 72:1467-73.

Van Zonneveld AJ, Veerman H, Pannkoek H, 1986. On the interaction of the finger and Kringle-2 domain of tissue-type plasminogen activator with fibrin. J Biol Chem 261:14214-18.

Varani J, McKeever PE, Fligiel SEG, Sitrin RG, 1987. Plasminogen activator production by human tumor cells: Effect on tumor cell-extracellular matrix interactions. Int J Cancer 40:772-77.

Vassalli J-D, Baccino D, Belin D, 1985. A cellular binding site for the M_r 55,000 form of the human plasminogen activator, urokinase. J Cell Biol. 100:86-92.

Vassalli J-D, Dayer J-M, Wohlwend A, Belin D, 1984. Concomitant secretion of prourokinase and of a plasminogen activator-specific inhibitor by cultured human monocytes. J Exp Med 159:1653-68.

Vassalli J-D, Hamilton J, Reich E, 1976. Macrophage plasminogen activator: modulation of enzyme production by antiinflammatory steroids, mitotic inhibitors, and cyclic nucleotides. Cell 8:271-81.

Vassalli J-D, Hamilton J, Reich E, 1977. Macrophage plasminogen activator: induction by concanavalin A and phorbol myristate acetate. Cell 11:695-705.

Verral S, Seeds NW, 1988. Tissue plasminogen activator binding to mouse cerebellar granule neurons. J Neuroscience Res 21:420-25.

Vihko KK, Kristensen P, Dano K, Parvinen M, 1988. Immu nohistochemical localization of urokinase-type plasminogen activator in Sertoli cells and tissue-type plasminogen activator in spermatogenic cells in the rat seminiferous epithelium. Devel Biol 126:150-55.

Wallen P, and Wiman B, 1970. Characterization of human plasminogen. I: On the relationship between dif ferent molecular forms of plasminogens demonstrated in plasma and found in purified preparations. Biochem Biophys Acta 221:20-30.

Wigler M, Defeo D, Weinstein, 1970. Induction of plas minogen activator in cultured cells by macrocyclic plant diterpene esters and other agents related to tumor promotion. Cancer Res 38:1434-37.

Wilmut I, Sales NI, Ashworth CJ, 1985. The influence of variation in embryo stage and maternal hormone profiles on embryo survival in farm animals. Theriogenology 23:107-19.

Wilson EL, Francis GE, 1987. Differentation-linked secretion of urokinase and tissue plasminogen activator by normal and hemopoietic cells. J Exp Med 165:1609-23.

Wilson EL, Reich E, 1978. Plasminogen activator in chick fibroblasts: induction of synthesis by retinoic acid; synergism with viral transformation and phorbol ester. Cell 15:385-92.

Wohlewend A, Belin D, Vassalli J-D, 1987. Plasminogen activator-specific inhibitors in mouse macrophages: in vivo and in vitro modulation of their synthesis and secretion. J Immunol 139:1278-84.

Wolbach SB, Howe PR, 1925. Tissue changes following depreivation of fat-soluble A vitamin. J Exp Med 42:753-77.

Wu J-T, 1987. Metabolism of progesterone by preimplantation mouse blastocysts in culture. Bio Reprod 36:54-56.