AN ABSTRACT OF THE THESIS OF

Susan E. Bartlett for the degree of Master of Science in Animal Sciences presented on June 11, 1992

Title: Characterization of the Plasminogen Activator Produced by Ovine Embryos and Evaluation of its Physiologic Role in Early Development.

Abstract Approved: _____
Alfred R. Menino, Jr.

Three experiments were conducted to determine: the type of plasminogen activator (PA) produced by cultured ovine embryos; differences in PA profiles of uterine flushings from pregnant and nonpregnant ewes; and the effects of extracellular matrices and the PA system on outgrowth of ovine inner cell masses (ICM). In Experiment 1, embryos were collected on days 7, 9 and 11 of pregnancy and cultured in alpha Miniumum Essential Medium + 0.15% bovine serum albumin (MEM + BSA) until equivalent gestational day (EGD) 15. Samples of conditioned medium were removed at 24-h intervals. Determination of PA activity, using a caseinolytic assay, revealed PA levels in the medium did not change up to EGD 12-13 but increased to peak levels by EGD 15. Incubation with anti-PA inhibitor-2 (PAI-2) increased PA activity ($P<0.05$), and incubation with amiloride (AMR), a specific

inhibitor of urokinase-type PA (uPA), reduced PA activity ($P<0.001$) in the medium in a dose-dependent fashion. Sodium dodecyl sulphate polyacrylamide gel electrophoresis with zymography revealed a plasminogen-dependent lytic zone (48-51 kD) within EGD 8-15 that was eliminated in the presence of AMR and precipitated by rabbit anti-bovine uPA. A second plasminogen-dependent lytic zone (79-83 kD) appeared after EGD 13. In Experiment 2, uterine flushings were obtained from pregnant and nonpregnant ewes on days 7, 9 and 11. Analysis by day revealed total protein concentration was not different between days 7, 9 and 11 in nonpregnant ewes, however, in pregnant ewes, total protein increased dramatically ($P<0.001$) on day 11. While plasmin inhibitor (PI) activity steadily decreased ($P<0.05$), PA and plasminogen activator inhibitor (PAI) activity remained constant in nonpregnant ewes. In uterine flushings from pregnant ewes, both PA and PAI activities decreased ($P<0.05$) on day 11, while PI activities remained unchanged. Polyacrylamide gel electrophoresis with zymography revealed the presence of 5 major plasminogen-dependent lytic zones. Day 11 pregnant samples exhibited a 53 kD protein and a 60 kD inibitor that were not present in any other samples. Presence of a 51 kD protein was consistently decreased in the presence of amiloride in all samples, suggesting it may be a uPA. In Experiment 3, ICM were isolated from day

7.5 embryos and cultured for 72 h on collagen, fibronectin or laminin in MEM + 1.5% BSA containing one of the following: 0 or 150 μg/ml human plasminogen; 0 or 100 IU/ml PA inhibitor-2 (PAI-2); or 10% normal rabbit serum or 10% rabbit anti-bovine uPA serum. Area of outgrowth was larger ($P<0.01$) in 0 μg/ml plasminogen than in 150 μg/ml plasminogen. Fibronectin supported the greatest outgrowth ($P<0.001$) and largest number of migrating cells ($P<0.001$). While addition of anti-uPA or PAI-2 to the culture medium had no effect on outgrowth area ($P>0.10$), fewer ($P<0.05$) cells migrated from ICM in 0 than 100 IU/ml PAI-2. Inner cell masses on fibronectin produced more ($P<0.01$) PA than on collagen or laminin, however, PA production was not correlated with areas of outgrowth ($r=0.06$; $P>0.77$) or cell numbers in the outgrowths ($r=0.02$; $P>0.90$). Results of these studies suggest 1) ovine embryos produce an urokinase-type PA, 2) total protein, PA, PAI and PI levels change with day and pregnancy status and 3) while fibronectin enhances cellular outgrowth from ovine ICM, however it is unlikely that PA plays a functional role in this cellular migration.

CHARACTERIZATION OF THE PLASMINOGEN ACTIVATOR PRODUCED BY OVINE EMBRYOS AND EVALUATION OF ITS PHYSIOLOGIC ROLE IN EARLY DEVELOPMENT

by

Susan E. Bartlett

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

Completed June 11, 1992
Commencement June 1993

APPROVED:

Redacted for Privacy

Professor of Animal Sciences in charge of major

Redacted for Privacy

Head of Department of Animal Sciences

Redacted for Privacy

Dean of Graduate School

Date thesis presented June 11, 1992

DEDICATION

This thesis is dedicated to my parents, Kathy and Ross Bartlett, whose constant love and support throughout my life made this thesis and other goals acheivable.

ACKNOWLEDGEMENTS

I would like to begin by thanking my committee members, Dr. Fred Stormshak, Dr. John Morris and Jim Moore, for their interest in my program and their careful analysis of my thesis. Sincere thanks go to my major professor and friend, Dr. Alfred Menino Jr., who encouraged me to challenge myself but also guided me through the past two years of my life. I am indebted to my lab colleagues Arwyn Coates and Nam Hyung Kim for the many hours they spent helping with injections, breedings and surgeries. Special thanks also go to Arywn for providing me an outlet and a sympathetic ear when the stress of graduate school seemed too much.

I would like to extend thanks to my roommate and friend, Elyshia Gardner for her help on graphs, and for putting up with me when my stress levels were high. Special thanks also go to my best friend, Jake Harwood for helping me realize there was life beyond the laboratory. Without his unending patience and support throughout my program, this thesis would not have been possible. And last but not least, my family. Thanks go to Kris, Gary, Bo, Gail, Greg, Mom, Dad and Shasta for providing constant encouragement, love and the strength I needed to complete this program.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHARACTERIZATION OF THE PLASMINOGEN ACTIVATOR PRODUCED BY OVINE EMBRYOS AND EVALUATION OF ITS PHYSIOLOGIC ROLE IN EARLY DEVELOPMENT

INTRODUCTION

Plasminogen activators (PA) are serine proteases that convert the serum zymogen plasminogen into plasmin, the enyzme responsible for the degradation of fibrin and turnover of the extracellular matrix (Christman et al., 1977). Two types of PA, each products of independent genes, have been identified based on molecular mass and functional differences: urokinase-type (uPA) and tissue-type (tPA) with molecular masses of 30-55 and 70 kD, respectively (Dano et al., 1985). Proteolytic actions of the PA/plasmin system are regulated by a family of serine protease inhibitors. These inhibitors are produced primarily by endothelial cells, placenta and platelets (Hart and Rehemtulla, 1987). Plasminogen activators are involved in a variety of reproductive processes including ovulation (Beers et al., 1975), spermatogenesis (Lacroix et al., 1977, 1981), trophoblastic invasiveness during implantation (Strickland et al., 1976; Sherman et al., 1976), parietal endoderm migration during embryogenEsis (Strickland et al., 1976) and facilitation of hatching (Menino and Williams, 1987).

Although PA is produced by a wide variety of embryos (rats, Leidholm and Astedt, 1975; mice, Strickland et al., 1976; swine, Mullins et al., 1980; cattle, Menino and Williams, 1987; and sheep, Menino et al., 1989), the

exact role it plays in embryonic development is unclear. Plasminogen activator has been implicated in cell migration and tissue differentiative events in mouse and swine embryos, however very little information regarding PA and its inhibitors in ovine embryonic development is available. Therefore, the objectives of these studies were to 1) characterize the type of PA produced by ovine embryos, 2) evaluate PA and PA-inhibitor profiles in uterine flushings from pregnant and nonpregnant ewes, and 3) to investigate the involvement of the PA system in the outgrowth of ovine inner cell masses on a variety of extracellular matrices.

REVIEW OF THE LITERATURE

CLASSIFICATION AND BIOCHEMISTRY OF PLASMINOGEN ACTIVATORS

Plasminogen activators (PA) are serine proteases that convert the proenzyme plasminogen into the active enzyme plasmin by hydrolysis of an arginine-valine peptide bond (Dano et al., 1985; Lijnen and Collen, 1988). Two types of PA have been characterized, urokinase-type PA (uPA) and tissue-type PA (tPA). These two enzymes differ in their molecular weight and functional and immunological properties. Based on their nucleotide sequences, it has been demonstrated that the two types of PA are independent gene products. Tissue-type PA has been purified from various sources including porcine heart, kidney and ovary, and human uterus and plasma (Dano et al., 1985). The protease is synthesized as a single-chain polypeptide proenzyme (sc-tPA) with a molecular mass of approximately 70 kD. The sc-tPA molecule is proteolytically modified to yield two polypeptide chains held together by disulfide bonds (Hart and Rehemtulla, 1988). The two chain form (tc-tPA) is the active enzyme and contains a small catalytic chain of 30 kD bound to a larger 40 kD chain. The 40 kD chain contains four structural domains: a "finger" domain which resembles a region in fibronectin; an epidermal growth factor (EGF) domain which shares homology with EGF and

two kringle domains (Hart and Rehemtulla, 1988). The only known protein substrate for tPA is plasminogen. Tissue-type PA is a major regulator of fibrinolysis and the finger domain and one of the kringle domains are responsible for the interaction of tPA with fibrin. Although tPA contains approximately 7% carbohydrate, removal of this moiety by glycosidases does not alter enzyme activity. However, in the presence of tunicamycin, tPA is not secreted from the cell suggesting glycosylation is required for transport of the protein out of the cell (Little et al., 1984).

Urokinase-type PA was first purified from human urine but has since been found in human plasma and in conditioned culture fluid of several types of cell lines (Dano et al., 1985). The enzyme is synthesized as a single chain molecule (sc-uPA) which must undergo proteolytic cleavage to yield an active disulfide linked two-chain molecule (tc-uPA) containing a catalytic and noncatalytic subunit. Unlike tPA, the noncatalytic region of uPA contains only the EGF domain and one kringle domain, however, the catalytic region of uPA is homologous with the 30 kD chain of tPA. While tPA functions in fibrinolysis, uPA appears to be involved in cellular migration and tissue remodeling (Vassalli et al., 1976). Sappino and co-workers (1989) found uPA mRNA in the invasive and migrating trophoblast cells of Day

5.5-8.5 mouse embryos. Like trophoblast, tumors are endowed with proliferative and migrating properties and have the ability to invade neighboring tissues. Transformed cell lines and tumors frequently produce uPA and experimental evidence suggests that the enzyme is essential to their invasive properties (Ossowski and Reich, 1983).

By the specific hydrolysis of an arginine-valine bond in its substrate plasminogen, uPA generates plasmin. Plasmin has broad trypsin-like specificity hydrolyzing lysine and arginine peptide bonds in several proteins, including casein (Remmert and Cohen, 1949), and immunoglobins (Christman et al., 1977). Due to its broad specificity, the generation of plasmin from plasminogen could cause unwanted proteolysis unless regulated. To control the location of PA-related events, certain cells express receptors specific for uPA that will bind either the single chain or two-chain form of the enzyme. The receptor-PA complex can confer PA activity to the cell surface and influence proteolysis in close proximity to the cell. The EGF domain on the noncatalytic chain of uPA is responsible for the enyzme-receptor interaction. Unlike uPA, no cell surface receptors for tPA have been reported. Because tPA is not associated with specific cellular functions, it has been hypothesized that the presence of receptors for tPA could interfere with the

primary function of the enzyme in fibrinolysis.

A low molecular weight form of uPA generated by extracellular cleavage exists at 30 kD. This molecule is comprised of the catalytic subunit of uPA and a small peptide of the non-catalytic subunit. Although enzymatically active, this low molecular weight species of uPA does not bind to cellular receptors, and a specific role in vivo is yet to be defined.

PLASMINOGEN ACTIVATOR INHIBITORS

The activities of PA in vascular and extracellular spaces may be regulated in a variety of ways: (a) by hormonal regulation of their biosynthesis; (b) by proteolytic conversion of single-chain PA into the two-chain active form of the enzyme; (c) by interaction with cell-surface receptors or with proteins that function as cofactors in the activation of plasminogen; and (d) by rapid clearance of PA via the liver. Also important in the regulation of PA activity are the serine protease inhibitors (SERPINS), a family of glycoproteins that covalently bind to and inhibit PA (Kruithof, 1988). Four inhibitors of tPA and/or uPA have been identified: PA inhibitor-1 (PAI-1); PA inhibitor-2 (PAI-2); PA inhibitor-3 (PAI-3); and protease nexin.

Plasminogen activator inhibitor-1 is the major PAI in plasma, and is derived from a variety of sources including endothelial cells, platelets, placenta and the

conditioned media of fibrosarcoma cells and hepatocytes. The inhibitor is a 52 kD glycoprotein which is capable of reacting with the single chain form of tPA. Plasminogen activator inhibitor 1 is not a stable compound and modification of its structure leads to spontaneous inactivation. Levels of PAI-1 are regulated by glucocorticoids, endotoxins, interleukin-1, transforming growth factor beta, tumor necrosis factor and thrombin.

Plasminogen activator inhibitor-2 is found in several tissues including the trophoblastic epithelium of the placenta, monocytes, leukocytes and fibrosarcoma cells. Unlike PAI-1, PAI-2 exists in two different forms. Intracellularly, PAI-2 exists as a nonglycosylated molecule with a molecular mass of 47 kD. The secreted form of PAI-2 is glycosylated and has a molecular mass of 60 kD. Although PAI-2 is capable of reacting with both types of PA, it has a higher affinity for uPA than tPA. Unlike PAI-1, PAI-2 is a stable inhibitor and it's biosynthesis and release are regulated by endotoxin and phorbol esters.

Plasminogen activator inhibitor-3 is present in human urine and plasma at a molecular mass of 51 kD. Although its physiological role is unkown, PAI-3 reacts with both tPA and the two-chain form of uPA.

Protease nexin is a glycoprotein of 47 kD which was first identified in the medium of fibroblasts. Unlike the

other PA inhibitors, protease nexin has a broad specificity and is capable of inhibiting thrombin, trypsin, plasmin and factor Xa.

EARLY EMBRYONIC DEVELOPMENT IN THE OVINE

Sheep are seasonally polyestrous and in the Northern hemisphere, exhibit 17 day estrous cycles during the months of September through January. Onset of estrus is defined as Day 0 and fertlization occurs on Day 1 in mated ewes. The fertilized ovum or zygote is surrounded by an acellular glycoprotein matrix called the zona pellucida which provides a protective covering during the first 8 days of development. Days 1-3 of development are characterized by a series of successive mitotic cleavages until Day 4 when the embryo reaches the morula stage and contains approximately 32-64 cells. At this stage, the embryo enters the uterus and begins to develop a fluid-filled cavity called the blastocoel. By Day 6, the blastocoel is enlarged, the embryo contains 300 cells and the embryo is termed a blastocyst. At the blastocyst stage, the first cellular differentiation occurs with the development of an outer trophectoderm surrounding a core of inner cell mass (Herbet and Graham, 1974; Denker, 1976). On Day 8, endodermal cells from the inner cell mass migrate along the blastocoelic side of the trophectoderm and form the trophoblast (Wintenberger-Torres and Flechon, 1974). Migration of endoderm occurs along an extracellular matrix containing among other

components, the glycoproteins type IV collagen, fibronectin and laminin. The inner cell mass will continue to differentiate into the embryo proper while the trophoblast will develop into the extraembryonic membranes of the placenta. Between Day 8 and 9, the embryo "hatches" and sheds the zona pellucida. The blastocyst continues to expand and by Day 10, mesures 0.4-0.9 mm in diameter and contains approximately 3000 cells (Wintenberger-Torres and Flechon, 1974). On day 11, the blastocyst begins elongating and by Day 12, reaches a length of 10-22 cm. By Day 15, the embryo is a blastodermic vesicle which begins to make contact with the uterine epithelium and on Day 16, contacts between blastocyst and uterus are numerous (Boshier, 1969).

PLASMINOGEN ACTIVATOR IN MAMMALIAN EMBRYONIC DEVELOPMENT

RODENT EMBRYOS

In 1975, Leidholm and Astedt first detected the presence of PA in the rat ovum. Their study showed that Day 1-4 ova exhibited fibrinolytic activity which was diminished in the presence of tranexamic acid, a competitive inhibitor of PA. By Day 6, when the ovum reached the uterus, the fibrinolytic activity had disappeared. Because proteolytic activity of cells in culture prevents their adhesion to surfaces and to each other, Leidholm and Astedt (1975) hypothesized that a decrease in fibrinolytic activity of the ovum may be a requirement for adhesion and implantation of the embryo.

Strickland et al. (1976) investigated PA production by mouse embryos. Plasminogen activator was produced in a biphasic manner and was first detectable on Day 6 of pregnancy. The first phase of PA production rose to maximum levels by Day 8 and then decreased. During the second phase, a larger amount of the enzyme accumulated and levels remained high until at least Day 15. By isolating the trophoblast and inner cell mass of the blastocyst, Strickland et al. (1976) were able to assay these tissues individually for PA production. Trophoblast was responsible for the first phase of enzyme synthesis, and the second phase of production was due to the inner cell mass.

Sherman et al. (1976) reported similar PA profiles when they isolated inner cell mass and trophoblast from Day 5 to 14 mouse embryos. Results of their study showed that PA production by the trophoblast appeared late on the fifth or early on the sixth day of gestation, peaked between Day 7-9 and declined to low levels thereafter. In contrast, parietal endoderm cells of the inner cell mass began producing PA on Day 7 and secreted increased amounts after Day 8.

The invasive period of mouse embryos is between Days 6 and 10 of embryonic development. This interval corresponds with the period of PA secretion from the trophoblast and suggests the enzyme may be involved in implantation. By Day 7, the parietal endoderm completely covers the inner surface

of the trophectoderm, and has begun secreting Reichert's membrane, the extracellular matrix between the endoderm and trophectoderm. In the studies by Sherman et al. (1976) and Strickland et al. (1976) levels of PA from isolated inner cell masses were detectable on Day 7, and synthesis of the enzyme continued for as long as the cells were viable. Thus, it appears inner cell mass may produce PA throughout its life span. Suggested roles for PA in the parietal endoderm include facilitating migration along the trophectoderm and involvement in the metabolism of Reichert's membrane.

Bode and Dziadek (1979) investigated PA production in both embryonic and extraembryonic mouse tissues. Plasminogen activator secretion was initiated progressively in different mouse embryo tissues during development from the 7th to 10th day of gestation. In Day 7 embryos, the only tissues that produced PA were parietal endoderm, extraembryonic ectoderm and ectoplacental cone. On Day 8, proliferation and migration of mesodermal cells occurs. Mesodermal PA production was high during this period, providing further evidence that this enzyme is associated with cell migration. Other tissues that begin secreting PA on Day 8 include embryonic ectoderm and visceral endoderm. By the 10th day of gestation, all tissues which were tested secreted PA: parietal endoderm, amnion, visceral yolk sac

endoderm and mesoderm, and also tissue from the embryo proper. Bode and Dziadek (1979) concluded that although it appears that PA may have different roles in different tissues at different stages of development, the patterns of its distribution are consistent with a role for this enzyme in morphogenic movement.

Marotti et al. (1982) examined cultured parietal endoderm, visceral endoderm and extraembryonic mesoderm cells from Day 12 mouse embryos for the presence of PA. Results indicated the presence of two types of PA at this stage of embryonic development; parietal endoderm produced tPA, while visceral and extraembryonic mesoderm produced uPA.

Sappino et al. (1989) investigated the cellular distribution of uPA and tPA mRNA during mouse oogenesis and embryo implantation. Over the years, evidence has accumulated suggesting a role for proteases in the ovulatory process (Espey, 1974; Beers et al., 1975). Sappino's study isolated tPA mRNA and uPA mRNA in cells from pre-ovulatory follicles. These findings are in agreement with a proteolytic role for PA in oogenesis and ovulation. Sappino et al. (1989) also located uPA mRNA in the invasive trophoblast cells of Day 5.5-7.5 embryos, providing further evidence for the involvement of PA in implantation.

PORCINE EMBRYOS

Unlike mice and rats, porcine embryos do not have an invasive form of implantation. Instead, porcine embryos exhibit placentation that involves interdigitation of microvilli between the epithelial surfaces of the trophoblast and endometrium. Mullins et al. (1980) undertook a study to investigate whether the absence of invasive implantation within the porcine uterus is possibly due to a failure of the conceptus to produce PA or the presence of an inhibitor. By isolating and culturing porcine embryos in the early stages of placentation (Day 12), Mullins et al. (1980) observed a time-dependent increase in PA activity in the conditioned medium. After examination of uterine flushings collected from Days 8-18 of pregnancy, Mullins et al. (1980) found low PA activity, particularly after Day 12. Because embryos isolated at this time and subsequently cultured in vitro secreted PA, Mullins et al. (1980) hypothesized that embryonic PA activity was suppressed in utero. Plasminogen activator levels in uterine flushings of gilts was also examined. Mullins et al. (1980) found high PA activity on Days 3 and 18 of the cycle, but much lower levels at Days 12 and 15. To determine whether there is an inhibitor of PA during mid-cycle, samples of Day 15 uterine flushings with negligible PA activity were mixed with samples of Day 3 and Day 18 flushings. The

results showed a significant decrease in PA activity of Day 3 and 18 uterine flushings in the presence of Day 15 flushings, suggesting the presence of a mid-cycle inhibitor. To determine whether the inhibitor was hormone induced, uterine flushings were obtained from ovariectomized gilts maintained on either progesterone or estrogen. An assay for PA activity revealed low levels of PA in progesterone-treated animals. These results indicated that the inhibitor was progesterone induced. Further assays revealed that this progesterone-induced inhibitor also reduced the release of PA from cultured blastocysts in a concentration-dependent manner. Because porcine embryos are not invasive in uterine tissues, but are invasive if transplanted to an ectopic site (Samuel, 1971), Mullins et al. (1980) concluded that the progesterone-induced inhibitor may possibly play a role in preventing invasive implantation.

Between Days 10-16, the porcine blastocyst elongates from a small sphere to a long, thread-like form. This process occurs by means of cellular reorganization and Fazleabas et al. (1983) observed PA release during this period. Similar to the findings of Strickland et al. (1976) in mouse embryos, Fazleabas et al. (1983) also observed that PA production by porcine embryos was biphasic. The initial phase occured between Days 10-12 and paralleled the early elongation phase, while the

secondary phase occurred on Days 14-16 and coincided with the time when increased DNA synthesis was occurring. Fazleabas et al. (1983) found that between the two phases of PA production (Days 12-13), the endometrium secreted high levels of an inhibitor of plasmin into the uterine lumen. This inhibitor may serve to protect the uterus against proteolysis by PA produced by the porcine blastocyst. Plasminogen activator produced by the porcine blastocyst is apparently not associated with implantation as is suggested for the mouse. Instead, it is associated with the period of intense tissue remodeling and proliferation occurring during the elongation phase of development.

BOVINE EMBRYOS

Menino and Williams (1987) were the first to investigate PA production and its role in bovine embryonic development. Sixteen-cell to early morula stage embryos were cultured in five concentrations of plasminogen ranging from 0-120 μg/ml. Plasminogen activator production was low for the first 48 h of culture, increased between 48-120 h, and plateaued thereafter. Percentages of embryos developing to the intiating hatching blastocyst, hatched blastocyst, attached blastocyst, and attached blastocyst with trophoblastic outgrowth stages did not differ among the five levels of plasminogen. However, as plasminogen

concentration increased in the culture medium, initiation and completion of hatching accelerated. Initiation of hatching ranged from 108.7-168.0 h and occurred during the time when the blastocyst was rapidly increasing its rate of plasminogen activation or the rate of activation had just plateaued. These results suggest that PA may facilitate hatching, perhaps by weakening the zona pellucida proteolytically so as to allow escape by the expanding blastocyst.

Kaaekuahiwi and Menino (1990) evaluated the relationship of PA production to cell stage, cell number and changes in overall diameter and zona pellucida thickness. Total PA production was correlated positively to embryonic size, developmental stage and cell number, and negatively, but weakly, correlated to zona pellucida thickness. Hatched embryos produced more total PA than embryos that did not hatch, supporting the hypothesis of Menino and Williams (1987) that PA is involved in the hatching process.

Dyk and Menino (1991) partially characterized the tissue source and type of PA produced by Day 12-14 bovine embryos. Using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (PAGE) and zymography, light (41.5-47.0 kD) and heavy (86.1-92.2kD) forms of PA were detected. Based on molecular mass classification, the light form was classified as a uPA while the heavy form,

being too heavy for tPA, was suggested to be a complex between uPA and a PAI. When blastocyst tissues were microdissected, Dyk and Menino (1991) found that the principal source of PA production was the trophoblast and not the embryonic disc.

Berg and Menino (1992) used antibodies against uPA and tPA and amiloride, a competitive inhibitor of uPA, to demonstrate that the light form of PA reported by Dyk and Menino (1991) was indeed uPA and not tPA. Treatment with anti-uPA abolished PA activity whereas anti-tPA had no effect on PA activity in embryonic samples suggesting that Day 12-14 bovine embryos produce only uPA. Addition of amiloride to embryonic samples completely eliminated PA activity, further confirming that Day 12-14 bovine embryos produce only uPA.

OVINE EMBRYOS

The only report to date of PA production by ovine embryos is from Menino et al. (1989). In this study, plasminogen activator production by ovine embryos and the effects of plasminogen on ovine embryo development and zona pellucida integrity were evaluated. When compared with embryos cultured in 0 μg/ml plasminogen, more blastocysts hatched in medium containing 60 and 120 μg/ml plasminogen. Zona pellucida solubility increased as plasminogen concentration in the medium increased, suggesting plasmin can alter zona pellucida integrity.

Plasminogen activator production was low until the morula stage, increased during the morula-blastocyst transition and remained elevated throughout blastocoelic expansion and hatching. This pattern of PA production may reflect transcriptional and translational events that are occurring during activation of the embryonic genome in sheep. Crosby et al. (1988) documented that the wave of transcriptional activity associated with activation of the ovine embryonic genome occurs during the fourth cell cycle or the period preceding the 16-cell stage. Plasminogen activator therefore may be one of the many gene products induced during the transition from maternal to embryonic control of early development. Menino et al. (1989) also reported that PA production and plasminogen conversion to plasmin were poorly correlated with zona pellucida solubility. The results of this study therefore indicated that although ovine embryos produce plasminogen activator and plasmin can increase zona pellucida solubility, other factors may also be involved in altering zona pellucida integrity prior to hatching.

OVINE EMBRYOS PRODUCE AN UROKINASE-TYPE PLASMINOGEN ACTIVATOR IN VITRO

ABSTRACT

The type of plasminogen activator (PA) produced by cultured ovine embryos was partially characterized. Day 7, 9 and 11 embryos were surgically collected from estrous-synchronized, superovulated and handmated crossbred ewes and cultured in alpha Minimum Essential Medium containing 0.15% BSA for 8, 6 and 4 days, respectively. At 24-h intervals, medium was recovered and frozen at -20°C and embryos were observed for developmental stage. Plasminogen activator concentrations in the medium were determined by a caseinolytic assay. Characterization of the type of PA produced by ovine embryos was conducted using SDS-PAGE and zymography, amiloride, a competitive inhibitor of urokinase-type PA (uPA), and antisera to human uPA (anti-uPA), tissue-type PA (anti-tPA) and PA inhibitors 1 (anti-PAI-1) and 2 (anti-PAI-2). Plasminogen activator levels in the medium did not change up to equivalent gestational days (EGD) 12-13 but increased to peak levels by EGD 15. Zymographic analysis revealed only one plasminogen-dependent lytic zone (48-51 kD) within EGD 8-13; however, a second plasminogen-dependent lytic zone appeared after EGD 13 (79-83 kD). When medium was incubated with anti-uPA, anti-tPA or anti-PAI-1, no differences ($P>0.10$) in PA activity were detected

compared to medium incubated with non-specific immunoglobulins. Plasminogen activator activity in the medium increased as anti-PAI-2 increased ($P<0.05$) and amiloride reduced PA activity ($P<0.001$) in a dose-dependent fashion. Ovine embryonic PA was succesfully immunoprecipitated with anti-rabbit bovine uPA serum. These data suggest that Day 7, 9 and 11 ovine embryos produce a uPA (48-51 kD) and possibly a PAI-2-like protein that complexes with the embryonic uPA and forms a high molecular mass PA complex (79-83 kD).

INTRODUCTION

Plasminogen activators (PA) are serine proteases responsible for converting the extracellular proenzyme plasminogen into the active serine enzyme plasmin (Christman et. al., 1977). Two major forms of PA exist that are characterized by molecular mass: urokinase-type PA (uPA; 31-55 kD) and tissue-type PA (tPA; 70 kD) (Dano et al., 1985). Plasminogen activators are associated with a variety of physiological processes including fibrinolysis, ovulation, spermatogenesis, and implantation (Strickland, 1980; Dano et al., 1985).

Tissue-type PA is the principal PA involved in clot lysis whereas uPA is involved in cell migration (Dano et al., 1985). Embryonic production of PA has been identified in a variety of species, including rats (Leidholm and Astedt, 1975), mice (Sherman et al., 1976;

Strickland et al., 1976), swine (Mullins et al., 1980; Fazleabas et al., 1983), cattle (Menino and Williams, 1987) and sheep (Menino et al., 1989), however, the physiological role of PA in early development is not well-defined. In cattle embryos, PA production is positively correlated with embryonic size, developmental stage and cell number (Kaaekuahiwi and Menino, 1990). In sheep embryos, PA production increases during the morula to blastocyst transition, and remains elevated through blastocoelic expansion and hatching (Menino et al., 1989). Because tPA and uPA participate in different physiologic processes, identifying the type of PA produced may afford a better understanding of the function of this enzyme in early embryogenesis. Also, because onset and maintenance of PA production appears to be related to embryonic cell stage, it could be a useful biochemical marker for investigating gene expression in early embryos. Therefore, to begin elucidating the role of PA in development, the objective of this study was to identify the type of PA produced by pre-attachment sheep blastocysts.

MATERIALS AND METHODS

EMBRYO COLLECTION AND CULTURE

Twenty-five multiparous, Polypay ewes were estrous synchronized with cloprostenol sodium (Estrumate; Haver; Shawnee, KS) and superovulated with porcine follicle

stimulating hormone (pFSH; Schering-Plough Animal Health Corp., Kenilworth, NJ). Ewes received two 100 μg i.m. injections of cloprostenol sodium 9 days apart (Day 0 = first cloprostenol sodium injection) and twice daily injections of pFSH i.m. at dosages of 4, 3 and 2 mg on days 8, 9 and 10, respectively, for a total of 18 mg per ewe. Estrus detection was initiated 24 h after the second injection of cloprostenol sodium and ewes were handmated by one of three rams starting at the onset of estrus and thereafter at 12-h intervals for as long as the ewe would accept the ram or for a total of at least 3 matings. Embryos were surgically collected from mated ewes 7, 9 or 11 days after onset of estrus. Anesthesia was induced in ewes by injection of 10 ml of 2.5% thiamylal sodium (Biotal; Boehringer Ingelheim Animal Health Inc.; St. Joseph, MO) into the jugular vein and was maintained during surgery via inhalation of halothane (Fluothane; Fort Dodge Laboratories Inc.; Fort Dodge, IA) and oxygen. The reproductive tract was exteriorized via ventral midline laparotomy and uteri were flushed in retrograde fashion with alpha Minimum Essential Medium (MEM; Sigma Chemical Co., St. Louis, MO), buffered with N′-2-hydroxyethyl piperazine-N′-ethanesulfonic acid (HEPES; MEM+H; Sigma). Flushings were examined with a dissecting microscope and embryos were recovered by aspiration. Embryos were washed in 5-10 ml of MEM+H with

15 mg/ml bovine serum albumin (MEM+H+BSA; Sigma) and transferred to screw-cap tissue culture tubes containing 10 ml of MEM+H+BSA maintained at 39°C. Culture tubes were transported to the laboratory and embryos were recovered from the tubes and washed three times in 50-μl microdrops of MEM+BSA. Embryos were evaluated for cell stage and overall morphology with an inverted-stage phase-contrast microscope at 100-200x magnification. Day 7, 9 and 11 embryos were cultured in 50, 75 or 125-μl microdrops, respectively, of MEM+BSA under paraffin oil (Fisher Scientific Co; Tustin, CA) in a humidified atmosphere of 5% CO_2 in air at 39°C, and were observed daily for stage of development. At 24-h intervals, starting at 24-h of culture and continuing through to an equivalent gestational age of 15 days, embryos were either transferred to fresh microdrops and the medium recovered (Day 7 embryos) or 50 and 75-μl aliquots of medium were withdrawn and replaced with fresh medium (Day 9 and 11 embryos, respectively).

At the end of culture, embryos and conditioned medium were recovered separately, placed in 1-ml snap cap tubes and stored at -20°C until evaluated for PA activity. To correct for any medium constituents that may contribute to the spontaneous activation of plasminogen, medium without embryos incubated under identical conditions was also recovered and frozen.

ELECTROPHORESIS AND ZYMOGRAPHY

To identify the type of PA produced by early sheep embryos, one dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and casein/plasminogen zymography (Granelli-Piperno and Reich, 1978; Vassalli et al., 1984) were conducted. Conditioned medium was combined with an equal volume of 2x SDS-PAGE sample buffer (5.0%; 20% glycerol; 0.0025% bromophenol blue in 0.125M Tris HCl buffer) and each polyacrylamide gel included lanes containing a urokinase standard (American Diagnostica Inc.; Greenwich, CT), and molecular mass markers ranging from 14.4 to 97.4 kD (Bio-Rad Laboratories; Richmond, CA). Conditioned medium, urokinase standards and molecular mass markers were aliquoted in 100 μl volumes into castellated wells in a 4% acrylamide stacking gel with 12% separating gel. Each sample of conditioned medium was analyzed in triplicate. Following electrophoresis, the polyacrylamide gels were gently shaken in 2.5% Triton X-100 (Sigma) for 45 min., rinsed with distilled water five times and shaken in phosphate buffered saline (PBS) for 45 min. The gel was removed from the PBS, placed on plastic wrap, and excess PBS removed by blotting gently with tissue paper. A casein-agar gel supported on a glass plate (zymograph) and containing purified human plasminogen (Sigma) at a final

concentration of 20 μg/ml was placed on the polyacrylamide gel.

Zymographs were prepared by combining equal volumes of 2% nonfat dry milk (Carnation Co., Los Angeles, CA) dissolved in buffer containing 0.0013M CaCl X $2H_2O$, 0.10M glycine, 0.038M Tris and 0.005M Na azide with a 2% solution of melted agarose (Sigma) at 65°C. Once the solution cooled to 45°C, human plasminogen was added and the mixture was cast onto a warmed glass plate and allowed to cool. Zymographs containing 0 μg/ml human plasminogen were used for detection of any nonspecific proteolytic activity. Polyacrylamide gels with zymographs were incubated at 39°C for 24-48 h and observed approximately every 5 h for lysis.

Protease migration was determined during incubation by measuring, to the nearest mm, the distance from the edge of the separating gel to the center of the clear caseinolytic bands in each lane. To terminate the incubation, zymographs were removed from the polyacrylamide gels and fixed with 3.0% acetic acid for 15 min. Zymographs were dried and stained for permanent storage (Lowenstein and Ingild, 1976). Polyacrylamide gels were stained with 0.05% Coomassie Brilliant Blue (Bio-Rad) in 65/25/10 water/isopropanol/acetic acid. For the amiloride experiments zymographs were prepared with the following modifications: equal volumes of 2.2%

agarose and 2.2% nonfat dry milk were combined and 1 ml containing either PBS or 100 mM amiloride (Sigma) and 200 μg human plasminogen was added to 9 ml of the melt. For all zymographs, each caseinolytic zone in lanes containing PA from conditioned medium, embryo tissues or urokinase and tPA standards served as an observation. Relative mobilities (Rm) of urokinase and tPA standards and embryonic PA were calculated by dividing the distance of migration from the point of origin on the separating gel to the center of the caseinolytic zone on the zymograph by the total length of the slab gel. Molecular masses of PA were determined by log molecular mass-Rm plots derived from the standards. Correlation-regression calculations provided the equation of the line and the correlation coefficients for the log molecular mass-Rm plots (Steel and Torrie, 1980). Unkown molecular masses were calculated from the equation of the line.

IMMUNOPRECIPITATION

For immunoprecipitation, embryo tissues in SDS solubilization buffer (Huarte et al., 1985) were used at a final volume of 10 μl. Embryo tissues were incubated overnight at 4°C with 10 μl of either rabbit anti-bovine uPA serum (American Diagnostica), or normal rabbit serum (NRS) diluted 1:2 with PBS. Thirty microliters of Protein-G bearing Streptococcus cells (OMNISORB, Cal-Biochem; La Jolla, CA) were added and the samples were

incubated for 2.5 h at room temperature. Samples were centrifuged using a microfuge (Beckman) for 5 min and the supernatant was removed and combined 1:2 with 1X SDS-PAGE sample buffer. The pellet was rinsed three times with 100 µl of PBS and combined 1:1 with 2X SDS-PAGE sample buffer. Samples were electrophoresed and polyacrylamide gels were overlaid with zymographs as previously described.

PLASMINOGEN ACTIVATOR ASSAYS

Caseinolytic-agar gel assays as described by Kaaekuahiwi and Menino (1990) were used to quantify PA activity in the medium. A modified caseinolytic assay was also performed using amiloride and a panel of antisera. In the modified assay, 30 µl of pooled medium from Day 7, 9 and 11 embryos were incubated with 15 µl of goat immunoglobulins (IgG) raised against human PA inhibitor-1 (anti-PAI-1), PAI-2 (anti-PAI-2), tPA (anti-htPA) or uPA (anti-huPA) (American Diagnostica), non-specific goat IgG (NSIgG; Sigma) or amiloride for 90 min at 39°C. Fifteen microliters of 240 µg/ml human plasminogen were added to each sample and incubated at 39°C for 15 min. Two, 25 µl aliquots of each reaction mixture were pipetted in 4 mm diameter wells in the casein agar plate and allowed to develop at room temperature for 24 h.

STATISTICAL ANALYSIS

Differences in PA activities in the culture medium were determined by analysis of variance and Duncan's Multiple Range Test. All analyses were conducted by using the NCSS statistical software program (Number Cruncher Statistical System, version 4.1, 1984, Hintze, J.L., Kaysville, UT, USA).

RESULTS

One hundred thirty-three ova were recovered from 24 ewes with a mean ovulation rate of 11.8 ± 1.7 corpora lutea. Cell stages of embryos recovered on each of the collection days are reported in table I-1. Thirty-eight, 21 and 21 morphologically normal embryos were recovered from ewes on days 7, 9 and 11, respectively. Typically, morulae to blastocysts were collected on Day 7, blastocysts to hatched blastocysts on Day 9 and hatched blastocysts on Day 11. As shown in figure I-1, PA activity in conditioned medium was unchanged until equivalent gestational day (EGD) 12-13 at which time activity increased ($P<0.05$) reaching a peak at EGD 15.

Zymographic analysis of conditioned medium revealed the presence of two plasminogen-dependent lytic zones (figure I-2). The lighter molecular mass form (49.9 ± 0.5 kD) was present from EGD 7-15, however, the heavier form (81.4 ± 1.2 kD) appeared from EGD 13-15 (table I-2).

No suppression or enhancement of PA activity

($P>0.05$) was observed after incubation of conditioned medium with antisera for human uPA and tPA or PAI-1, respectively (figure I-3). Increasing concentrations of anti-PAI-2 in the reaction mixture increased ($P<0.05$) PA activity (figure I-3). Incubation with amiloride reduced ($P<0.001$) PA activity in the conditioned medium in a dose-dependent fashion (figure I-3).

Addition of amiloride to the zymographs completely inhibited plasminogen-dependent caseinolysis by conditioned medium at 49.9 kD and urokinase standards, whereas caseinolysis by the tPA standards persisted (figure I-4). As shown in figure I-5, rabbit antiserum for bovine uPA precipitated both the heavy and low molecular mass forms of PA present in the ovine embryonic samples whereas no caseinolysis was observed in samples treated with NRS.

DISCUSSION

Results from the present study suggest that the PA activity observed in early ovine embryos in a previous report (Menino et al., 1989) is due to a uPA. Zymographic analysis revealed that ovine embryos produced a plasminogen-dependent protease (49.9 kD) during EGD 8-15 and a second plasminogen-dependent band (81.4 kD) during EGD 13-15. The 49.9 kD species has many of the characteristics of uPA and it is likely that the 81.4 kD form is a uPA-PAI complex. Several results support these

conclusions. First, the molecular mass of the 49.9 kD species corresponds to the values reported by Dano et al. (1985) for uPA of 31-55 kD. Second, PA activity significantly decreased in dose-dependent fashion when amiloride, a specific, competitive inhibitor of uPA (Vassali and Belin, 1987), was added to the conditioned medium. Third, plasminogen-dependent caseinolysis by ovine embryonic samples, but not tPA standards, was reduced with the addition of this inhibitor to the zymograph. Fourth, antiserum to bovine uPA successfully precipitated both forms of PA in the conditioned medium and embryonic tissues. Lastly, when antisera to PAI-2 was added to conditioned medium, PA activity increased. Plasminogen activator inhibitor-2 is a more selective inhibitor of uPA whereas PAI-1 has a higher binding affinity for tPA (Hart and Rehemtulla, 1988). An increase in PA activity was not observed when anti-PAI-1 was added to embryonic samples.

Addition of either anti-htPA or anti-huPA had no effect on sheep embryo PA activity. Considering the effect of amiloride, the observation that PA activity did not change after anti-huPA was added to the embryonic samples was unexpected. Berg and Menino (1992) observed complete suppression of PA activity in Day 12-14 cattle embryonic extracts and conditioned medium with anti-huPA. Sufficient antigenic differences must exist between the

cattle and sheep embryonic PA that prevent cross-reactivity with anti-huPA. However, there must also be sufficient homology to allow for immunoprecipitation of sheep embryonic PA with anti-bovine uPA.

Dano et. al. (1985) reported values for the molecular mass of tPA of approximately 70 kD. This value is too low for the higher molecular mass band observed in this study, and from the experimental evidence presented, we propose that it may instead be a uPA complexed with a PAI. Three PAI have been purified, PAI-1, PAI-2, and PAI-3. All have the ability to form SDS-stable complexes with PA that can be recognized zymographically (Kruithof, 1988). The molecular masses of PAI range between 47-60 kD and when added to the molecular mass of the uPA band results in a total mass similar, but somewhat higher than that observed for the high molecular mass form of PA. Typically, complexes between serine proteases and serine protease inhibitors (serpins) are of lower molecular mass than would be mathematically expected, due to release of a cleavage fragment upon association. Wiman and Collen (1979) reported that dissociation of plasmin-α2-antiplasmin complexes with NH_4OH resulted in regeneration of plasmin activity and an inhibitor with a lower molecular mass (60 kD) than the uncomplexed inhibitor (70 kD). The 81.4 kD band observed in our study is somewhat consistent with the findings of Rehemtulla et al. (1987)

who observed an 85 kD complex between high molecular mass uPA (52 kD) and a PA binding protein (33 kD) in human bladder carcinoma cells. Rehemtulla et al. (1990) also observed a 72 kD PA in human K562 cells. When Rehemtulla et al. (1990) added excess of urokinase (52 kD) to the culture medium, the 72 kD band disappeared and a 92 kD band appeared suggesting that the molecular mass of the binding protein was 40 kD. However, reverse zymography revealed a PAI with a molecular mass of 45 kD. Collectively, these findings suggest that upon interacting with the PA, some portion of the PAI may be cleaved, thus leaving a complex of lower molecular mass than would be expected.

Similar to the results of this study, Dyk and Menino (1991) reported that Day 12-14 cattle embryos also produce two plasminogen-dependent lytic zones, a light form of 41.5-47 kD and a heavy form of 86.1-92.2 kD. Berg and Menino (1992) demonstrated that PA activity in Day 12-14 cattle embryos could be completely suppressed by amiloride and anti-huPA whereas PA activity was unchanged with anti-htPA, suggesting the heavy form observed by Dyk and Menino (1991) was not a tPA. Coates and Menino (1991) demonstrated complete elimination of both the heavy and light forms of PA in cattle embryos in zymographs containing amiloride, further supporting the concept of an uPA-PAI complex comprising the heavy form.

The role PA plays in embryonic development is still unclear. However, Menino et al. (1989) demonstrated that PA produced by ovine embryos increased zona pellucida solubility in the presence of plasminogen thus effecting a sublysis of the zona pellucida and facilitating hatching. Plasminogen activator has also been suggested as having a role in cellular migration. Studies by Strickland et al. (1976) demonstrated that blastocysts secrete PA coincident with the time when trophoblast cells become invasive. Sappino et al. (1989) found uPA mRNA in invasive and migrating trophoblast cells of Day 5.5 and 6.5 mouse embryos. At Day 7.5, uPA mRNA was localized in those trophoblast cells implanted deep in the endometrium. Another migratory event occurs when parietal endoderm from the inner cell mass migrates along the trophectoderm to line the blastocoel (Sherman et al., 1976; Strickland et al., 1976). The fact that parietal endoderm begins secreting PA during this period suggests this protease may play a role in cellular migration.

In summary, results of the present study have demonstrated that ovine embryos produce a uPA and possibly a uPA-PAI complex. Further work is needed to resolve the role of PA in early embryonic development and the identity of the PAI-like molecule.

Figure I-1. Plasminogen activator (PA) production by Day 7, 9 and 11 ovine embryos. Day 7 embryos = ●—●; Day 9 embryos = o---o; and Day 11 embryos = o-·-o.

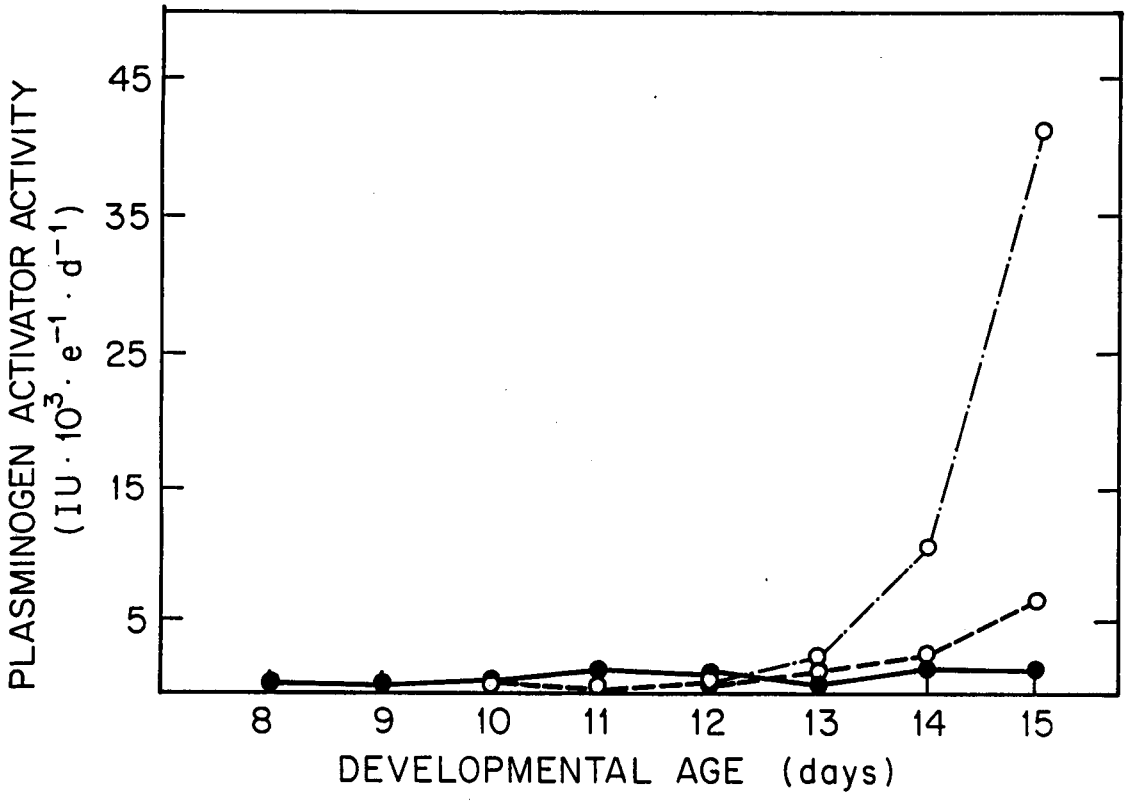

Figure I-2. Zymographic analysis of conditioned medium from ovine embryos collected on Day 7 of pregnancy, and cultured for 192 h. Numbers at top of figure indicate hours of culture.

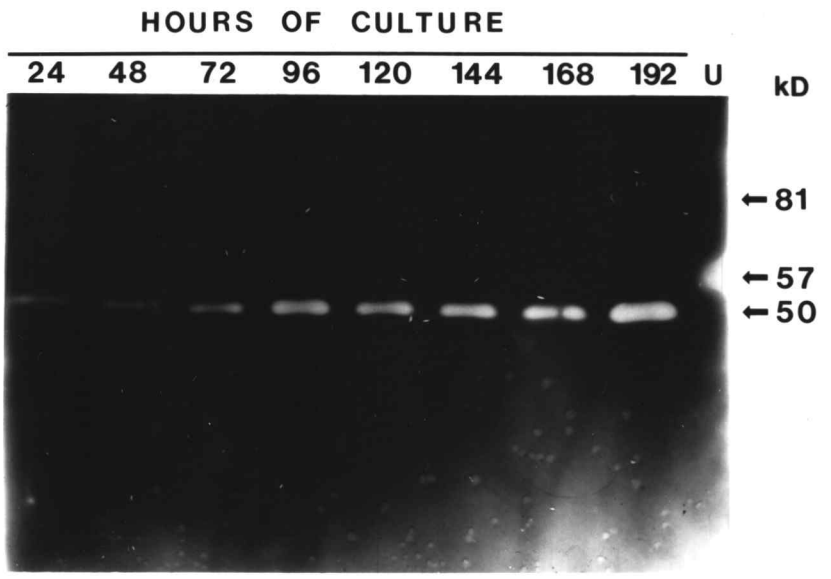

Figure I-3. Plasminogen activator (PA) activity in ovine embryonic samples following incubation with anti-tPA, anti-uPA, amiloride, anti-PAI-1 or anti-PAI-2.

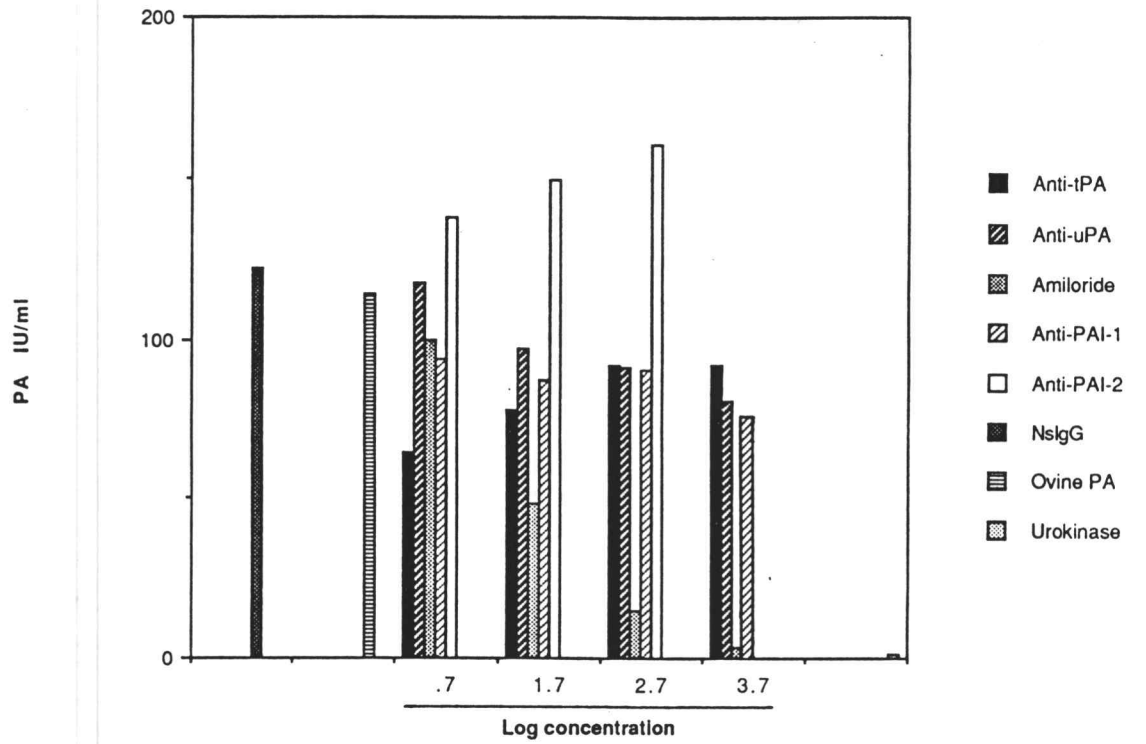

Figure I-4. Zymographic analysis of ovine embryonic samples in the presence or absence of amiloride, a competitive inhibitor of urokinase-type plasminogen activator. Ovine embryonic samples, lanes 1 and 8; control medium samples, lanes 2 and 7; tissue-type plasminogen activator standards, lanes 3 and 6; and urokinase standard, lanes 4 and 5.

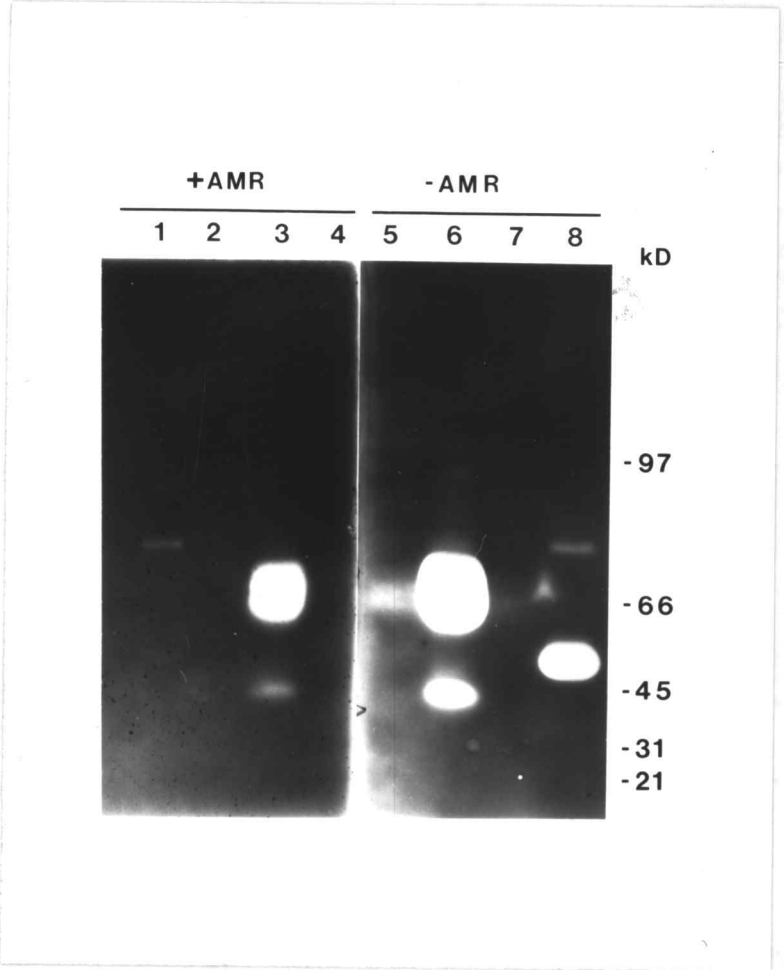

Figure I-5. Immunoprecipitation and zymographic analysis of ovine embryonic samples. Anti-bovine uPA + PBS, lane 1 and 6; NRS + PBS, lane 2 and 7; ovine embryonic sample + NRS, lane 3 and 8, ovine embryonic sample + anti-bovine uPA, lane 4 and 9, ovine embryonic sample + PBS, lane 5 and 10.

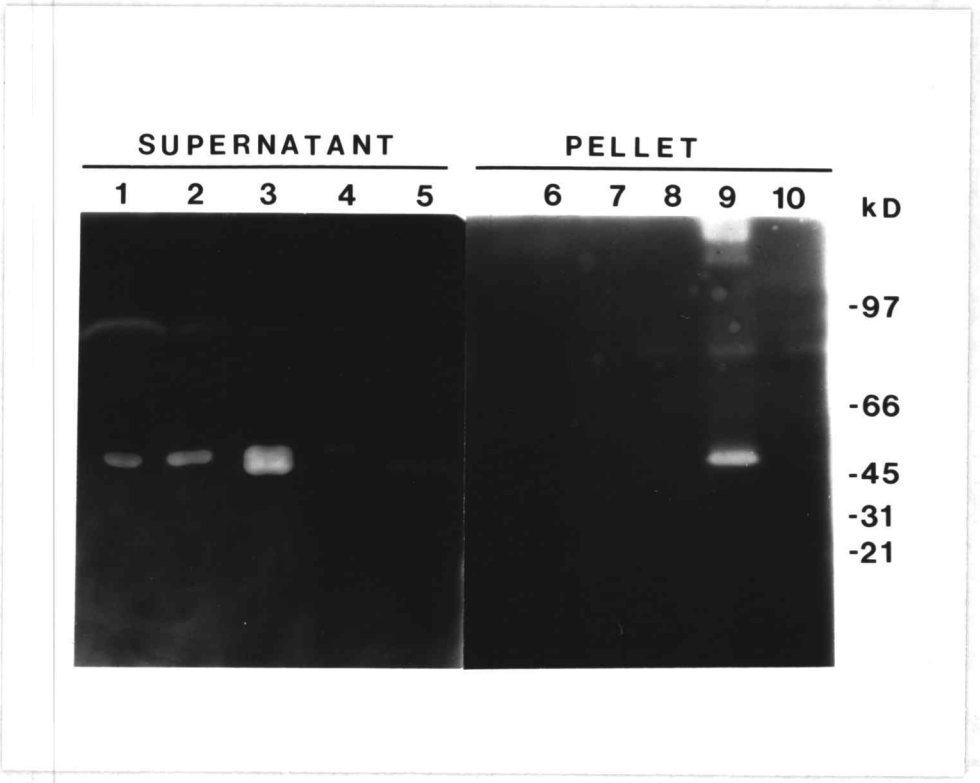

Table I-1. Cell stage of embryos recovered surgically from ewes on days 7, 9 and 11 of pregnancy.

Day of pregnancy	Developmental stage[a]								
	D	UFO	C	M	EBL	BL	X	I	H
7	0	2	2	9	19	10	0	0	0
9	4	20	6	0	0	3	1	3	16
11	1	15	0	0	0	0	0	0	22

a Developmental stages are abbreviated as: degenerate (D), unfertilized (UFO), cleavage stage (C), morula (M), early blastocyst (EBL), blastocyst (BL), expanded blastocyst (X), hatching blastocyst (I) and hatched blastocyst (H).

Table I-2. Molecular masses (kD) of plasminogen activators produced by days 7,9 and 11 ovine embryos.

		Molecular Mass (kD)	
Form	n	Mean	SE
Heavy	8	81.4	1.2
Light	37	49.9	0.5

REFERENCES

Berg, D.A. and A.R. Menino, Jr. 1992. Bovine embryos produce a urokinase-type plasminogen activator. Mol. Repro. Dev. 31:14-19.

Christman, J.K., S.C. Silverstein and G. Acs. 1977. Plasminogen activators. In: Barrett A.J. (ed.), Proteinases in mammalian cells and tissues. Amsterdam: Elsevier/North-Holland Biomedical Press, pp 91-149.

Coates, A.A. and A.R. Menino, Jr. 1991. Identification of the plasminogen activator produced by bovine embryos in vitro. J. Anim. Sc. 69 (Suppl. 1): 407 (Abstract).

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

Dyk, A.R. and A.R. Menino, Jr. 1991. Electrophoretic characterization of the plasminogen activator produced by bovine blastocysts. J. Reprod. Fert. 93:483-489.

Fazleabas, A.T., R.D. Geisert, F.W. Bazer and R.M. Roberts. 1983. Relationship between release of plasminogen activator and estrogen by blastocyst and secretion of plasmin inhibitor by uterine endometrium in the pregnant pig. Biol. Reprod. 29:225-238.

Granelli-Piperno, A. and E. Reich. 1978. A study of proteases and protease-inhibitor complexes in biological fluids. J. Exp. Med. 148:223-234.

Hart, D.A. and A. Rehemtulla. 1988. Plasminogen activators and their inhibitors: Regulators of extracellular proteolysis and cell function. Comp. Biochem. Physiol. 9013:691-708.

Kaaekuahiwi, M.A. and A.R. Menino, Jr. 1990. Relationship between plasminogen activator production and bovine embryo development in vitro. J. Anim. Sci. 68:2009-2014.

Kruithof, E.K.O. (1988). Plasminogen activator inhibitors - A review. Enzyme 10:113:121.

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

Lowenstein, J. and A. Ingild. 1976. A micromethod for determination of proteolytic enzymes in the PH range of 2.8 to 4.8. Analyt. Bioch. 71:204-208.

Menino, A.R., Jr., A.R. Dyk, C.S. Gardiner, M.A. Grobner, M.A. Kaaekuahiwi and J.S. Williams. 1989. The effects of plasminogen on in vitro ovine embryo development. Biol. Reprod. 41:899-905.

Menino, A.R., Jr., and J.S. Williams. 1987. Activation of plasminogen by the early bovine embryo. Biol. Reprod. 36:1289-1295.

Mullins, D.E., F.W. Bazer and M.R. Roberts. 1980. Secretion of a progesterone induced inhibitor of plasminogen activator by the porcine uterus. Cell. 20:865-872.

Rehemtulla, A., A. Arndt and D.A. Hart. 1990. Induction of plasminogen activator inhibitor type 2 expression during differentiation of human K562 cells towards a macrophage phenotype. Bioch. Cell Biol. 68:1377-1343.

Rehemtulla, A., R. Smith and D.A. Hart. 1987. Regulation of plasminogen activator and plasminogen activator-inhibitors production by tissue culture cells: Evidence for independent induction and regulation. Fibrinolysis. 1:109-116.

Sappino, A.P., J. Huarte, D. Belin and J.D. Vassalli. 1989. Plasminogen activators in tissue remodeling and invasion: mRNA localization in mouse ovaries and implanting embryos. J. Cell Biol. 109:2471-2479.

Steel, R.D.G. and J.H. Torrie. 1980. Principles and procedures of statistics. McGraw-Hill Book Company, New York.

Sherman, M.I., S. Strickland and E. Reich. 1976. Differentiation of early mouse embryonic and teratocarcinoma cells in vitro: plasminogen activator production. Cancer Res. 36:4208-4216.

Strickland, S., E. Reich and M.I. Sherman. 1976. Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm.

Cell. 9:231-240.

Vassali, J.-D. and D. Belin. 1987. Amiloride selectivly inhibits the urokinase-type plasminogen activator. FEBS Lett. 214:187-191.

Vassali, J.-D., J.-M. Dayer, A. Wohlwend and D. Belin. 1984. Concomitant secretion of pro-urokinase and of a plasminogen activator-specific inhibitor by cultured human monocytes-macrophages. J. Exp. Med. 159:1653-1668.

Wiman B. and D. Collen. 1979. On the mechanism of the reaction between human 2-antiplasmin and plasmin. J. Biol. Chem. 254:9291-9297.

DIFFERENCES IN PLASMINOGEN ACTIVATOR, PLASMINOGEN ACTIVATOR INHIBITOR AND PLASMIN INHIBITOR IN UTERINE FLUSHINGS FROM PREGNANT AND NONPREGNANT EWES

ABSTRACT

Uterine flushings (UTF) were examined for differences in relative amounts of total protein, plasminogen activator (PA), PA inhibitors (PAI) and plasmin inhibitors (PI). Uterine flushings (UTF) were surgically collected on Days 7, 9 and 11 from estrous synchronized, pregnant (P) and nonpregnant (NP) ewes. Recovered UTF were concentrated by ultrafiltration and total protein was determined using the Bio-Rad protein assay. Plasminogen activator, PAI and PI activities were quantified using a caseinolytic assay. Sodium dodecyl sulfate polyacrylamide gel electrophoresis with zymography was performed to analyze molecular masses of PA activators and inhibitors present in UTF. Analysis by day revealed total protein concentration was not different between Days 7, 9 and 11 in UTF from NP ewes, however in UTF from P ewes, total protein increased dramatically ($P<0.001$) on Day 11. Although PA and PAI activity remained constant in UTF from NP ewes, PI activity steadily decreased ($P<0.05$) reaching low levels on Day 11. While PI activities did not change in UTF from P ewes, both PA and PAI activities decreased ($P<0.05$) on Day 11. Polyacrylamide gel electrophoresis with zymography revealed 5 major plasminogen-dependent lytic zones. When amiloride was added to zymographs,

activity in the 51 kD protein was depressed in all days and was not dependent on pregnancy status. Presence of a 53 kD protein and a 60 kD inhibitor was only detected in UTF from P ewes on Day 11. These results suggest: 1) total protein levels in UTF increase with stage of pregnancy; 2) levels of PA, PI and PAI fluctuate based on day and pregnancy status and 3) five major molecular mass classes of plasminogen-dependent proteins are present in ovine UTF possibly including a urokinase-type PA (51 kD) and a Day 11 pregnancy-specific protease (53 kD).

INTRODUCTION

Uterine luminal fluid (UTF) and its constituents play a major role during early embryonic development. A variety of proteases and protease inhibitors have been found in the uterine lumen. Katz et al. (1976) reported the presence of an estrogen-dependent trypsin-like protease in the rat uterus which they classified as a plasminogen activator (PA). Dabitch and Owens (1978) demonstrated that levels of a trypsin-like inhibitor were high in uterine secretions from Day 6 pregnant mice. Similarly, Fazleabas et al. (1982) observed a progesterone-induced protease inhibitor in the uterine secretions of pregnant pigs.

The serine protease plasmin is the active product of cleavage of the zymogen, plasminogen, by PA. Plasminogen activators can be classified into two types based on

molecular mass; urokinase-type PA (uPA, 30-55 kD), and tissue-type PA (tPA, 70 kD). Based on its proteolytic capabilities, a role for PA is in the degradative processes of tissue remodeling and reorganization of extracellular structures. In early embryonic development, examples of intense tissue remodeling occur with the differentiation and migration of developing tissues within the blastocyst and during implantation of the embryo into the endometrium (Strickland, 1980).

During the period of invasive implantation in mice, increased levels of PA are associated with the trophoblast, suggesting a correlation between this enzyme and implantation (Strickland et al., 1976). In contrast, the uterus of the pig secretes high levels of a protease inhibitor during this comparative stage of development (Mullins et al., 1980). Unlike the mouse, swine embryos do not invasively implant, therefore, the presence of the protease inhibitor may serve to protect the uterus from unwanted proteolysis by the blastocyst.

Plasminogen activator production and plasminogen conversion to plasmin increases during blastocoelic expansion and hatching in sheep embryos (Menino et al., 1989). To date, no information regarding this protease or its inhibitors in the UTF of sheep has been reported. Therefore, the purpose of this study was to investigate UTF from ewes for the presence of PA and related

inhibitors and determine if differences in these parameters existed due to stage of pregnancy.

MATERIALS AND METHODS

COLLECTION OF UTERINE FLUSHINGS

Twenty multiparous Polypay ewes were randomly assigned to pregnant (P) or nonpregnant (NP) groups and were estrous synchronized with two, 100 μg i.m. injections of cloprostenol sodium (Estrumate; Haver; Shawnee, KS), given 9 days apart (Day 0 = first cloprostenol sodium injection). Those ewes assigned to pregnant groups were superovulated with twice daily i.m. injections of porcine follicle stimulating hormone (pFSH; Schering-Plough Animal Health Corp., Kenilworth, NJ) at dosages of 4, 3 and 2 mg on Days 8, 9 and 10, respectively. Estrus detection was initiated 24 h after the second injection of cloprostenol sodium, and continued at 12-h intervals. All ewes were exposed to an intact ram to detect estrus, but only superovulated ewes were allowed to be handmated. Seven, 9 and 11 days after onset of estrus, ewes were anesthetized by injection of 10 ml of 2.5% thiamylal sodium (Bio-tal; Boehringer Ingelheim Animal Health, Inc.; St. Joseph, MO) into the jugular vein and maintained via inhalation with halothane (Flouthane, Fort Dodge, Inc.; Fort Dodge, IA) and oxygen. The reproductive tract was exteriorized via ventral midline laparotomy and uteri were flushed in a retrograde

fashion with 60 ml of alpha Minimum Essential Medium (MEM; Sigma Chemical Co., St. Louis, MO) buffered with N'-2-hydroxyethyl piperazine-N'-ethanesulfonic acid (HEPES/MEM+H; Sigma). Flushings from both P and NP ewes were examined with a dissecting microscope (10-40x) and embryos recovered from P ewes were used for an ongoing culture experiment. Uterine flushings from all ewes were centrifuged at 5,000 x g for 10 min at room temperature. The pellet was discarded and the supernatant was stored at -20°C.

ULTRAFILTRATION AND TOTAL PROTEIN DETERMINATION

Uterine flushings were thawed and concentrated to 3-5 ml using a stirred ultrafiltration cell with a YM2 membrane (1,000 D molecular weight cut-off; Amicon Corporation; Danvers, MA). The retentate was stored at -20°C.

Protein concentrations in UTF were determined using the Bio-Rad protein assay (Bio-Rad Laboratories; Richmond, CA). Standard curves were constructed using 0, 200, 400, 600, 800 and 1000 μg/ml BSA (Sigma). Protein concentrations of UTF were calculated using the equation of the line for the standard curve. Each UTF was assayed in triplicate.

ELECTROPHORESIS AND ZYMOGRAPHY

One dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was

conducted under nonreducing conditions with casein/plasminogen zymography (Granelli-Piperno and Reich, 1978; Vassali et al., 1984). Twenty-five microliters of concentrated UTF was combined with 75μl of 1X SDS-PAGE sample buffer (2.5% SDS; 10% glycerol; 0.00012% bromophenol blue in 0.125 M Tris HCl buffer) and each polyacrylamide gel included lanes containing a urokinase standard (E.C. 3.4.21.31; American Diagnostica Inc; Greenwich, CT), and molecular mass markers ranging from 14.4 kD to 97.4 kD (Bio-Rad). Concentrated UTF, urokinase standards and molecular mass markers were aliquoted in 100 μl volumes into castellated wells in a 4% acrylamide stacking gel with a 12% separating gel. Following electrophoresis, the polyacrylamide gels were gently shaken in 2.5% Triton X-100 (Sigma) for 45 min, rinsed with distilled water five times and shaken in phosphate buffered saline (PBS) for 45 min. The gel was removed from the PBS, placed on plastic wrap, and excess PBS removed by blotting gently with tissue paper. A casein-agar gel supported on a glass plate (zymograph) and containing 20 μg/ml human plasminogen, was placed on the polyacrylamide gel. Zymographs were prepared by combining equal volumes of 4% nonfat dry milk (Carnation Co.; Los Angeles, CA), dissolved in buffer containing 0.0013 M $CaCl_2$ x $2H_20$, 0.10 M glycine, 0.038 M tris and 0.005 M sodium azide with a 2% solution of melted agarose

(Sigma) at 65°C. Once the solution had cooled to 45°C, human plasminogen was added. The mixture was then cast onto a warmed glass plate and allowed to cool. Zymographs containing 0 μg/ml human plasminogen were used for detection of any nonspecific proteolytic activity. Polyacrylamide gels with zymographs were incubated at 39°C for 24-48 h and observed every 5 h for lysis. To terminate the incubation, zymographs were removed from the polyacrylamide gels and fixed with 3.0% acetic acid for 15 min. Zymographs were dried and stained for permanent storage (Lowenstein and Ingild, 1976). Polyacrylamide gels were stained as described by Dyk and Menino (1991). For the amiloride zymographs, UTF were electrophoresed as previously described, and the zymographs were prepared with the following modifications: equal volumes of 2.2% agarose + 4.2% nonfat dry milk were combined; 1 ml of either PBS or 100 mM amiloride (Sigma) and human plasminogen to a final concentration of 20 μg/ml were added to 9 ml of the agarose-nonfat dry milk mixture. The zymograph was allowed to cool and placed on the polyacrylamide gel. For all zymographs, measurements and calculations were made as described by Dyk and Menino (1991).

ASSAYS FOR PLASMINOGEN ACTIVATOR, PLASMINOGEN ACTIVATOR INHIBITOR (PAI) AND PLASMIN INHIBITOR (PI)

The caseinolytic assay as described by Kaaekuahiwi and Menino (1990) was used to determine PA levels in UTF.

To investigate levels of PAI and PI in UTF, modifications of this technique were used. Briefly, for both assays, casein-agar plates were prepared as described by Kaaekuahiwi and Menino (1991). For the PAI assay, 45 µl of UTF sample or MEM control were incubated for 30 min with 30 µl of 10 IU/ml urokinase or PBS. Fifteen microliters of 360 µg/ml plasminogen was then added, and the samples were allowed to incubate for an additional 15 min at 39°C. Three 25 µl aliquots from each sample were then pipetted into wells in the casein-agar plates and allowed to develop at room temperature for 24 h. For the PI assay, 60 µl of 900 units human plasmin (E.C. 3.4.21.7; Sigma) were incubated with 30 µl of UTF sample or MEM for 15 min at 39°C. Three 25 µl aliquots from each sample were pipetted onto the plates and allowed to develop as described above. The assay was terminated by fixing the plates with 3% acetic acid for 15 min. After rinsing with tap water, areas of lysis were measured with an electronic digital caliper. A lytic zone area of 1 mm^2 was equal to 1 protease unit and the inhibition of 1 protease unit was equal to 1 PAI unit (PAIU) or 1 PI unit (PIU). Calculations for inhibitor activities in UTF were performed using the following equations derived from Leprince et al. (1989): 1) total inhibitor (PAI/PI) = [(area of 10 IU/ml urokinase - area of 0 IU/ml urokinase) - (area of 10 IU/ml urokinase with conditioned medium -

area of conditioned medium)]; 2) plasmin inhibitor (PI) = (area of PBS/control medium with plasmin standard - area of conditioned medium with plasmin standard); and 3) PAI = (PAI/PI) - (PI).

DENSITOMETRIC ANANLYSIS

Zymographs were densitometrically scanned using a model 1650 Transmittance/Reflectance Scanning Densitometer (Bio-RAD) and scans were plotted using the GS 350H software system (Hoefer Scientific Instruments, San Fransisco, CA). A compensating polar planimeter (model L-20-M; LASICO; Los Angeles, CA) was used to trace the densitometric scans and determine areas of the lytic zones. Areas of the lytic zones were expressed as relative percentages of the entire densitometric scan.

STATISTICAL ANALYSIS

Total protein, activities of PA, PAI and PI in μg total protein, concentration of PA, PAI and PI and relative percentages of plasminogen-dependent lytic zones were analyzed using multi-way analysis of variance. Differences due to day, amiloride or pregnancy status were analyzed using Duncans multiple range test. All analyses were conducted using the NCSS statistical software program (Number Cruncher Statistical Systerm, version 4.1, 1984, Hintze, J.L., Kaysville, UT, USA).

RESULTS

Total protein in UTF from Day 7, 9 and 11 P and NP

ewes is shown in Figure II-1. Day of the estrous cycle had no effect on total protein in NP ewes, however, after remaining constant between Days 7 and 9, levels increased dramatically ($P<0.001$) on Day 11 of pregnancy. Although protein levels on Day 11 of pregnancy appear higher than on Day 11 of the estrous cycle, no significant differences ($P>0.05$) were observed for pregnancy status.

Figure II-2 shows PA activities based on day and pregnancy status. Plasminogen activator activities in UTF from nonpregnant ewes were not different ($P>0.05$) by day of the estrous cycle. Although not different from Day 9, PA in UTF from Day 7 of pregnancy was significantly higher ($P<0.05$) than on Day 11.

While levels of PI remained constant, levels of PAI were lower ($P<0.05$) on Day 11 than on Day 7 or 9 in UTF from P ewes (Figure II-3). Although, PAI activity in UTF from NP ewes was not different by day, PAI activity was lower ($P<0.05$) on Day 7 and higher ($P<0.05$) on Day 11 when compared to respective UTF from P ewes. Plasmin inhibitor activities in UTF from NP ewes steadily decreased ($P<0.05$) each day, reaching lowest levels on Day 11.

Table II-1 reports concentrations of PA, PAI and PI in UTF collected from P and NP ewes. Plasminogen activator concentrations were lowest ($P<0.05$) in Day 11 P UTF. The only differences in PAI and PI concentrations

were due to pregnancy status with levels on Day 11 and Day 7, respectively, being lower ($P<0.05$) in samples from pregnant than from nonpregnant ewes.

Zymographic analysis revealed 5 major molecular mass classes of plasminogen dependent-lytic zones (Figure II-4). Absence of plasminogen in zymographs failed to support any caseinolysis, therefore confirming the absence of nonspecific proteases. Samples from Day 11 P ewes also exhibited a PAI or PI that was was not observed in other samples from P or NP ewes. Relative percents of the 5 major plasminogen-dependent lytic zones are reported in Table II-2. Amiloride treatment depressed PA activity in the 51 kD protein, however, a significant reduction in relative percentage was observed only in samples from Day 7 P ewes. Amiloride treatment also shifted the relative percentages of the 77 kD protein in Day 11 P and the 47 kD protein in Day 7 NP and Day 9 P ewes. Differences in relative percentages by pregnancy were observed in the 77 kD band for Days 7 and 9 and the 53 kD band was detected only in Day 11 P ewes ($P<0.10$). The relative percentage of the 47 kD band was greater ($P<0.10$) in samples from Day 9 P ewes but less ($P<0.10$) in samples from Day 11 P ewes when compared to corresponding samples from NP ewes. Relative percentages of the bands also varied by day of collection. Day 9 NP had greater ($P<0.05$) relative percentages of the 97 kD

and 77 kD bands than Day 11 but lower ($P<0.05$) relative percentages of the 47 kD band than either Day 7 NP or Day 11 NP ewes. The 77 and 47 kD bands were greater ($P<0.05$) in Day 9 P ewes compared to Day 11 P ewes.

DISCUSSION

Total protein levels in UTF from P and NP ewes were similar to those reported by Segerson (1981). Values increased dramatically on Day 11 of pregnancy, after remaining fairly constant between Days 7 and 9. In the pig uterus, levels of protein in UTF do not differ between P and NP animals until about Day 11 (Geisert et al., 1982). At this time, conceptuses induce release of secretory vesicles from glandular epithelial cells. Although not significantly different, total protein levels in Day 11 P UTF in the present study were higher than in samples from Day 11 NP ewes.

Although PA activities remained unchanged in NP ewes, PA activity in Day 7 P ewes were higher than in Day 11 animals. One explanation for this could be that ewes which represented Day 7 P UTF were carrying 18 embryos at the time samples were collected. Menino et al. (1989) reported ovine embryos secrete PA during the morula-blastocyst transition which corresponds to approximately Day 6 of embryonic development in sheep. The presence of 18 embryos in the uterus could potentially lead to high amounts of PA in the uterine lumen. Another explanation

is the presence of the inhibitor in Day 11 P ewe UTF. Although in this study the zymographs did not discriminate between PAI or PI, the presence of either would have depressed interpretable PA activity. The caseinolytic assay is an indirect assay that quantifies PA activity relative to the amount of plasmin generated. Therefore, either a PAI or PI would cause the measured PA activity to be reduced. When evaluating PA activities, however, one must consider that activities were expressed relative to mg of total protein. Because protein levels were higher on Day 11 of pregnancy, the relative amount of protein actually due to PA is, as a result, mathematically lower. The fluctuations observed in PAI and PI activity no doubt represent changes in protein composition in UTF.

Five major molecular mass classes of PA were revealed by zymography. No activity was present in zymographs containing 0 μg/ml plasminogen indicating all lytic zones were due to plasminogen-dependent caseinolysis. Additon of amiloride, a competitive inhibitor of urokinase-type PA (uPA) to the zymographs shifted activity in the 47, 51 and 77 kD proteins. Although not significant, activities in the 51 kD protein were consistently lower in the presence of amiloride. Caution must be exercised in interpreting the changes in relative percentages because elimination or reduction in

one protein would shift the relative percentages of other proteins upward. Because activity of the 51 kD protein was lower or completely diminished in the presence of amiloride, a shift in the relative percentage of the remaining bands occurred. This may account for the increases observed in the 77 kD band in Day 11 P ewe UTF and the 47 kD protein in Day 7 NP ewe UTF in the presence of amiloride. The fact that amiloride caused a consistent suppression of the 51 kD protein suggests that this band may be a uPA. The observed molecular mass of uPA ranges from 30-55 kD (Dano et al., 1985), further suggesting this protein may be a uPA.

Two Day 11 P specific proteins were observed zymographically. One was a 53 kD protein and the second, a 60 kD inhibitor. Mullins et al. (1980) reported the presence of a progesterone-induced inhibitor of PA in Day-15 swine UTF. Mullins et al. (1980) suggested that the inhibitor may play a role in preventing invasive implantation in the pig. While the sheep, like the pig does not exhibit invasive implantation, it is unclear whether the inhibitor present in sheep UTF plays a similar preventative role. Further studies on the characteristics of this Day-11 P specific inhibitor need to be undertaken before conclusions can be made about the inhibitor's specific function.

Uterine proteins may play a variety of roles in

embryonic development. Secretions of a number of progesterone responsive proteins have been reported, some of which appear to play a nutritional role (Roberts and Bazer, 1988). Other functions of uterine proteins may include immunoprotective or antiviral functions. The present study reports activities of PA, PAI and PI in UTF from P and NP ewes and also reports the presence of a pregnancy-specific PA and inhibitor. Further studies need to be undertaken to investigate a role for these proteins in early embryonic development.

Figure II-1. Total protein in uterine flushings from Day 7, 9 and 11 pregnant and nonpregnant ewes.
a,b Values without common letters are different (P<0.05).

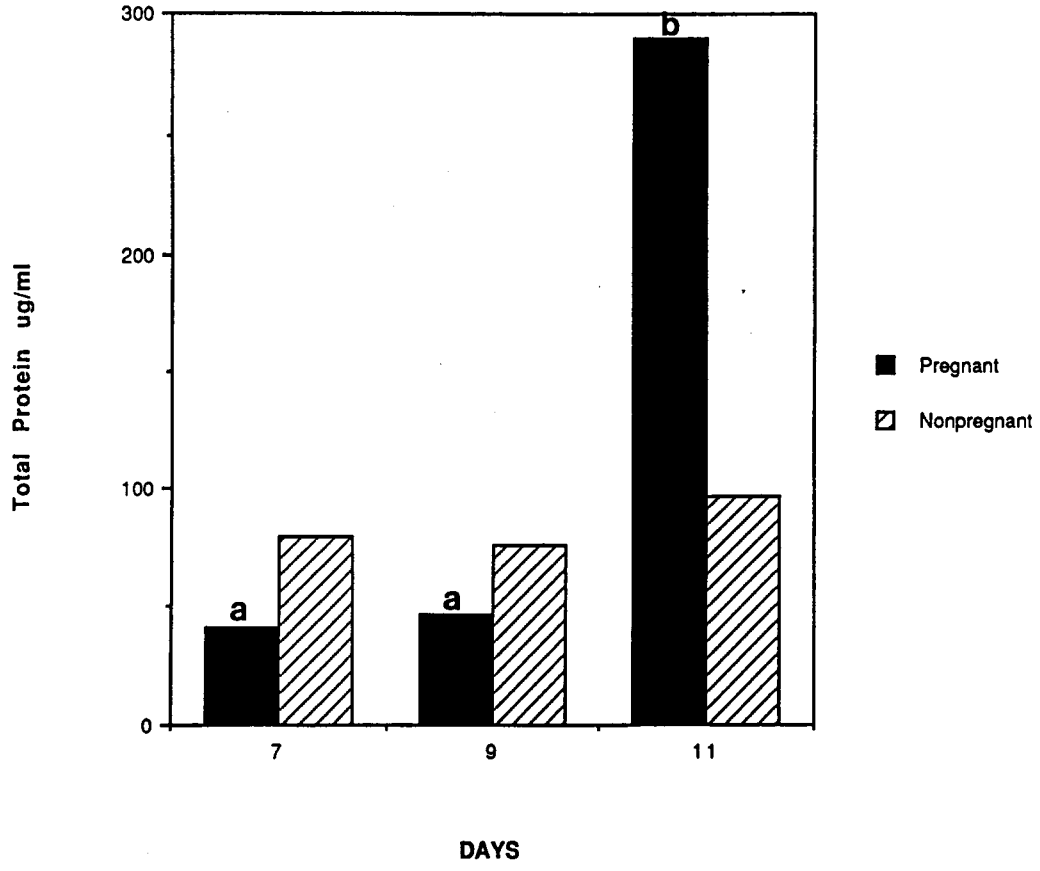

Figure II-2. Plasminogen activator activities in uterine flushings from Day 7, 9 and 11 pregnant and nonpregnant ewes.
*Different from corresponding pregnant sample (P<0.05).
a,b Values without common letters are different (P<0.05).

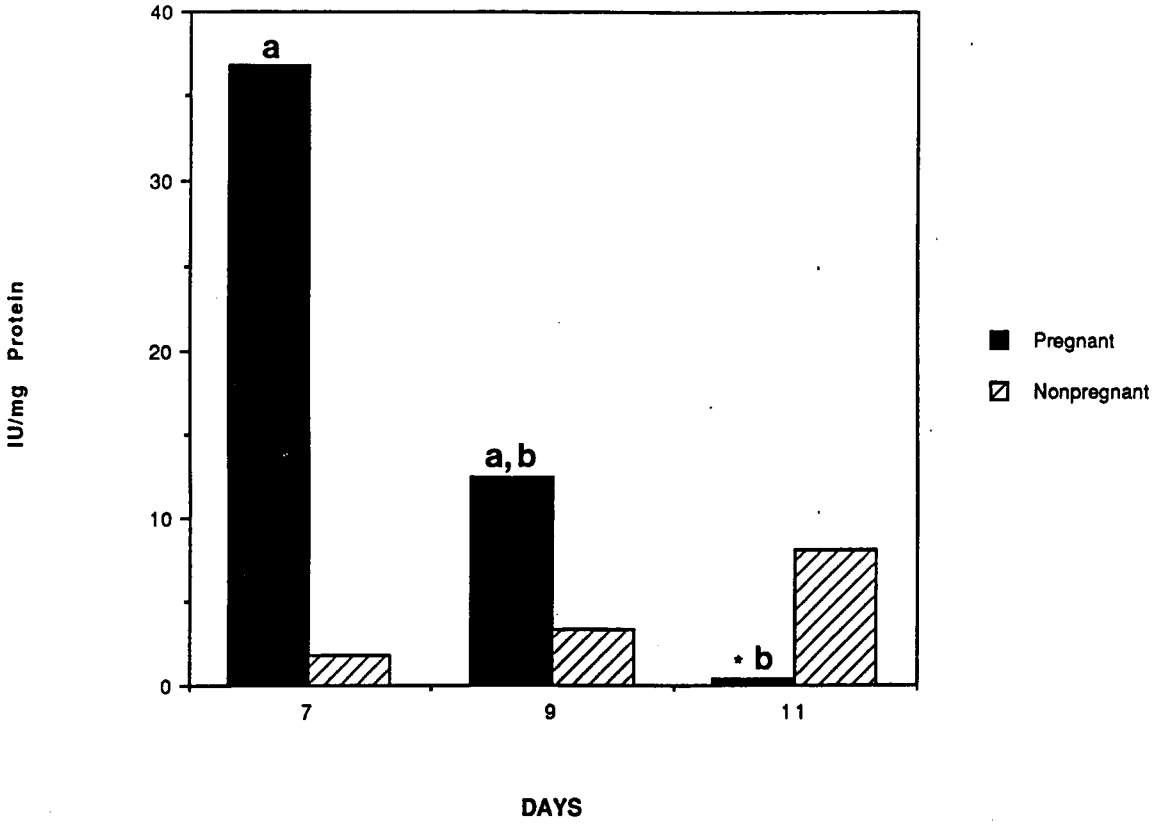

Figure II-3. Plasminogen activator inhibitor (PAI) and plasmin inhibitor (PI) acivities in uterine flushings from Day 7, 9 and
11 pregnant and nonpregnant ewes.
*Different from corresponding pregnant sample.
a,b,c Values without common letters are different (P<.05).

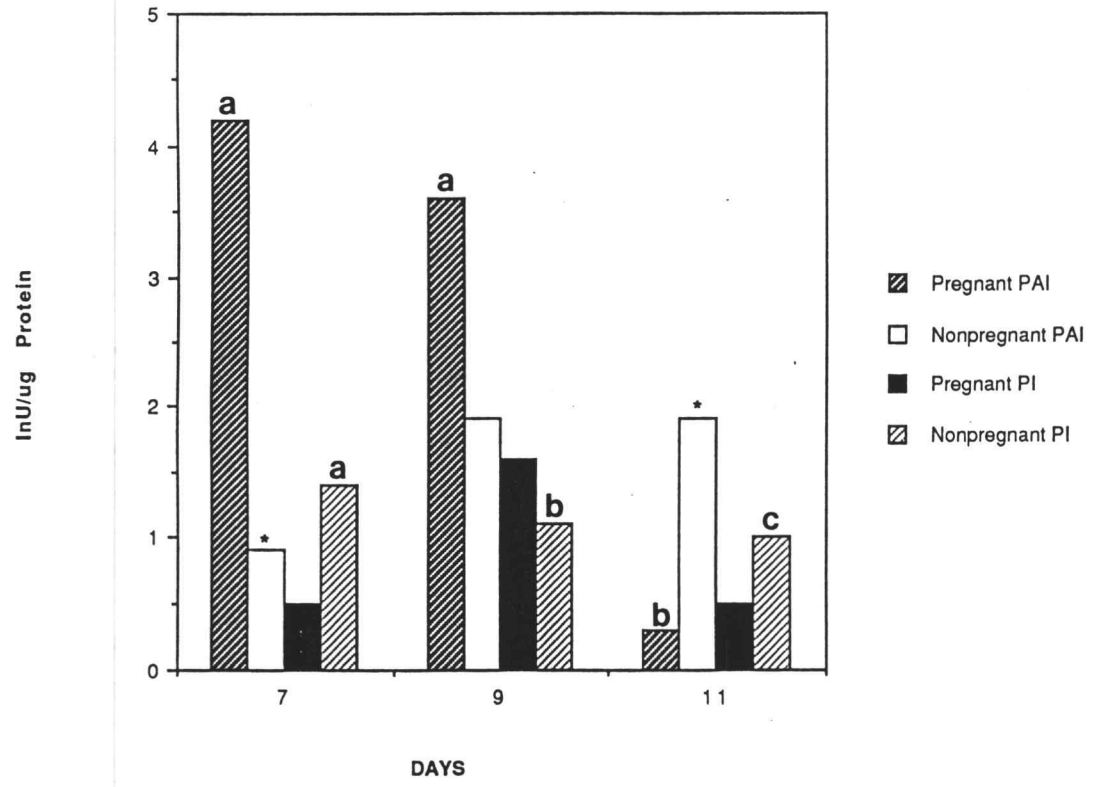

Figure II-4. Zymographic analysis of uterine flushings in the presence (a) or absence (b) of amiloride from pregnant and nonpregnant ewes. Lane 1, Day 7 pregnant, Lane 2 Day 7 nonpregnant, Lane 3, Day 9 pregnant, Lane 4, Day 9 nonpregnant, Lane 5 Day 11 pregnant, Lane 6 Day 11 nonpregnant.

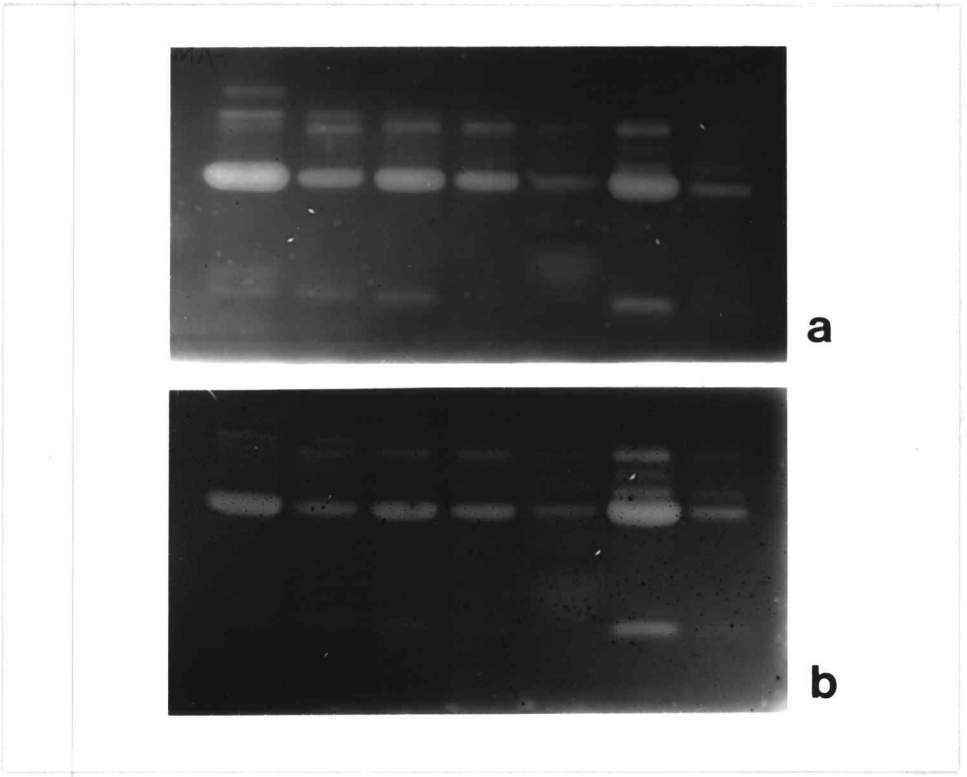

Table II-1. Concentrations of plasminogen activator (PA), plasminogen activator inhibitor (PAI) and plasmin inhibitor (PI) in uterine flushings collected from pregnant (P) and nonpregnant (NP) ewes.

	Day of collection and pregnancy status						
	7P	7NP	9P	9NP	11P	11NP	EMS[c]
PA							
IU/ml	2.02_{a}	0.16	$0.61_{a,b}$	0.24	0.07_{b}*	0.76	0.69
PAI							
InU/μl	171.5	76.1	172.5	140.1	68.5*	179.0	5103.8
PI							
InU/μl	26.2*	113.7	69.7	84.4	135.5	92.0	3131.4
n	2	2	6	2	5	2	-

* Different from corresponding nonpregnant sample ($P<0.05$)
a,b Values with different subscripts differ from corresponding days of collection ($P<0.05$).
c Error mean square

Table II-2. Relative percentages of major molecular mass classes of plasminogen activators in uterine flushings collected from pregnant and nonpregnant ewes 7, 9 and 11 days after onset of estrus.

Molecular mass (kD)	- amiloride						+ amiloride						Pooled SE
	7NP	7P	9NP	9P	11NP	11P	7NP	7P	9NP	9P	11NP	11P	
97 ± 0.4	$18.8_{a,b}$	10.8_{a}	30.0_{b}	21.8_{a}	15.5_{a}	14.8_{a}	$25.5_{a,b}$	20.2_{a}	35.0_{b}	24.8_{a}	17.0_{a}	18.8_{a}	4.0
77 ± 0.3	40.0^{+}_{a}	$46.0_{a,b}$	68.2^{+}_{b}	48.0_{b}	41.0_{a}	33.8^{*}_{a}	39.8^{+}_{a}	49.8_{a}	65.0^{+}_{b}	49.8_{a}	40.5_{a}	49.0_{a}	4.0
53 ± 0.0	0.0	0.0_{a}	0.0	0.0_{a}	0.0+	28.2_{b}	0.0	0.0_{a}	0.0	0.0_{a}	0.0++	32.0_{b}	4.8
51 ± 0.5	11.5^{+}_{a}	25.8^{**}_{a}	0.0_{a}	0.0_{a}	7.8_{a}	23.5_{a}	0.0	5.8_{a}	0.0	0.0_{a}	0.0	0.0_{a}	5.6
47 ± 0.3	9.8^{**}_{a}	5.5_{a}	0.0^{+}_{b}	19.8^{*}_{b}	21.0^{++}_{c}	0.0_{a}	17.5_{a}	10.2_{a}	0.0^{++}_{b}	10.5_{a}	19.2^{++}_{a}	0.0_{a}	2.2

** Different from corresponding amiloride treated sample ($P<0.05$)
* Different from corresponding amiloride treated sample ($P<0.10$)
++ Different from corresponding pregnant sample ($P<0.05$)
+ Different from corresponding pregnant sample ($P<0.10$)
a,b,cPercentages with different subscripts differ from corresponding days of collection ($P<0.05$)

REFERENCES

Dabitch, D. and M.S. Owens. 1978. Trypsin-like inhibitor activity in mouse uteri during early gestation and delayed implantation. Proc. Soc. Exp. Biol. and Med. 157:175-179.

Dano, K., P.A. Andreason, J. Grondahl-Hansen, P. Kristenson, L.S. Nielson and L. Skriver. 1985. Plasminogen activators tissue degradation, and cancer. Adv. Cancer Res. 44:139-266.

Dyk, A.R. and A.R. Menino, Jr. 1991. Electrophoretic characterization of the plasminogen activator produced by bovine blastocysts. J. Reprod. Fert. 93:483-489.

Fazleabas, A.T., F.W. Bazer and M.R. Roberts. 1983. Purification and properties of a progesterone-induced plasmin/trypsin inhibitor from uterine secretions of pigs and its immunocytochemical localization in the pregnant uterus. J. Biol. Chem. 257:6886-6897.

Geisert, R.D., R.H. Renegar, W.W. Thatcher, R.M Roberts and F.W. Bazer. 1982. Establishment of pregnancy in the pig: I. Interrelationships between preimplantation development of the the pig blastocyst and uterine endometrial secretions. Biol. Reprod. 27:925-949.

Granelli-Piperno, A. and E. Reich. 1978. A study of proteases and protease-inhibitor complexes in biological fluids. J. Exp. Med. 148:223-234.

Kaaekuahiwi, M.A. and A.R. Menino, Jr. 1990. The relationship between plasminogen activator production and various aspects of bovine embryo development in vitro. J. Anim. Sci. 68:2009-2014.

Katz, J., W. Troll, M. Levy, K. Filkins, J. Russo and M. Levitz. 1976. Estrogen-dependent trypsin-like activity in the rat uterus: Localization of activity in the 12,000g pellet and nucleus. Arch. Biochem. Bioph. 173:347-354.

LePrince, P., B. Rogister and G. Moonen. 1989. A colorimetric assay for the simultaneous measurement of plasminogen activators and plasminogen activator inhibitors in serum-free conditioned media from cultured cells. Anal. Bioch. 177:341-346.

Lowenstein, H. and Ingild, A. 1976. A micromethod for determination of proteolytic enzymes in the pH range of 2.8 to 4.8. Anal. Biochem. 71:204-208.

Menino, Jr., A.R., A.R Dyk, C.S Gardiner, M.A. Grobner, M.A Kaaekuahiwi and J.S. Williams. 1989. The effects of plasminogen on in vitro ovine embyro development. Biol. Reprod. 41:899-905.

Mullins, D.E., F.W Bazer and M.R. Roberts. 1980. Secretion of a progesterone-induced inhibitor of plasminogen activator by the porcine uterus. Cell 20:865-872.

Roberts, R.M. and F.W. Bazer. 1988. The functions of uterine secretions. J. Reprod. Fert. 82:875-892.

Segerson, E.C. 1981. Immunosuppressive effect of ovine uterine secretory protein upon lymphocytes in vitro. Biol. Reprod. 25:77-84.

Strickland, S. 1980. Plasminogen activator in early embryo development. In: Johnson MH (ed.), Development in Mammals, Vol 4. Amsterdam: Elsevier/North-Holland Biomedical Press, pp.81-100.

Strickland, S., E. Reich and M.I. Sherman. 1976. Plasminogen activator in early embryogenesis: Enzyme production by trophoblast and parietal endoderm. Cell 9:231-240.

Vassali, J.-D., J.-M. Dayer, A. Wohlwend and D. Belin. 1984. Concomitant secretion of pro-urokinase and of a plasminogen activator-specific inhibitor by culture human monocytes-macrophages. 159:1653-1668.

EVALUATION OF EXTRACELLULAR MATRICES AND THE PLASMINOGEN ACTIVATOR SYSTEM IN OVINE INNER CELL MASS OUTGROWTH IN VITRO

ABSTRACT

Effects of extracellular matrices (ECM) and the role of the plasminogen activator (PA) system in outgrowth of ovine inner cell masses (ICM) in vitro were investigated. Inner cells masses were isolated by immunosurgery from Day 7.5 embryos and cultured for 72 h in alpha Mininum Essential Medium with 15 mg/ml BSA on ECM of collagen, fibronectin or laminin. At 24-h intervals, areas of the ICM and outgrowth were measured and the numbers of migrating cells were counted. Experiment 1 evaluated the effects of plasminogen (PGN) and ECM-type on ICM outgrowth. Inner cell masses were cultured in ECM-coated, glass Lab-Tek chamber slides in medium containing 0 or 150 μg/ml human PGN. Experiment 2 evaluated the effects of 100 IU/ml PA inhibitor-2 (PAI-2) or 10% rabbit antiserum to bovine urokinase-type PA (anti-uPA) on ICM outgrowth in fibronectin-coated, glass Lab-Tek slides. Experiment 3 evaluated the relationship between PA production and ECM-type on ICM outgrowth. Inner cell masses were cultured in 25-μl microdrops under paraffin oil on ECM-coated 60 x 15 mm plastic culture plates. In Experiment 1, ICM in 0 μg/ml PGN had a larger ($P<0.01$) mean area of outgrowth than in 150 μg/ml PGN. Fibronectin supported greater ($P<0.001$) numbers of migrating cells than did either collagen or laminin. In Experiment 2, addition of anti-uPA or PAI-2 to the

culture medium had no effect on outgrowth area ($P>0.10$). However, whereas the numbers of migrating cells in anti-uPA did not differ compared to normal rabbit serum, fewer ($P<0.001$) cells migrated from ICM in 0 than in 100 IU/ml PAI-2. In Experiment 3, ICM on fibronectin produced more ($P<0.01$) PA than on collagen or laminin, however, PA production was not correlated with areas of ICM outgrowths ($r=0.06$;$P>0.77$) or cell numbers in the outgrowths ($r=0.02$; $P>0.90$). Inner cell mass area was correlated with PA production ($r=0.41$; $P<0.01$). These results suggest that fibronectin enhances cellular outgrowth from ovine inner cell masses, however, it is unlikely that PA plays much of a functional role in this cellular migration.

INTRODUCTION

Plasminogen activators (PA) are serine proteases that convert the zymogen plasminogen into plasmin, the enzyme active in clot lysis (Cristman et. al., 1977). Reproductive processes PA are involved in include ovulation (Dano et al., 1985), blastocyst escape from the zona pellucida (Menino et al., 1989), and implantation (Dano et al., 1985). Strickland et al. (1976) have suggested that PA may be involved in the tissue remodeling and cellular proliferation occurring in early embryogenesis. Evidence supporting this concept in other systems has been demonstrated in that high levels of PA

are associated with migrating tumor cells (Dano et al., 1985) and the epithelium of mammary glands undergoing involution (Ossowski et al., 1979). Embryonic PA production has been identified in several species and is produced in a biphasic manner by mouse and swine embryos (Sherman et. al., 1976; Fazleabas et. al., 1983; Menino and Williams, 1987; Menino et al., 1989). In mouse embryos, the first phase of PA production is by the trophoblast and is believed to be involved in implantation. The second phase is attributed to parietal endoderm and is possibly involved in cellular migration (Sherman et. al., 1976; Strickland et. al., 1976).

In early embryonic development, as the blastocyst expands, endoderm migrates from the inner cell mass (ICM) to line the blastocoel. This migration is along an extracellular matrix (ECM) on the basal surface of the trophectoderm. Interactions between cells and components of the ECM are essential for tissue differentiation and regulation of embryonic growth (Mullins and Rohrlich, 1983). In ovine embryos, PA production is elevated through blastocoelic expansion, hatching and the period corresponding to endodermal cell migration (Menino et al., 1989). In an effort to determine if PA is involved in the mechanisms of endodermal migration, we investigated the PA system and the effects of the extracellular matrices type IV collagen, fibronectin and

laminin on the outgrowth of ovine inner cell masses in vitro.

MATERIALS AND METHODS

EMBRYO COLLECTION AND CULTURE

Forty cross-bred ewes were estrous-synchronized with cloprostenol sodium (Estrumate, Haver, Shawnee, KS) and superovulated with porcine follicle stimulating hormone (pFSH; Shering Corporation, Kenilworth, NJ). Ewes received two 100 μg i.m. injections of cloprostenol sodium 9 days apart (Day 0 = first cloprostenol sodium injection) and twice daily injections of pFSH i.m. at dosages of 4, 3 and 2 mg on Days 8, 9 and 10, respectively for a total of 18 mg per ewe. Estrus detection was initiated 24 h after the second injection of cloprostenol sodium and ewes were handmated by one of 3 rams starting at the onset of estrus and thereafter at 12-h intervals for as long as the ewe would except the ram, or for a total of 3 matings. Seven to 7.5 days after onset of estrus, anesthesia was induced in ewes by injection of 10 ml of 2.5% thiamylal sodium (Bio-tal; Boehringer Ingelheim Animal Health, Inc., St. Joseph, MO) into the jugular vein and was maintained during surgery via inhalation of halothane (Flouthane, Fort Dodge, IA) and oxygen. The reproductive tract was exteriorized via ventral midline laparotomy and the uteri were flushed in a retrograde fashion with alpha Minimum Essential Medium

(MEM) buffered with N-2-hydroxyethyl piperizine-N'-ethanesulfonic acid (MEM+H; Sigma, St. Louis, MO). Uterine flushings were examined by means of a dissecting microscope. Morula to hatched blastocysts were recovered from the uterine flushings by aspiration. Embryos were transferred to screw-cap tissue culture tubes containing 5 ml of MEM+H and 15 mg/ml bovine serum albumin (MEM+BSA;Sigma) maintained at 39°C and transported to the laboratory. Embryos were recovered from the tubes, washed three times in MEM+BSA, and morphologically evaluated using phase-contrast microscopy (200x). Embryos were cultured overnight in groups of 1 to 10 in 50-μl microdrops of MEM+BSA under paraffin oil in a humidified atmosphere of 5% CO_2 in air at 39°C. Conditioned medium was removed from the drops and stored at -20°C until assayed for PA activity and evaluated for inhibition by PA inhibitor-2 (PAI-2).

TROPHECTODERM AND INNER CELL MASS ISOLATION

For embryos which failed to complete the hatching process during the overnight culture, zonae pellucidae were removed using acidified PBS (pH 2). Mechanical separation of trophectoderm (TE) and inner cell mass (ICM) tissues was performed by bissecting the blastocyst using a finely drawn glass needle and a dissecting microscope. The glass needle was laid over the top of the blastocyst and was positioned in a manner that would

confine the ICM to either the right or left half. The blastocyst was gently rolled as the needle was moved in a sawing motion to separate the blastocyst into two trophectodermal vesicles (TV). The TV were washed in MEM+BSA and returned to culture for 6-12 h to permit resealing of the TE and re-expansion of the blastocoel (Figure III-1a and b). Trophectodermal vesicles possessing the ICM were subjected to immunosurgery using the techniques of Carnegie and Cabaca (1991) and Solter and Knowles (1975). Embryos were sequentially incubated in microdrops of rabbit anti-sheep antiserum (diluted 1 to 4 with MEM+BSA) for 1 h, three changes of MEM+BSA and guinea pig complement (diluted 1 to 4 with MEM+BSA). All incubations were conducted at 39°C in a humidified atmosphere of 5% CO_2 in air. Trophectodermal cells and debris were removed by use of a siliconized micropipette, and isolated ICM were placed in MEM+BSA until allocation into appropriate treatment (Figure III-2). To estimate the number of cells in an ICM at initiation of culture, immediately after immunosurgical isolation 8 ICM were washed twice in 2.0% sodium citrate, transferred to 0.7% sodium citrate for 3 min., fixed on microscope slides with 25% acetic acid in ethanol, air-dried (McGaughey and Chang, 1969) and stained with hematoxylin and eosin.

EXPERIMENT 1

In Experiment 1, the effects of human plasminogen

(hPGN) and ECM-type on ICM and TE outgrowth were evaluated. Inner cell masses and TE were cultured in glass, 8-chamber, Lab-Tek chamber slides (Nunc, Inc., Naperville, IL) coated with type IV collagen, fibronectin or laminin (Sigma). Chamber slides were coated by overnight incubation with 150 μl of 10 μg/ml solutions of type IV collagen, fibronectin or laminin in sterile PBS with 1% antibiotic (Sigma). The solutions were removed the following morning and each well was rinsed three times with MEM and then overlaid with MEM+BSA containing 0 or 150 μg/ml hPGN (Sigma). Inner cell masses and TE were cultured for 72 h in a humidified atmosphere of 5% CO_2 in air at 39°C. At 24-h intervals ICM and TE were observed for attachment and cellular outgrowth with an inverted stage phase-constrast microscope and a photomicrograph of each ICM was taken. Numbers of cells migrating away from the ICM core were also counted, and length and width measurements of the ICM and outgrowth were determined using an ocular micrometer. At the end of culture, medium was recovered and stored at -20°C until assayed for PA and plasmin activity. Areas of the ICM and outgrowth were determined by tracing the photomicrograph with a compensating polar planimeter (model L-20-M, LASICO, Los Angeles, CA) and computing the actual microscopic area.

EXPERIMENT 2:

Effects of PAI-2 or rabbit antiserum to bovine urokinase-type PA (anti-uPA) on ICM outgrowth were evaluated. Previous work in our laboratory (Bartlett and Menino, 1991) demonstrated that Day 7-11 ovine embryos produced a urokinase-type PA (uPA) that was immunoprecipitated with anti-uPA (American Diagnostica, Inc., Greenwich, CT). Because PAI-2 is the most effective inhibitor of uPA (Hart and Rehemtulla, 1988) it was also selected to suppress PA activity. Inner cell masses were cultured in 8-chamber, Lab-Tek chamber slides coated with fibronectin as described in Experiment 1. After overnight incubation, the slides were rinsed with MEM and overlaid with MEM+BSA containing one of the following: 0 or 100 IU/ml PAI-2 (American Diagnostica) or 10% normal rabbit serum (NRS) or anti-uPA (American Diagnostica, Inc.). Inner cell masses and their outgrowth were evaluated at 24-h intervals and medium was recovered at the end of culture as described in Experiment 1.

EXPERIMENT 3:

The relationship between PA production and ECM-type on ICM and TE outgrowth was evaluated. Inner cell masses and TE were cultured for 72 h on type IV collagen, fibronectin, or laminin coated 60 x 15mm plastic tissue culture dishes (Becton Dickinson and Company, Lincoln

Park, New Jersey). Tissue culture dishes were coated by aliquoting 16, 25-μl microdrops of collagen, fibronectin or laminin onto the surface of the dish and covering the drops with paraffin oil. Dishes were incubated overnight at 39°C in a humidified atmosphere of 5% CO_2 in air. Solutions were removed the following morning and each drop was rinsed three times with MEM and overlaid with MEM+BSA. At 24-h intervals, 15 μl of conditioned medium was removed from each microdrop, and replaced with 15 μl of MEM+BSA. Conditioned medium was placed in snap-cap vials and stored at -20°C until assayed for PA activity.

CASEINOLYTIC ASSAYS FOR PLASMINOGEN ACTIVATOR AND PLASMIN

Plasminogen activator activities in conditioned medium were analyzed using a caseinolytic-agar gel assay as described for bovine embryos by Kaaekuahiwi and Menino (1990). Conditioned medium from PGN-containing cultures were assayed for plasmin as described by Menino and Willimas (1987). Standards for the PA and plasmin assays were human urokinase (E.C. 3.4.21.31; American Diagnostica) and human plasmin (E.C. 3.4.21.7; Sigma), respectively.

STATISTICAL ANALYSIS

All morphometric data were analyzed by multi-way analysis of variance. Differences in ICM and outgrowth areas, cell numbers and PA production were tested for significance by Duncan's multiple range test.

Correlation-regression analysis was performed on PA production vs areas of ICM, and outgrowth and cell numbers.

RESULTS

Three hundred and thirty ova were recovered from 39 ewes. Two hundred and fifteen embryos were considered morphologically normal and used in the study. Mean number of ovulations per ewe was 13.5 ± 1.1. Mean number of cells per ICM was found to be 31.6 ± 2.8.

EXPERIMENT 1

Effects of plasminogen and ECM-type on ICM outgrowth were examined. Although cell numbers in outgrowths were not significantly different, ICM cultured in 0 μg/ml plasminogen grew to be larger ($P<0.01$) and had greater ($p<0.01$) areas of outgrowth than those cultured in 150 μg/ml plasminogen (table III-1, figure III-3). When evaluated for main effects, fibronectin supported greater areas of outgrowth and numbers of migrating cells ($P<0.01$) than did either collagen or laminin (table III-2). While ICM area did not change ($P>0.10$), outgrowth area and cell numbers increased ($P<0.01$; $P<0.08$, respectively), with time (table III-3). When compared to collagen and laminin, ICM cultured on fibronectin produced low and intermediate levels of PA and plasmin, respectively, however, no statistically significant differences were observed (table III-4). Plasminogen

activator and plasmin activities for trophectodermal vesicles are reported in table III-5. Trophectodermal vesicles cultured on laminin produced high levels of PA.

EXPERIMENT 2

Plasminogen activator inhibitor-2 and rabbit anti-bovine uPA were used to evaluate effects of inhibitors of PA on ICM outgrowth on fibronectin. Ability of PAI-2 to inhibit PA activity is shown in figure III-4. When 100 IU/ml PAI-2 was incubated with conditioned medium, PA activities were diminished ($P<0.05$).

Table III-6 shows individual means over time of ICM cultured in the presence or absence of PAI-2. As a main effect, addition of PAI-2 to the culture medium had no effect ($P>0.10$) on ICM area (6,534.7 μ^2 vs 5,031.9 μ^2) or area of outgrowth (25,682.4 μ^2 vs 22,422.2 μ^2). However, those ICM cultured in 100 IU/ml PAI-2 had greater ($p<0.01$) numbers of migrating cells than those cultured with 0 IU/ml PAI-2 (33.9 vs. 17.0). Individual means over time of ICM cultured in anti-uPA or NRS are presented in table III-7. Anti-uPA had no effect on ICM and outgrowth area, or cell number. When analyzed for main effects with time, ICM at 72 h were larger ($p<0.05$) than those at 0 h (7,974 μ^2 vs. 4,859.4 μ^2). When compared to activities in 100 IU/ml PAI-2 and 10% anti-uPA, plasminogen activator activities were greater in conditioned medium from ICM cultured in 0 IU/ml PAI-2 and

10% NRS, respectively (Table III-8).

EXPERIMENT 3

The relationship between PA production and ECM-type on ICM outgrowth was evaluated. When analyzed by main effects, ICM on fibronectin produced more ($P<0.01$) PA (16.8×10^{-3} IU/d) than those cultured on collagen (1.5×10^{-3} IU/d) or laminin (2.9×10^{-3} IU/d). Plasminogen activator production on collagen, fibronectin and laminin by time is presented in table III-9. Inner cell masses cultured on fibronectin grew to be larger ($P<0.05$; 11,771.7 μ^2) than those on laminin (3,925.0 μ^2), but were not different from those cultured on collagen (7,308.4 μ^2). When analyzed by time, ICM area was largest ($P<0.05$) at 48 h (13,273.1 μ^2), but not different ($P>0.05$) from areas at 24 and 72 h (8,443.8 μ^2 and 10,219.7 μ^2 respectively). Area of outgrowth for ICM on fibronectin was larger ($p<0.01$; 17,3347.3 μ^2) than those cultured on laminin (no outgrowth) but not different ($P>0.05$) from those on collagen (96,544.3 μ^2). Outgrowth at 72 h had larger area (242,844.6 μ^2; $P<0.01$) than at 0, 24 or 48 h (0 μ^2, 17,888.2 μ^2 and 99,122.7 μ^2, respectively). Number of cells that migrated on collagen or fibronectin per day is presented in table III-10. When analyzed for main effects, no differences ($P>0.05$) in number of cells migrating were found between collagen and fibronectin (48.2 and 65.4, respectively).

Although PA production and outgrowth were highest for ICM cultured on fibronectin, there was no correlation between amount of PA produced and area of outgrowth ($r=0.06$; $P<0.01$) or cell number ($r=0.02$; $P<0.90$) over all ECM evaluated. However, ICM area was correlated with PA production ($r=0.41$; $p<0.01$).

This experiment also evaluated PA production by trophectodermal vesicles cultured on collagen, fibronectin or laminin. No differences in PA production were observed with respect to time or ECM (Table III-11).

DISCUSSION

Our results demonstrate that fibronectin enhances outward migration of ovine ICM in vitro via a mechanism independent of the PA system. In previous reports, fibronectin has been shown to support attachment and outgrowth of mouse blastocysts (Armant et al., 1986) and ICM (Carnegie and Cabaca, 1991). In contrast to our findings however, in those studies, laminin and/or collagen supported equal if not greater amounts of cell migration. Although unclear, these differences in affinity for collagen, fibronectin and laminin may be due to species effects. Reichert's membrane is a thick basement membrane formed between the trophoblast and parietal endoderm of mice and rats. Using polyacrylamide gel electrophoresis to analyze the structural components, Smith and Strickland (1981) found Reichert's membrane to

be comprised mainly of the glycoproteins, collagen and laminin with very low levels of fibronectin. Although no similar studies have been reported in sheep, it is possible that a different combination of glycoproteins exist in the ECM along which the ICM migrates. In agreement with our results, Coopman et al., (1991) found that while fibronectin and collagen IV supported cellular outgrowth, laminin arrested migration of a human breast cell line. The authors suggested that laminin functioned as a stop signal for cell migration. Results from the present study support this hypothesis. While fibronectin and collagen both supported large amounts of outgrowth, ICM cultured on laminin did not support outgrowth.

Addition of plasminogen to the culture medium significantly reduced area of outgrowth. Although Varani et al. (1987) reported partial inhibition of attachment and outgrowth of human tumor cell lines cultured on fibronectin in the presence of plasminogen, because of wide spread implication of PA having a role in cell migration, these results were not expected. Reich and co-workers (1988) reported that malignant human and murine cell lines are able to invade through basement membrane via a protease cascade which results in the breakdown of collagenase. Using serine protease inhibitors and antibodies to PA, inhibition of different steps in the cascade reduced invasion of the tumor cells

through the basement membrane. Similar results were reported by Axelrod et al. (1989) when they observed that transformed NIH 3T3 cells expressing PA can rapidly invade a basement membrane. This invasion was suppresed with the addition of antibodies blocking the activity of human PA. Erikson and Isseroff (1989) reported neural crest cells isolated from quail neural tubes produced high levels of PA after 8 days in culture. When added to the culture medium, inhibitors of PA and plasmin significantly reduced neural crest cell motility in vitro supporting the hypothesis that proteases are important in cell migration. Interestingly, in contrast to these results, Sanders and Prasad (1989) found that PA was not involved in avian gastrulation, a period when epithelial cells ingress and invade underlying tissue after penetration through the basement membrane. One possible explanation for the inhibitory effects of plasminogen on outgrowth is the levels of protease generated in the cultures containing 150 μg/ml plasminogen may have been too high. This seems unlikely, however, because this concentration resembles plasminogen concentrations normally found in plasma (2 μM; Dano et al., 1985).

Further evidence against the involvement of the PA system in ICM outgrowth is the observation that addition of the PA inhibitors anti-uPA and PAI-2 had no effects on outgrowth. Previous reports from our laboratory show

rabbit anti-bovine uPA and anti-PAI-2 are effective inhibitors of the uPA produced by ovine embryos (Bartlett and Menino, 1991). Although PAI-2 binds with greater affinity to uPA, PAI-1 is also an effective inhibitor of this PA. Using PAI-1, Cajot et al., (1990) reported PAI-1 regulated ECM breakdown by fibrosarcoma cells. Addition of PAI-1 to culture medium inhibited matrix degradation whereas antibodies against PAI-1 increased it. Additional studies need to be performed to examine the effects of PAI-1 on outgrowth of isolated ovine ICM before ruling out possible inhibition of ICM outgrowth by inhibitors of PA. Despite the observation that ICM cultured on fibronectin produced more PA than ICM cultured on collagen or laminin, PA production was not correlated with outgrowth area or cell numbers in outgrowths. This suggests further that the mechanism of endodermal migration in sheep occurs independent of plasminogen activation. However, additional work is needed to determine why fibronectin supported greater PA production.

Two events that are important in early embryonic development are the migration of cells and remodelling of the extracellular matrix. Complex interactions occur between migrating cells and the matrix constituents they encounter during these processes. Collectively, our results suggest that the ECM, fibronectin, and to lesser

extent collagen, can support migration of endodermal cells from isolated ovine ICM. Although our data strongly suggest that this migration occurs independently of the PA system, further investigations using other proteolytic systems need to be conducted to elucidate the mechanism of endodermal migration in ovine embryos.

Figure III-1a. Trophectodermal vesicle containing trophectoderm and inner cell mass after mechanical microsurgery.

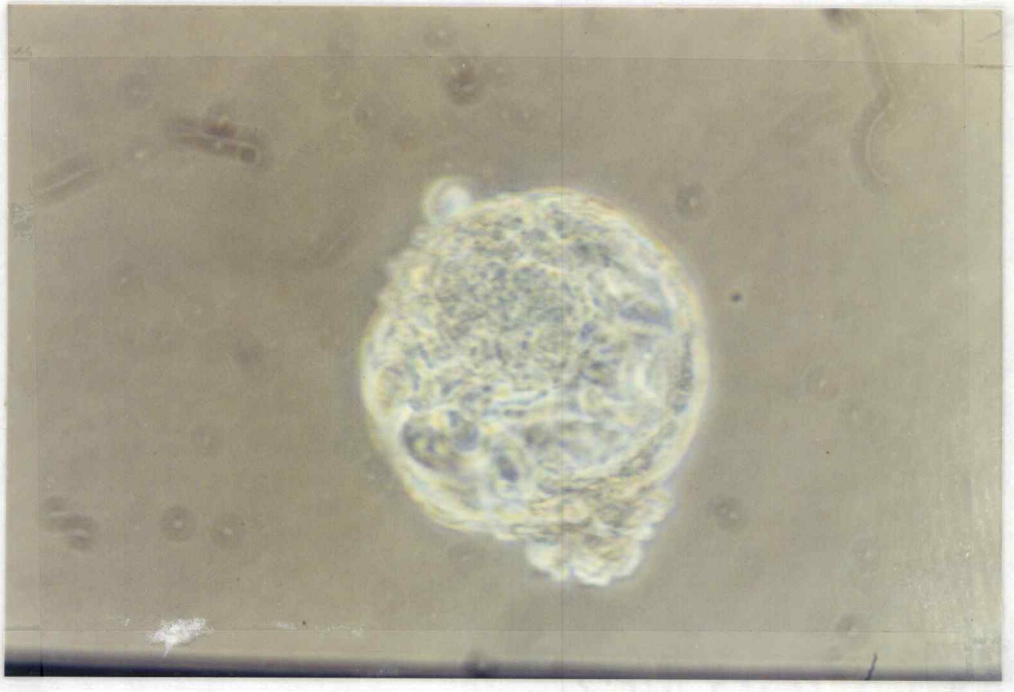

Figure III-1b. Trophectodermal vesicle containing trophectoderm only after mechanical microsurgery.

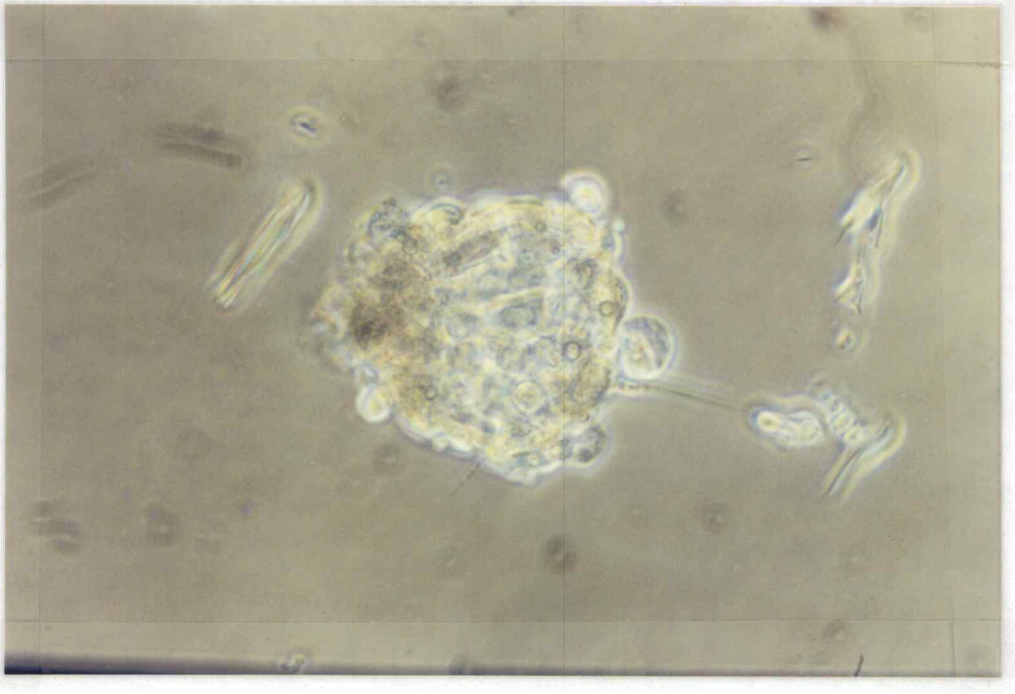

Figure III-2. Inner cell mass isolated immunosurgically.

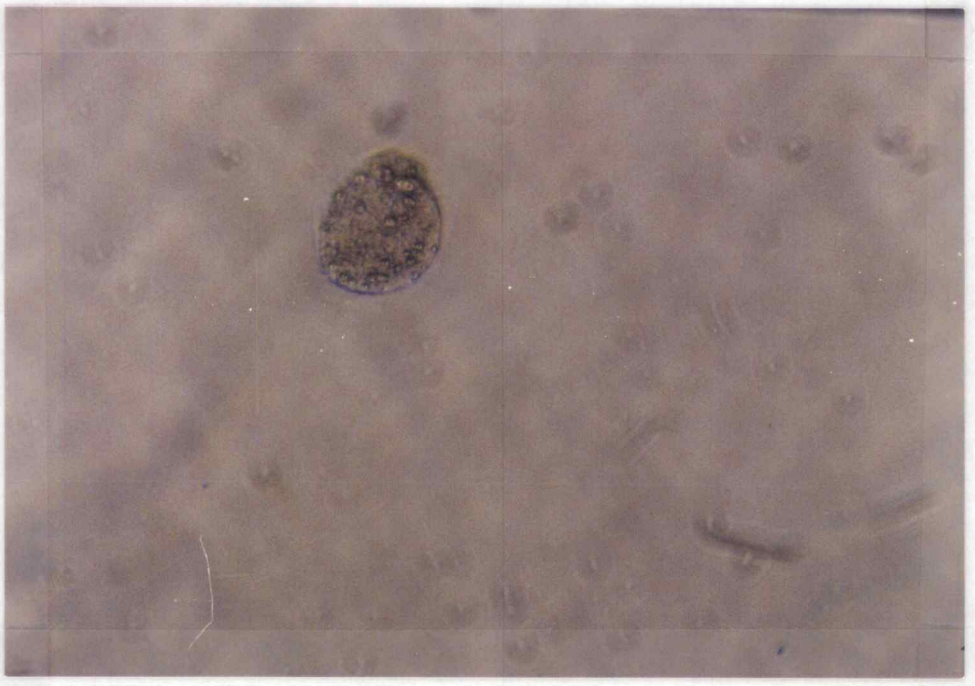

Figure III-3. Inner cell mass and accompanying outgrowth after 48 h on Fibronectin.

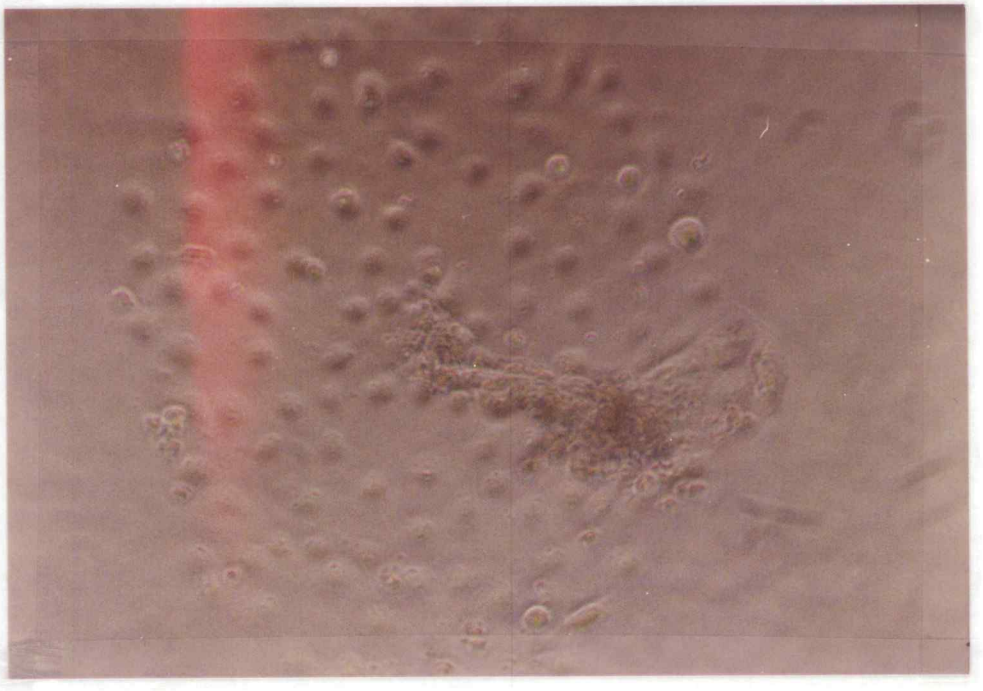

Figure III-4. Inhibition of ovine embryonic plasminogen activator (PA) by PA inhibitor-2 (PAI-2).

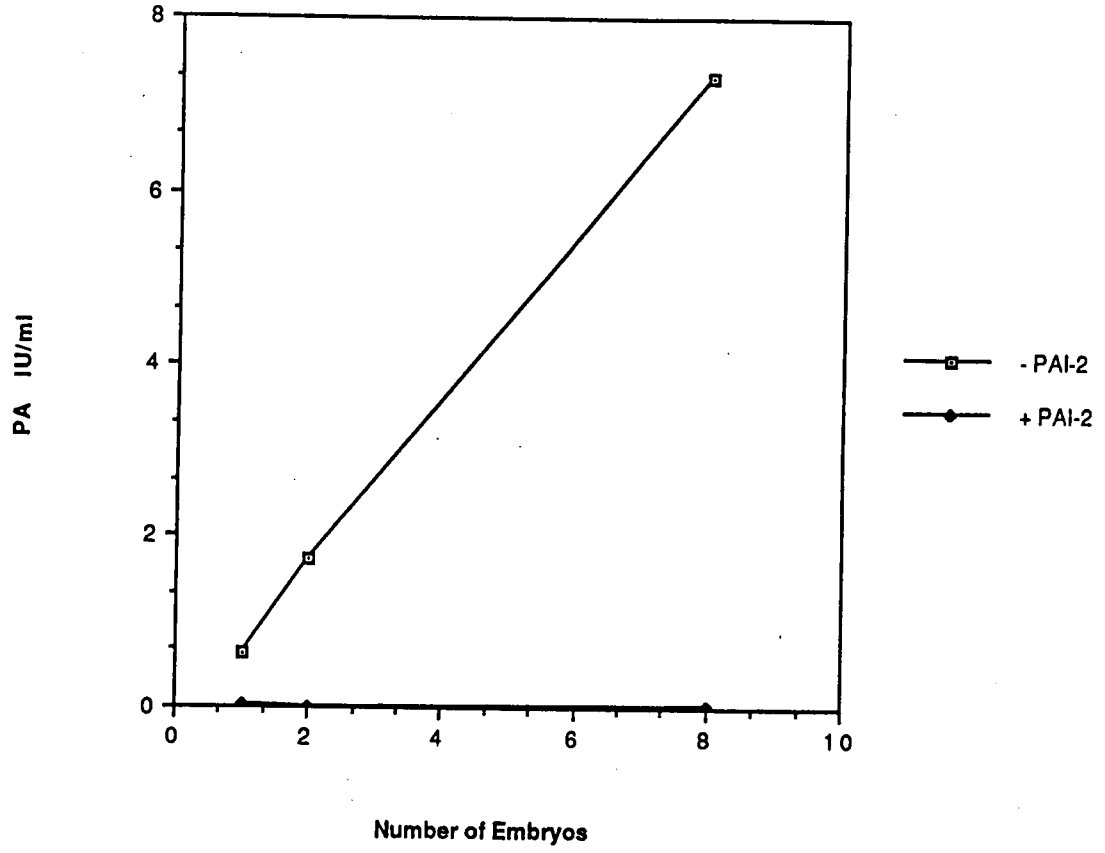

Table III-1. Overall means of ICM, outgrowth and total areas (μ^2) and numbers of migrating cells for ICM cultured in 0 or 150 μg/ml plasminogen.

Level of plasminogen μg/ml	ICM area	Outgrowth area	Total area	Cell#
0	5523.5[a]	16521.7[a]	21715.5[a]	21.9[a]
150	4408.4[b]	1732.5[b]	6066.8[b]	10.1[a]

a,b Values in the same column without common superscripts are different ($P<0.05$).

Table III-2. Overall means of ICM, outgrowth and total areas (μ^2), and numbers of migrating cells for ICM cultured on collagen, fibronectin or laminin.

Extracellular matrix	ICM area	Outgrowth area	Total area	Cell#
Collagen	5215.9[a,b]	1653.2[a]	6746.3[a]	11.1[a]
Fibronectin	5749.6[b]	28841.7[b]	34081.9[b]	28.8[b]
Laminin	4146.1[a]	11.9[a]	4111.4[a]	1.5[a]

a,b Values in the same column without common superscripts are different ($P<0.05$).

Table III-3. Overall means of ICM, outgrowth and total areas (μ^2) and numbers of migrating cells for ICM at 0, 24, 48 and 72 h of culture.

Time	ICM area	Outgrowth area	Total area	Cell#
0	5499.1^{a}	0.0^{a}	5499.1^{a}	-
24	5103.3^{a}	3169.7^{ab}	8120.7^{ab}	7.6^{a}
48	4690.2^{a}	17797.2^{abc}	22328.4^{bc}	21.0^{ab}
72	4157.3^{a}	26922.4^{c}	30499.6^{c}	23.0^{b}

a,b,c Values in the same colum without common superscripts are different ($P<0.05$).

Table III-4. Plasminogen activator activities (IU/ml) and plasmin concentrations (μg/ml) in conditioned medium from ovine inner cell masses cultured for 72 h on collagen, fibronectin or laminin.

Extracellular matrix	plasminogen activator		plasmin	
	n	mean + SE	n	mean + SE
Collagen	8	.653 ± 0.386[a]	7	1.25 ± 1.01[a]
Fibronectin	10	.133 ± 0.386	9	1.68 ± 0.89
Laminin	8	.442 ± 0.345	8	2.40 ± 0.95

Table III-5. Plasminogen activator activities (IU/ml) and plasmin concentrations (μg/ml) in conditioned medium from ovine trophectodermal vesicles cultured for 72h on collagen, fibronectin or laminin.

Extracellular matrix	plasminogen activator		plasmin	
	n	mean + SE	n	mean + SE
Collagen	7	0.152 ± 0.418[a]	7	1.83 ± 1.81[a]
Fibronectin	8	0.200 ± 0.391	9	5.13 ± 1.60
Laminin	9	0.924 ± 0.368	11	5.20 ± 1.45

Table III-6. Individual mean inner cell mass (ICM), outgrowth and total areas (μ^2) and cell numbers of ovine ICM cultured on fibronectin in medium containing 0 or 100 IU/ml plasminogen activator inhibitor-2 (PAI-2).

	PAI-2							
	0 IU/ml				100 IU/ml			
Area (x100)	0	24	48	72	0	24	48	72
Inner cell mass and outgrowth	50.2	188.4	637.7	205.2	47.0	299.8	511.9	353.2
Inner cell mass	50.2	51.9	58.3	40.9	47.0	70.6	48.7	95.0
Outgrowth	0.0	140.9	579.4	176.6	0.0	244.3	473.7	309.4
Cell number	-	11.0	21.3	19.0	-	37.9	38.7	25.1

Table III-7. Individual mean inner cell mass (ICM), outgrowth and total areas (μ^2) and cell numbers of ovine inner cell masses cultured on fibronectin in medium containing 10% rabbit antibovine urokinase-type plasminogen activator serum (anti-uPA) or 10% normal rabbit serum (NRS).

	10% NRS				10% anti-uPA			
Area (x100)	0	24	48	72	0	24	48	72
Inner cell mass and outgrowth	49.6	128.7	129.6	87.1	47.6	78.9	81.7	72.4
Inner cell mass	49.6	57.6	63.7	87.1	47.6	59.2	62.8	72.4
Outgrowth	0.0	80.7	65.9	0.0	0.0	19.7	18.9	0.0
Cell number	0.0	37.0	11.0	2.5	0.0	6.0	16.0	0.0

Table III-8. Mean plasminogen activator (PA) activities (IU/ml) in conditioned medium from ovine inner cell masses containing PA inhibitor-2 (PAI-2) or rabbit anti-bovine urokinase-type PA serum (anti-uPA).

Treatment	n	Mean	S.E.
PAI-2			
0 IU/ml	12	0.567[a]	0.253
100 IU/ml	14	0.069[b]	0.048
Anti-uPA			
10%	7	0.012[c]	0.002
Normal rabbit serum			
10%	6	0.070[d]	0.016

a,b Values without similar superscripts are different ($P<0.05$).
c,d Values without similar superscripts are different ($P<0.05$).

Table III-9. Mean plasminogen activator production ($IUx10^3$) by ovine inner cell masses cultured in microdrops on collagen, fibronectin or laminin.

Extracellular matrix	n	Time 24	48	72
Collagen	6	1.21	2.05	1.35
Fibronectin	6	11.09	16.45	22.92
Laminin	5	3.94	3.69	1.20

Table III-10. Mean cell numbers in outgrowths generated by ovine inner cell masses cultured in microdrops on collagen or fibronectin.

Extracellular matrix	n	Time 24	48	72
Collagen	6	7.7	42.0	94.8
Fibronectin	6	24.5	60.0	111.7

Table III-11. Mean plasminogen activator production (IU x 10^3) by ovine trophectodermal vesicles cultured in microdrops on collagen, fibronectin or laminin

Extracellular matrix	n	Time		
		24	48	72
Collagen	6	12.8	28.6	10.2
Fibronectin	6	13.2	18.3	17.4
Laminin	6	15.2	8.7	19.8

REFERENCES

Armant, D.R., H.A. Kaplan and W.J. Lennarz. 1986. Fibronectin and laminin promote in vitro attachment and outgrowth of mouse blastocysts. Dev. Biol. 116:519-523.

Axelrod, J.H., R. Reich and R. Miskin. 1989. Expression of human recombinant Plasminogen activators invasion and experimental metastasis of H-ras-transformed NIH 3T3 cells. Mol. Cell. Biol. 9:2133-2141.

Bartlett, S.E. and A.R. Menino, Jr. 1991. Ovine embryos produce an urokinase-type plasminogen activator in vitro. Abst.

Cajot, J.F., J. Bamat, G.E. Bergonzelli, E.K.O. Kruithof, R.L. Medcalf, J. Testuz and B. Sordat. 1990. Plasminogen-activator inhibitor type 1 is a potent natural inhibitor of extracellular matrix degradation by fibrosarcoma and colon carcinoma cells. Proc. Natl. Acad. Sci. 87:6939-6943.

Carnegie, J.A. and O. Cabaca. 1991. The influence of extracellular matrix components on the proliferation and migration of inner cell mass-derived parietal endodermal cells. Biol. Reprod. 45:572-580.

Christman, J.K., S.C. Silverstein and G. Asc. 1977. Plasminogen activators. In proteinases in mammalian cells and tissues, pp.91-149. Ed. A.J. Barret. Elsevier/North Holland Biomedical Press. Amsterdam.

Coopman, P.J., M.E. Bracke, J.C. Lissitzky, G.K. De Bryune, F.M. Van Roy, J.M. Foidart and M.M. Mareel. 1991. Influence of basement membrane molecules on directional migration of human breast cell lines in vitro. J. Cell Sc. 98:395-401.

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

Erickson, C.A. and R.R. Isseroff. 1989. Plasminogen activator activity is associated with neural crest cell motility in tissue culture. J. Exper. Zool. 251:123-133.

Fazleabas, A.T., R.D Geisert, F.W. Bazer and R.M. Roberts. 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. 29:225-238.

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

Kaaekuahiwi, M.A. and A.R. Menino Jr. 1990. Relationship between plasminogen activator production and bovine embryo development in vitro. J. Anim. Sci. 68:2009-2014.

Mcgaughey, R.W. and M.C. Chang. 1969. Meiosis of mouse eggs before and after sperm penetration. J. Exp. Zool. 1709:397-410.

Menino, A.R.,Jr., A.R. Dyk, C.S. Gardiner, M.A. Grobner, M.A. Kaaekuahiwi and J.S Williams. 1989. The effects of plasminogen on in vitro ovine embryo development. Biol. Reprod. 41:899-905.

Menino, A.R., Jr. and J.S. Williams. 1987. Activation of plasminogen by the early bovine embyro. Biol. Reprod. 36:1289-1295.

Mullins, D.G. and S.T. Rorlich. 1983. The role of proteinases in cellular invasiveness. Biochem. Biophys. Acta. 695:177-214.

Ossowski, L., D. Biegel and E. Reich. 1979. Mammary plasminogen activator: Correlation with involution, hormonal modulation and comparison between normal and neoplastic tissue. Cell 16:929-940.

Reich, R., E.W. Thompson, Y. Iwamota, G.R. Martin, J.R. Deason, G.C. Fuller and R. Miskin. 1988. Effects of plasminogen activator, serine proteinases, and collagenase IV on the invasion of basement membranes by metastatic cells. Cancer Res. 48:3307-3312.

Sanders, E.J. and S. Prasad. 1989. Evidence that plasminogen activator is not involved in basement membrane penetration at avian gastrulation. Exper. Cell Res. 185:394-398.

Sherman, M.I., S. Strickland and E. Reich. 1976. Differentiation of early mouse embyronic and teratocarcinoma cells in vitro: Plasminogen activator production. Cancer Res. 36:4208-4216.

Smith, K.K. and S. Strickland. 1981. Structural components and characteristics of Reichert's membrane, an extra-embryonic basement membrane. J. Biol. Chem. 9:4654-4661.

Solter, D. and B.B. Knowles. 1975. Immunosurgery of mouse blastocyst. Proc. Nat. Acad. Sci. 72:5099-5102.

Strickland, S., E. Reich and M.I. Sherman. 1976. Plasminogen activator in early embryogenisis: Enzyme production by trophoblast and parietal endoderm. Cell 9:231-240.

Varani, J., P.E. McKeever, S.E.G. Fligiel and R.G. Sitrin. 1987. Plasminogen activator production by human tumor cells: Effect on tumor cell-extracellular matrix interactions. Int. J. Cancer 40:772-777.

BIBLIOGRAPHY

Armant, D.R., H.A. Kaplan and W.J. Lennarz. 1986. Fibronectin and laminin promote in vitro attachment and outgrowth of mouse blastocysts. Dev. Biol. 116:519-523.

Axelrod, J.H., R. Reich and R. Miskin. 1989. Expression of human recombinant plasminogen activators enhances invasion and experimental metastasis of H-ras-transformed NIH 3T3 cells. Mol. Cell. Biol. 9:2133-2141.

Bartlett, S.E. and A.R. Menino, Jr. 1991. Ovine embryos produce an urokinase-type plasminogen activator in vitro. Biol. Reprod. 44 (Suppl. 1):154 (Abstract).

Beers, W.H. 1975. Follicular plasminogen and plasminogen activator and the effect of plasmin on ovarian follicle wall. Cell 6:379-872.

Berg, D.A. and A.R. Menino Jr. 1992. Bovine embryos produce a urokinase-type plasminogen activator. Mol. Reprod. Dev. 31:14-19.

Bode, V.C. and M.A. Dziadek. 1979. Plasminogen activator secretion during mouse embryogenesis. Dev. Biol. 73:272-289.

Boshier, D.P. 1969. An histological and histochemical examination of implantation and early placentome formation in sheep. J. Reprod. Fert. 19:51-61.

Cajot, J.R., J. Bamat, G.E. Bergonzelli, E.K.O. Kruithof, R.L. Medcalf, J. Testuz and B. Sordat. 1990. Plasminogen-activator inhibitor type 1 is a potent natural inhibitor of extracellular matrix degradation by fibrosarcoma and colon carcinoma cells. Proc. Natl. Acad. Sci. 87:6939-6943.

Carnegie, J.A. and O. Cabaca. 1991. The influence of extracellular matrix components on the proliferation and migration of inner cell mass-derived parietal endodermal cells. Biol. Reprod. 45:572-580.

Christman, J.K., S.C. Silverstein and G. Acs. 1977. Plasminogen activators. In: Barret AJ (ed). Proteinases in mammalian cells and tissues. Amsterdam: Elsevier/North-Holland Biomedical Press, pp.91-149.

Coates, A.A. and A.R. Menino, Jr. 1991. Identification of the plasminogen activator produced by bovine embryos in vitro. J. Anim. Sci. 69 (Suppl. 1): 407 (Abstract).

Coopman, P.J., M.E. Bracke, J.C. Lissitzky, G.K. De Bryune, F.M. Van Roy, J.M. Foidart and M.M. Mareel. 1991. Influence of basement membrane molecules on directional migration of human breast cell lines in vitro. J. Cell Sci. 98:395-401.

Crosby, I.M., F. Gandolfi and R.M. Moor. 1988. Control of protein synthesis during early cleavage of sheep embryos. J. Reprod. Fert. 82:769-75.

Dabitch, D. and M.S. Owens. 1978. Trypsin-like inhibitor activity in mouse uteri during early gestation and delayed implantation. Proc. Soc. Exp. Biol. and Med. 157:175-179.

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

Denker, H.W. 1976. Formation of the blastocyst: Determination of the trophoblast and embryonic knot. Current Topics in Pathology. 62:59-79.

Dyk, A.R and A.R. Menino Jr. 1990. Electrophoretic characterization of the plasminogen activator produced by bovine blastocysts. J. Reprod. Fert. 93:483-489.

Epsey L.L. 1974. Ovarian proteolytic enzymes and ovulation. Biol. Reprod. 10:216-235.

Erickson, C.A. and R.R. Isseroff. 1989. Plasminogen activator activity is associated with neural crest cell motility in tissue culture. J. Exper. Zool. 251:123-133.

Fazleabas, A.T., R.P. Geisert, F.W. Bazer and R.M. Roberts. 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. 29:225-238.

Geisert, R.D., R.H. Renegar, W.W. Thatcher, R.M. Roberts and F.W. Bazer. 1982. Establishment of pregnancy in the pig: I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. Biol. Reprod. 27:925-949.

Granelli-Piperno, A. and E. Reich. 1978. A study of proteases and protease-inhibitor complexes in biological fluids. J. Exp. Med. 148:223-234.

Hart, D.A. and A. Rehemtulla. 1988. Mini Review: Plasminogen activators and their inhibitors: Regulators of extracellular proteolysis. 90B:691-708.

Herbet, M.C. and C.F. Graham. 1974. Cell determination and biochemical differentiation of the early mammalian embryo. Current Topics in Developmental Biology. 8:151-178.

Kaaekuahiwi, M.A. and A.R. Menino Jr. 1990. Relationship between plasminogen activator production and bovine embryo development in vitro. J. Anim. Sc. 68:2009-2014.

Katz, J., W. Troll, M. Levy, K. Filkins, J. Russo and M. Levitz. 1976. Estrogen-dependent trypsin-like activity in the rat uterus: Localization of activity in the 12,000g pellet and nucleas. Arch. Bioch. Bioph. 173:347-354.

Kruithof, E.K.O. 1988. Plasminogen activator inhibitors: A review. Enzyme. 40:113-121.

Lacroix, M., F.E. Smith and I.B. Fritz. 1977. Secretion of plasminogen activator by Sertoli cell enriched cultures. Mol. Cell. Endoc. 9:227-236.

Lacroix, M., M. Parvinen and I.B. Fritz. 1981. Localization of testicular plasminogen activator in discrete portions (stages VII and VIII) of the seminiferous tubule. Biol. Reprod. 25:143-146.

Lazarowitz, S.G., A.R. Goldberg and P.W. Choppin. 1973. Proteolytic cleavage by plasmin of the HA polypeptide of influenza virus: Host cell activation of serum plasminogen. Virology. 56:172-180.

Leidholm P. and B. Astedt. 1975. Fibrinolytic activity of the rat ovum. Appearance during tubal passage and disappearance at implantation. Int. J. Fertil. 20:24-26.

Leprince, P., B. Rogister and G. Moonen. 1989. A colorimetric assay for the simultaneous measurement of plasminogen activators and plasminogen activator inhibitors in serum-free conditioned media from cultured cells. Anal. Bioch. 177:341-346.

Lijnen, H.R. and P. Collen . 1988. Mechanism of plasminogen activation by mammalian plasminogen activators. Enzyme. 40:90-96.

Little, S., N. Bang, C. Harms, C. Marks and L. Mattler. 1984. Functional properties of carbohydrate-depleted tissue plasminogen activator. Biochemistry. 23:6191-6195.

Lowenstein, H. and Ingild, A. 1976. A micromethod for determination of proteolytic enzymes in the pH range of 2.8 to 4.8. Analyt. Biochem. 71:204-208.

Marotti, K.R., D. Belin and S. Strickland. 1982. The production of distinct forms of plasminogen activator by mouse embryonic cell. Dev. Biol. 90:154-159.

Mcgaughey, R.W. and M.C. Chang. 1969. Meiosis of mouse eggs before and after sperm penetraiton. J. Exp. Zool. 1709:397-410.

Menino, A.R., Jr., A.R. Dyk, C.S. Gardiner, M.A. Grobner, M.A. Kaaekuahiwi and J.S. Williams. 1989. The effects of plasminogen on in vitro ovine embyro development. Biol. Reprod. 44:899-905.

Menino, A.R., Jr. and J.S. Williams. 1987. Activation of plasminogen by the early bovine embryo. Biol. Reprod. 36:1289-1295.

Mullins, D.E., F.W. Bazer and M.R. Roberts. 1980. Secretion of a progesterone-induced inhibitor of plasminogen activator by the porcine uterus. Cell. 20:865-872.

Ossowski, L., D. Biegel and E. Reich. 1979. Mammary plasminogen activator: Correlation with involution, hormonal modulation and comparison between normal and neoplastic tissue. Cell 16:929-940.

Ossowski, L. and E. Reich. 1983. Antibodies to plasminogen activator inhibit human tumor metastasis. Cell 35:611-619.

Reich, R., E.W. Thompson, Y. Iwamota, G.R. Martin, J.R. Deason, G.C. Fuller and R. Miskin. 1988. Effects of plasminogen activator, serine proteinases, and collagenase IV on the invasion of basement membranes by metastatic cells. Cancer Res. 48:3307-3312.

Rehemtulla, A., A. Arndt and D.A. Hart. 1990. Induction of plasminogen activator inhibitor type 2 expression during differentiation of human K562 cells towards a macrophage phenotype. Bioch. Cell Biol. 68:1377-1343.

Rehemtulla, A., R. Smith and D.A. Hart. 1987. Regulation of plasminogen activator and plasminogen activator-inhibitor production by tissue culture cells: Evidence for independent induction and regulation. Fibrinolysis. 1:109-116.

Remmert, L.F. and P.P Cohen. 1949. Partial purification and properties of a proteolytic enzyme of human serum. J. Biol. Chem. 181:431-448.

Roberts, R.M. and F.W. Bazer. 1988. The functions of uterine secretions. J. Reprod. Fert. 82:875-892.

Samuel, C.A. 1971. The development of pig trophoblast in ectopic sites. J. Reprod. Fert. 27:494-495.

Sanders, E.J. and S. Prasad. 1989. Evidence that plasminogen activator is not involved in basement membrane penetration at avian gastrulation. Exper. Cell Res. 185:394-398.

Sappino, A.P., J. Huarte, D. Belin and J.-D. Vassalli. 1989. Plasminogen activator in tissue remodeling and invasion: mRNA localization in mouse ovaries and implanting embryos. J. Cell. Biol. 109:2471-2479.

Segerson, E.C. 1981. Immunosuppressive effect of ovine uterine secretory protein upon lymphocytes in vitro. Biol. Reprod. 25:77-84.

Sherman, M.I., S. Strickland and E. Reich. 1976. Differentiation of early mouse embryonic and teratocarcinoma cells in vitro: plasminogen activator production. Cancer Res. 36:4208-4216.

Smith, K.K. and S. Strickland. 1981. Structural components and characteristics of Reichert's membrane, an extra-embryonic basement membrane. J. Biol. Chem. 9:4654-4661.

Solter, D. and B.B Knowles. 1975. Immunosurgery of mouse blastocyst. Proc. Nat. Acad. Sci. 72:5099-5102.

Steel, R.D.G. and J.H. Torrie. 1980. Principles and procedures of statistics. McGraw-Hill Book Company, New York.

Strickland, S., E. Reich and M.I. Sherman. 1976. Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm. Cell 9:231-240.

Varani, J., P.E. McKeever, S.E.G. Fligiel and R.G. Sitrin. 1987. Plasminogen activator production by human tumor cells: Effect on tumor cell-extracellular matrix interactions. Int. J. Cancer 40:772-777.

Vassalli, J.-D. and D. Belin. 1987. Amiloride selectively inhibits the urokinase-type plasminogen activator. FEBS Lett. 214:187-191.

Vassalli, J.-D., J.-M. Dayer, A. Wohlwend and D. Belin. 1984. Concomitant secretion of pro-urokinase and of a plasminogen activator-specific inhibitor by cultured human moncytes-macrophages. J. Exp. Med. 159:1653-1668.

Vassalli, J.-D., J. Hamilton and E. Reich. 1976. Macrophage plasminogen activator: Modulation of enzyme production by anti-inflammatory steriods, mitotic inhibitors, and cyclic nucleotides. Cell. 8:271-279.

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

Wiman B. and D. Collen. 1979. On the mechanism of the reaction between human α2-antiplasmin and plasmin. J. Biol. Chem. 254:9291-9297.

Wintenberger-Torres, S. and J.E. Flechon. 1974. Ultrastructural evolution of the trophoblast cells of the pre-implantation sheep blastocyst from day 8 to day 18. J. Anat. 118:143-153.