

AN ABSTRACT OF THE THESIS OF

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Title: Identification of the Plasminogen Activator Produced by  
Microdissected Day 12 to 14 Bovine Embryonic Tissues.

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The type of plasminogen activator (PA) secreted by bovine embryos was identified. Day 12 to 14 blastocysts were collected at slaughter from estrous-synchronized, superovulated and naturally mated crossbred beef cows. Embryos were either left as intact embryos (E) or microdissected into component embryonic discs (ED) and trophoblastic vesicles (TV). Intact embryos, ED and TV were pre-cultured for 2 d in  $\alpha$  Minimum Essential Medium (MEM) with 10% heat-treated fetal calf serum, washed in serum-free MEM $\alpha$  and cultured individually for 5 d in 50- $\mu$ l microdrops of MEM with 15 mg/ml bovine serum albumin. At 24-h intervals, E, ED and TV were observed for tissue morphology and transferred to fresh microdrops, and media were recovered and frozen at -20°C. At the end of culture, blastocoelic fluid (BF) and embryonic tissues were recovered and frozen at -20°C. Plasminogen activator concentrations in media, tissues and BF were determined using a caseinolytic assay. Antibodies to urokinase-type PA (anti-uPA) and tissue-type PA (anti-tPA), and the urokinase inhibitor, amiloride (AMR), were used to identify the type of PA produced by bovine embryonic

tissues. Intact embryos and TV released more PA ( $P < .05$ ) than ED, and tissues exhibiting expanded blastocoels released less PA ( $P < .05$ ) than tissues with collapsed blastocoels. Blastocoelic fluid from TV exhibited more PA ( $P < .05$ ) activity than from ED. Anti-uPA decreased PA activity ( $P < .05$ ) in media from TV, ED and E compared to controls with nonspecific immunoglobulins and anti-tPA. Amiloride completely eliminated PA activity ( $P < .05$ ) in tissues, media and BF from TV, ED and E. These results suggest that the PA secreted by the bovine embryo is the urokinase-type.

IDENTIFICATION OF THE PLASMINOGEN ACTIVATOR  
PRODUCED BY MICRODISSECTED DAY 12 TO 14  
BOVINE EMBRYONIC TISSUES

by

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### DEDICATION

To the most wonderful father in the world who believes all things are possible when you set your mind to it and make an effort. There have been many times in my life when I wanted to take the easy way out, but you have always been able to make me look over the horizon and tough things out. Thank you for 25 years of wisdom, patience, kindness, support, understanding, encouragement, wonderful memories, and most of all - love. I love you, Pops, and without you I would not be where I am today!

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BOVINE EMBRYONIC TISSUES

**Introduction**

Plasminogen activator (PA) is a serine protease that converts the zymogen, plasminogen, to the active enzyme plasmin (Dano et al., 1985). Plasminogen activators are found in a wide variety of tissues and participate in several proteolytic functions, including follicular disruption at ovulation (Beers et al., 1975), blastocyst invasion of the endometrium (Sherman et al., 1976; Strickland et al., 1976), involution of the rodent mammary gland (Ossowski et al., 1979), tissue remodelling and cell migration during embryogenesis (Bode and Dziadek, 1979; Fazleabas et al., 1983; Dano et al., 1985), and facilitation of embryonic hatching (Menino and Williams, 1987). Bovine and ovine embryos release PA during blastocoelic expansion and initiation and completion of hatching (Menino and Williams, 1987; Menino et al., 1989). Recently Kaaekuahiwi and Menino (In Press) reported positive correlations between PA production and cell number, developmental stage and embryonic size in the bovine suggesting a relationship between in vitro embryo viability and PA production. Bovine embryo transfer could become more efficient with the development of a rapid and dependable method to determine embryo viability prior to a transfer. However, before such a method can be realized using PA, the biochemistry of embryonic PA and its physiologic relevance to the embryo must first be determined. In an effort to answer some of the biochemical questions concerning bovine embryonic PA, the objective of this research was to

evaluate PA production by cultured embryonic tissues and identify the type of PA secreted.

## Review of the Literature

### Embryonic Development in the Bovine

In the bovine, estrus is defined as d 0 with fertilization being completed on d 1. The fertilized ovum or zygote is surrounded by the zona pellucida, which protects and aids in development of the embryo. Early preimplantation development is characterized by a series of rapid mitotic divisions known as cleavages. By d 5 to 6 the embryo becomes a morula, containing 16 to 32 cells, and is typified by a "mulberry-like" appearance. Between d 7 to 9, the morula is transformed into a blastocyst, which has a fluid-filled cavity known as a blastocoel and an inner cell mass (ICM) surrounded by a single layer of cells known as trophectoderm. The earliest apparent differentiation of cells into two distinct types occurs in the early blastocyst when the ICM and trophectoderm become distinguishable (Herbert and Graham, 1974; Denker, 1976; Massip et al., 1981; and Betteridge and Flechon, 1988). In the cow, endodermal cells spread from beneath the ICM on d 8 and line the trophectoderm to form the trophoblast by d 10. By this stage the embryo has lost its totipotency, that is the ability of each cell to form a viable embryo. Inner cell mass will eventually develop into the fetus, while the trophoblast will develop into the fetal membranes of the placenta. The blastocyst expands and then hatches from the zona pellucida between d 9 to 11. Elongation of the embryo begins between d 12 to 14. On approximately d 14 the ICM differentiates into the embryonic disc and mesodermal cells from the ICM begin to migrate between the trophectoderm and endoderm to form the trilaminar yolk sac.

### Biology of Trophoblastic Vesicles

Obvious morphological differences exist between bovine ICM and trophoctoderm: ICM is discoidal while trophoctodermal cells are flat and elongated with tight intercellular junctions which maintain the blastocoelic environment (Massip et al., 1981). Because dissected trophoblast readily reforms into vesicles, trophoblastic vesicles (TV) can be used as a model to study cell-to-cell interactions during embryonic development. Kuzan and Wright (1980) reported on the morphological features of TV produced from d 12 porcine embryos. Embryos were enzymatically dispersed using trypsin and TV or trophoblast monolayers formed after 24 h in culture. Trophoblast monolayers also produced TV after 3 to 4 d in culture. Morphology of the TV resembled that of in vivo blastocysts that lacked an ICM. Histologically, some TV were comprised of a trilaminar membrane resembling the chorioallantois which contained an outer trophoctodermal cell layer, a middle vascular mesenchyme and an inner epithelial cell layer. Other TV contained an area resembling the embryonic disc in addition to the trilaminar membrane. Heyman and Menezo (1987) observed that cultured d 14 bovine embryonic fragments of trophoblast formed vesicles composed of only two cell layers: trophoctoderm and endoderm.

Signals inhibiting luteolysis are thought to be produced during the elongation phase of the trophoblast in cattle. Trophoblastic vesicles made from microdissected d 14 cow embryos can prolong the lifespan of the corpus luteum (Heyman et al., 1984). Of the heifers which received TV in the uterine horn ipsilateral to the corpus luteum, 66 percent exhibited prolonged estrous cycles of 25 d or more, as compared to the control group which had a 21 d estrous cycle. These

results suggest that the antiluteolytic and/or luteotrophic substance(s) are of trophoblastic origin. Heyman et al. (1987b) have shown improved pregnancy rates when frozen bovine blastocysts were transferred with TV; presumably due to the TV contributing to improved luteal maintenance.

Numerous workers have had low success rates with the in vitro development of early bovine embryos (1- to 8-cell stages) to blastocysts, since development is usually arrested at the 8- to 12-cell stage or "block stage." Camous et al. (1984a,b), Heyman and Menezo (1987), and Heyman et al. (1987a) have demonstrated development beyond the block stage in early bovine embryos when co-cultured with TV. Trophoblastic vesicles are thought to promote development either by release of unknown compound(s) normally present in vivo, or by removing detrimental metabolites from the medium, thereby promoting the cleavage of early bovine embryos.

#### Biochemistry of Plasminogen Activator

Plasminogen activators are serine proteases which convert the proenzyme, plasminogen, to the active enzyme plasmin (Dano et al., 1985; Lijnen and Collen, 1987). Two types of PA have been indicated by differences in function, molecular weight and immunological reactivity. The two types of PA are independent gene products and are classified primarily according to molecular weight (MW): urokinase-type PA (uPA) and tissue-type (tPA). Molecular weights for uPA and tPA are 30 to 50 kilodaltons (kD) and 70 kD, respectively. The 50 kD MW uPA occurs in a one-polypeptide chain form as the inactive proenzyme, and a two-polypeptide chain form held together by a disulfide bond as the active enzyme. Dano et al. (1985) speculated that uPA is released as an inactive proenzyme and converted to the active enzyme by limited

proteolysis. The heavy chain or B-chain has a MW of 30 kD and contains the active site comprised of serine, histidine and aspartic acid residues, while the light chain or A-chain has a MW of 20 kD. Variation in MW of the A- and B-chains exists among the different species. There is also an enzymatically active form of uPA with a MW of 30 kD. The 30 kD form is formed by proteolytic conversion of the native 50 kD form, and contains an intact heavy chain and a light chain consisting of only the C-terminal 21 residues of the light chain of the 50 kD form.

As with uPA, tPA has a one-polypeptide proenzyme chain form and a two-polypeptide chain active enzyme form, held together by a disulfide bond. One-chain tPA appears to be the inactive proenzyme form that is catalyzed by plasmin to become the two-chain form, the active enzyme. Like uPA, the B-chain (30 kD) contains the active site comprised of serine, histidine and aspartic acid residues.

### Plasminogen Activator Inhibitors

The primary function of tPA appears to be a major regulator of plasma fibrinolysis, while the primary function of uPA appears to be related to cellular movements and remodelling (Hart and Rehemtulla, 1988). The generalization is supported by differences in components regulating the two types of PA. Certain cells express receptors for uPA that are specific for the 50 kD form and will bind either the single-chain (sc) or two-chain (tc) form. Thus, the receptor-PA complex can confer PA activity to the cell surface which may facilitate cell migration by allowing cell surface-mediated proteolysis (Vassalli et al., 1985). There are no known cell surface receptors for tPA. Tissue-type PA is activated by binding to fibrin and requires plasmin catalysis to convert sctPA to tctPA.



Plasminogen activators are involved in critical biological functions, such as plasma fibrinolysis and tissue remodeling; therefore, specific inhibitors of PA have evolved to regulate these enzymes (Hart and Rehemtulla, 1988). Kruithof (1988) has identified four proteins that react with tPA and/or uPA: PA inhibitor 1 (PAI-1), PA inhibitor 2 (PAI-2), protease nexin (PN), and PA inhibitor 3 (PAI-3).

In normal human plasma, PAI-1 (52 kD) is the primary inhibitor of both tPA and uPA. Plasminogen activator inhibitor 1 has been identified in a wide variety of sources: plasma, platelets, placenta, and conditioned media from human endothelial cells, rat hepatoma cells, fibrosarcoma cells and hepatocytes. Plasminogen activator inhibitor 1 interacts with the single-chain form of tPA and the two-chain forms of both tPA and uPA.

Human placenta contains PAI-2 where it is localized to the trophoblastic epithelium and found in pregnancy plasma. Plasminogen activator inhibitor 2 is also secreted by leukocytes, leukocyte-derived cell lines and fibrosarcoma cells. There are two forms of PAI-2, a 47 kD nonglycosylated form found intracellularly and a 60 kD glycosylated form found extracellularly. Both have similar PA inhibitor characteristics. They react with two-chain uPA, two-chain tPA and react weakly with single-chain tPA and plasmin.

Two-chain tPA, uPA, thrombin, trypsin, plasmin, and factor Xa are all inhibited by PN, a 47 kD glycoprotein found in fibroblasts and a variety of other cells. Plasminogen activator inhibitor 3 has a MW of 51 kD, reacts with two-chain uPA and was originally purified from urine.

### Plasminogen Activator in Embryos

Rat Embryos. Plasminogen activator was first found in the embryo by Liedholm and Astedt (1975) in the rat. Fibrinolytic activity was determined by placing d 1 to 5 embryos on fibrin slides, which were then cultured for 30 min at 23°C in a moist chamber, fixed with formaldehyde and stained with Giemsa solution and Harris' hematoxylin. Also, d 6 uteri were removed, flushed with saline and frozen in liquid nitrogen. Serial cryostat sections were used to identify the site of implantation which was then analyzed for fibrinolytic activity. Nonpregnant rat oviducts and endometrium were also examined for fibrinolytic activity, with some of the experiments using the PA inhibitor tranexamic acid (AMCA). Fibrinolytic activity appeared on d 1 to 4, decreased on d 5, and disappeared at implantation on d 6. The AMCA quenched the fibrinolytic activity indicating that activity was due to a PA. Other than the oviducts, the tissues had no activity. Liedholm and Astedt (1975) suggested that the fibrinolytic activity of the embryo prevented adhesion to fibrin deposits on the oviductal mucosa and the loss of activity in the uterus may be necessary for implantation. Liu and Hsueh (1987) evaluated PA activity in cumulus-oocyte complexes produced during the periovulatory period from gonadotropin treated rats. Female rats were superovulated with pregnant mare's serum gonadotropin (PMSG) and ovulation was induced with human chorionic gonadotropin (hCG). Cumulus-oocyte complexes were aspirated from large follicles (0.6 to 1.0 mm in diameter) and washed three times in McCoy's 5a medium. Some of the oocyte-cumulus complexes were denuded with hyaluronidase and movement through a fine pipette. Hormonal responsiveness of the oocyte-cumulus complexes was tested in the presence or absence of follicle stimulating

hormone (FSH) or hCG. The type of PA was identified by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) followed by a fibrin overlay, a technique known as zymography. Liu and Hsueh (1987) found only tPA, which increased with time, in rats prior to and after gonadotropin treatment. Increased tPA activity accompanied cumulus cell expansion and dispersion prior to ovulation. And, both FSH and hCG increased tPA activity in PMSG-treated rats. Liu and Hsueh (1987) concluded that gonadotropins stimulated the secretion of tPA from oocytes, which may be involved in cumulus cell expansion and dispersion during ovulation.

Mouse Embryos. Strickland et al. (1976) researched PA production by cultured mouse embryos during the early stages of development and differentiation utilizing a fibrin-agar overlay assay and a  $^{125}\text{I}$ -labeled fibrin assay. Mouse embryos displayed a biphasic pattern of PA secretion. In the first phase, enzyme production began on d 6, peaked between d 8 to 9, then declined and increased again beginning on d 11. Isolated trophoblast and ICM were then obtained to determine the cell type(s) responsible for PA secretion. Trophoblast was produced by two methods: (1) four- to eight-cell embryos were separated into one or two blastomeres and cultured, or (2) the ICM of blastocysts were selectively destroyed using 5-bromo-2'-deoxyuridine (BUdR). Secretion of PA began on d 7, increased until d 9 and then gradually decreased in the preparations of pure trophoblast. Pure ICM was obtained by an immunosurgical method using mouse antiserum and complement to selectively destroy the trophectoderm layer (Solter and Knowles, 1975). Isolated ICM cultures began secreting PA about d 9 and production increased thereafter. However, at the onset of PA production, the ICM

has differentiated into a variety of cell types. The immunosurgical technique was used on the ICM a second time to remove parietal and visceral endoderm, leaving only the inner core. The two fibrin assays clearly identified the endoderm as the source of PA. To determine the endodermal source of PA, d 10 embryos were dissected into parietal endoderm, yolk sac (visceral endoderm and mesoderm), amnion, and embryo proper for assay. The parietal endoderm was the only source identified to have substantial PA activity in this study. Strickland et al. (1976) concluded that the first rise in PA was due to trophoblast and the second rise was due to parietal endoderm. The first phase of PA production corresponds to implantation where there is maximum tissue disruption and the trophoblast cells invade the uterine wall, suggesting PA as a contributing factor. The second phase has been suggested to facilitate cell migration and metabolism of Reichert's membrane. This later phase coincides with the migration of parietal endoderm cells along the trophectoderm to form Reichert's membrane, which is subject to rapid remodelling as the yolk sac enlarges. Using the same two fibrinolytic assays, Sherman et al. (1976) compared the PA production of normal mouse embryos with F9 embryonal carcinoma cells. Embryos were collected, cultured and assayed to measure PA synthesis from equivalent gestational ages of d 5 to 14. Trophoblast and ICM were isolated by use of BUdR and immunosurgery, respectively, as described by Strickland et al. (1976) with similar results. In tumors and under ordinary culture conditions, embryonal carcinoma cells from teratocarcinoma cell line F9 exhibit no PA activity; however, the F9 embryonal carcinoma cells differentiated into cells that had morphological and histological properties of parietal endoderm and exhibited enzymatic activity when

cultured on bacteriological dishes. Sherman et al. (1976) concluded that PA is associated with cells that have the ability to migrate and degrade connective tissue, and that PA production, which is under hormonal control in normal cells, appears to be internally programmed in trophoblast cells. Bode and Dziadek (1979) analyzed embryonic tissues from d 7 to 10 microdissected mouse embryos for PA secretion using either a caseinolytic-agar assay or a fibrin-agar assay. Decidua containing the embryos were incubated for 2 h to facilitate tissue separation by microdissection. In the d 10 embryo, incubation in pancreatin/trypsin was used to ease separation of visceral yolk sac mesoderm from endoderm and EGTA in PBS was used to ease separation of trophoblast from parietal endoderm. In d 7 embryos, PA secretion was observed in parietal endoderm, extraembryonic ectoderm and ectoplacental cone tissues and by d 8, embryonic ectoderm, visceral endoderm and some embryonic mesoderm tissues began PA secretion. Visceral extraembryonic endoderm tissue exhibited PA secretion on d 9. All of the tissues tested maintained PA secretion through d 10. Bode and Dziadek (1979) found PA secretion to be more widespread in the embryo than Strickland et al. (1976); thereby limiting the use of PA as a tissue-specific biochemical marker, but consistent with the supposition of this protease playing a role in rapid embryonic growth and morphogenetic movement. Kubo and Spindle (1980) examined proteolytic activity in cultured d 4 mouse blastocysts. Soybean trypsin inhibitor was found to decrease embryonic attachment to the decidual cell monolayer, while low concentrations of trypsin enhanced attachment. Fibrin-agar overlay assays indicated no enzyme activity until after attachment, with enzyme activity increasing during growth of trophoblast cells. The potent PA

inhibitor nitrophenyl-p-guanidino benzoate completely inhibited trophoblastic enzyme activity. Based on these results, Kubo and Spindle (1980) concluded that during implantation, attachment is associated with trypsin-like protease activity and trophoblast outgrowth is associated with fibrinolytic activity. Sherman (1980) studied the correlation between PA secretion and embryonic development in d 1 to 5 mouse embryos using a fibrin-agar assay. Fibrinolytic activity was detected in all stages of preimplantation embryonic development and activity was found to be PA as activity decreased when plasminogen-depleted serum was used. Sherman (1980) also noted that: (1) nondeveloping, zona-enclosed embryos exhibited fibrinolytic activity; (2) empty zonae, or zona-bound degenerating embryos showed fibrinolytic activity; (3) no fibrinolytic activity occurred in embryos that had been treated with pronase to dissolve the zona pellucida; and (4) upon manual separation of zona and embryo, empty zona tested positive and embryos tested negative or slightly positive for fibrinolytic activity. Sherman (1980) concluded that the zona pellucida, not the embryo, was the source of PA activity. Furthermore, he suggested that PA production and secretion by trophoblast cells was internally programmed. Plasminogen activator may be involved with the movement of trophoblast cells between adjacent uterine epithelial cells, penetration of uterine basement membranes and digestion of debris caused by degenerating cells and/or migration into the endometrium. Marotti et al. (1982) characterized the PA secreted by cultured d 12 embryonic mouse tissues with the use of a  $^{125}\text{I}$ -fibrinolytic assay and zymography. Plasminogen activator activity was detected in parietal endoderm, visceral endoderm and extraembryonic mesoderm in serum-free media, but only in parietal endoderm tissue in

serum supplemented media. Two types of PA were implicated when activity was completely inhibited in visceral endoderm and extraembryonic mesoderm and partially inhibited in parietal endoderm when samples were exposed to acid-treated serum or mixed with serum and then acid-treated prior to  $^{125}\text{I}$ -fibrinolytic assay. Plasminogen activator activity was completely inhibited in all tissue types upon sample culture with actinomycin D or cycloheximide, suggesting tissue synthesis of PA. Upon zymographic analysis, the visceral endodermal enzyme migrated as a single form of 48 kD, the extraembryonic mesoderm activity appeared as a doublet at 48 kD and the parietal endoderm was identified to have two bands of activity: a major species at 79 kD and a minor one at 45 kD. Further analysis to distinguish PA types yielded the following results: (1) both forms from parietal endoderm were inhibited by anti-tPA antibodies (Ab) and no effect was observed on visceral endodermal or extraembryonic mesodermal enzymes; (2) the exact opposite occurred when tissues were analyzed with the addition of anti-uPA Ab. Treatment of F9 teratocarcinoma stem cells with retinoic acid and dibutyryl cAMP (RA-DBC-treated cells) yielded a differentiated cell type identical to parietal endoderm. The enzyme produced by RA-DBC-treated cells was only partially inhibited by acid-treated serum. Proteolytic activity was inhibited with anti-tPA Ab while remaining unaffected by anti-uPA Ab. Zymography revealed two molecular weight forms: a major form at 79 kD and a minor form at 48 kD. Marotti et al. (1982) concluded that at least two types of PA can be characterized in mouse embryos and used as tissue specific markers: tPA from parietal endoderm and uPA from visceral endoderm and extra embryonic mesoderm. With the use of antibodies and zymography, Huarte et al. (1985) analyzed PA production

by primary and secondary mouse oocytes and d 1 to 4 mouse embryos. Tissue-type PA (MW 72 kD) was present only in secondary oocytes that had undergone germinal vesicle breakdown (GVBD). When GVBD was prevented with either isobutyl-methyl-xanthine or dibutyryl-cAMP, proteolytic activity was undetected. Formation of secondary oocytes and tPA activity were halted with the addition of cycloheximide, but actinomycin D and  $\alpha$ -amanitin had no effect on enzymatic activity. These results imply that PA synthesis in the secondary oocyte comes from pre-existing mRNA. Cytochalasin B and colchicine, which prevent emission of the first polar body but do not effect meiotic maturation, and dexamethasone, retinoic acid and phorbol ester, which modulate PA activity in a variety of cell types, do not effect the pattern of PA production by oocytes. This indicates that PA is produced in conjunction with meiotic maturation after GVBD. Enzymatic activity decreased with age but was found in all embryos from d 1 to 4 of development. Upon removal of the zona pellucida with pronase, secondary oocytes retained PA activity while the zygote lost PA activity. Huarte et al. (1985) concluded that meiotic maturation of the primary oocyte into the secondary oocyte induces the translation of tPA from pre-formed mRNA. By the time fertilization occurs the enzyme has been released into the extracellular milieu where its presence may aid in fertilization. Menino and O'Claray (1986) compared the effects of media supplemented with various levels of plasmin, plasminogen, trypsin or pronase on the development of cultured two-cell murine embryos by measuring proteolytic activity with a caseinolytic-agar gel assay. Only in plasminogen supplemented medium was proteolytic activity observed to increase following exposure to embryos, validating embryonic production



of PA. Pronase and the high levels of trypsin were found to decrease blastocyst formation and subsequent embryonic growth and development, while high levels of plasmin and plasminogen were found to be beneficial. Menino and O'Claray (1986) attributed the enhanced embryonic development in media supplemented with plasmin and plasminogen to the provision of an additional trophic effect. Based on the findings of Huarte et al. (1985), Huarte et al. (1987) evaluated tPA mRNA activation due to GVBD in mouse oocytes utilizing Northern blot analysis. The addition of either actinomycin D or  $\alpha$ -amanitin had no effect on the increase in size of tPA mRNA which accompanies meiotic maturation. Cycloheximide, puromycin and pactamycin all prevented growth of the tPA mRNA, and the treatment of secondary oocyte RNA with RNase H in the presence of oligo (dT) stopped the growth of the poly(A) tail on tPA mRNA. Further analysis with RNase H showed the size increase at the 3' end of the molecule. Huarte et al. (1987) concluded that the process of GVBD activates the growth of the poly(A) tail at the 3' end of the dormant tPA mRNA. Strickland et al. (1988) evaluated the effects of microinjecting antisense RNA (asRNA) on tPA expression in mouse oocytes by Northern blot analysis. Antisense RNA developed against the 3' region of tPA mRNA was the most effective in causing proteolytic inhibition. The addition of asRNA at the 3' noncoding region was sufficient to prevent the polyadenylation, translational activation and destabilization of maternal tPA mRNA necessary for tPA production. Strickland et al. (1988) suggests that the events leading to production of oocyte tPA from dormant mRNA are under control of maternal mRNA. Sappino et al. (1989) observed the presence of uPA mRNA during the implantation phase of d 5 to 10 murine embryos with

hybridization studies on cryostat tissue sections using a  $^3\text{H}$ -labelled cRNA probe. All stages of embryos exhibited uPA mRNA demonstrating that in vivo synthesis of uPA by trophoblastic cells is concurrent with trophoblastic invasion of the uterus.

Pig Embryos. Mullins et al. (1980) detected PA production by cultured d 12 porcine blastocysts using the  $^{125}\text{I}$ -fibrin plate assay. Plasminogen activator activity was found to increase in a time-dependent manner during the 48 h culture period. Samples of uterine flushings showed fairly high activity at the beginning and end of the estrous cycle, but low activity during mid-cycle or the luteal phase. Uterine flushings from pregnant animals had peak PA activity from d 10 to 12, where it then decreased and remained low. Samples with high activity (d 3 to d 18) were mixed with low activity mid-cycle (d 15) samples, with the resulting mixtures having low PA activity; whereas a mixture of d 3 and d 18 samples had an additive effect on PA activity. These data suggest the presence of an inhibitor(s) in the mid-cycle flushings. Uterine flushings assayed from ovariectomized gilts treated with progesterone, estrogen, progesterone and estrogen, or corn oil verified the presence of a progesterone-induced inhibitor. To test for direct inhibition of PA activity, a flurometric assay with glutaryl-glycyl-L-arginine-4-methyl-coumarinyl-7-amide as the substrate was used to measure PA activity from both d 12 blastocysts and urokinase. Unlike the rodent, placentation in the pig is noninvasive, therefore, Mullins et al. (1980) concluded that in the pig uterus PA proteolysis is controlled by a progesterone-induced PA or plasmin inhibitor. Fazleabas et al. (1983) examined the relationship between release of PA and estrogen by blastocysts and the secretion of plasminogen and plasmin

inhibitor by uterine endometrium in the pregnant pig. Day 10 to 16 blastocysts were collected and assayed for PA production with the  $^{125}\text{I}$ -fibrin plate assay. Plasminogen activator was assayed in both washed and unwashed d 13 to 16 filamentous blastocysts. Low levels of PA were noted in both groups of d 13 blastocysts. Day 14 and 15 blastocysts had a marked increase in PA production after being washed, and by d 16, both groups of conceptuses released large amounts of PA. Release of PA by washed blastocysts at 6, 12, 24, 36 and 48 h of culture was in a linear time-dependent manner for about 24 h. During blastocyst expansion, d 10 to 11.5, PA production and DNA content remained fairly constant. In the early filamentous stages, d 12 and 13, PA activity was very low, however, a marked increase in PA release and in DNA content was observed in d 14 to 16 conceptuses. Affinity chromatography on L-lysyl sepharose followed by electrophoresis was used to detect the presence of plasminogen in uterine flushings. Plasminogen content was highest on d 12 with little or none present in d 14 and 16 flushings. Samples eluted from the affinity column were combined with urokinase and analyzed using the  $^{125}\text{I}$ -fibrin assay. Day 10 to 12 flushings had significant activity while d 14 and 16 showed very low levels, confirming the electrophoresis results. Analysis of d 10 to 16 uterine flushings for inhibitors showed a 7-fold increase in inhibitor activity between d 10 and 12 followed by a drop to approximately one-fifth the previous levels by d 14. Ouchterlony double immunodiffusion analysis and two-dimensional electrophoresis (NEPHGE) also gave strong evidence for the presence of an inhibitor(s) between d 11.5 and 13. Uterine flushings from cycling gilts treated with either estradiol valerate or corn oil on d 11 were recovered during the subsequent 72 h and analyzed for the presence of

the inhibitor(s). The estrogen treated females had a 6- to 10-fold higher amount of plasmin inhibitor, which mimicked the pattern found in the pregnant d 10 to 12 uterus. Fazleabas et al. (1983) suggested a biphasic pattern of PA production for the pig blastocyst. The first phase, d 10 to 12, was associated with intense tissue remodelling and the second phase, d 14 to 16, with tissue proliferation. Fazleabas et al. (1983) concluded that blastocyst estrogen triggered the release of endometrial plasmin inhibitor(s) which resulted in a protective adjustment of the uterine environment to accommodate the growing and potentially invasive conceptus.

Cow Embryos. Menino and Williams (1987) used a caseinolytic-agar gel assay to evaluate PA production in early bovine embryos. Adding plasminogen to the culture medium had no significant effect on in vitro embryo development, however, initiation and completion of hatching was accelerated with the higher levels of plasminogen. Plasminogen activator production by embryos was low or undetectable until blastocoelic expansion and initiation of hatching when PA production increased and plateaued upon hatching. Menino and Williams (1987) suggested that PA production by the bovine blastocyst may cause a sublysis of the zona pellucida and thereby facilitate hatching. In a similar study, Kjerulf et al. (1989) observed that plasmin added to the culture medium reduced the incidence of hatching, had little effect on cell number and enhanced zona pellucida solubility. Kjerulf et al. (1989) suggested that the added plasmin induced detrimental effects on embryo survivability which accounted for the reduced development despite the enhanced zona pellucida solubility. Dyk and Menino (1989) characterized the PA produced by bovine embryos using SDS-PAGE and

zymography. Day 12 to 14 blastocysts were collected at slaughter and cultured for 24 h. Blastocysts were found to secrete a tPA and an uPA. Embryos were then microdissected and cultured to determine the tissue source(s) of PA. Embryonic disc was void of PA while trophoblast and undissected blastocysts produced both uPA and tPA. Dyk and Menino (1989) suggested a potential role for PA in the tissue remodelling and trophoblastic elongation occurring during d 12 to 14 of bovine embryo development. Kaaekuahiwi and Menino (In Press) investigated the correlation between embryonic viability and PA production in the bovine. Late morulae to blastocysts were nonsurgically collected and cultured for microscopic analysis and PA determination. Total PA production was found to be positively correlated to changes in zona pellucida thickness. Hatched embryos produced more total PA than nonhatched embryos. Kaaekuahiwi and Menino (In Press) concluded that PA activity was representative of embryo viability and may be able to serve as an indicator of success in pregnancy establishment.

Sheep embryos. Menino et al. (1989) utilized a caseinolytic assay to determine PA production by ovine embryos and the effects of plasminogen on ovine embryo development. The addition of plasminogen to the medium decreased hatching time by increasing zona pellucida solubility. Levels of PA production increased during the morula-blastocyst transition and then plateaued throughout blastocoelic expansion and hatching. Based on these findings, Menino et al. (1989) postulated that PA produced by the ovine embryo increased zona pellucida solubility through activation of plasminogen and may participate in hatching.

## Materials and Methods

### Embryo Collection and Culture

Seven crossbred beef cows were estrous-synchronized with prostaglandin  $F_{2\alpha}^1$  ( $PGF_{2\alpha}$ ) and superovulated with porcine follicle-stimulating hormone<sup>2</sup> (pFSH). Two 25-mg injections of  $PGF_{2\alpha}$  were administered i.m. 12 d apart (d 0 = first  $PGF_{2\alpha}$  injection) to all cows. Cows received twice-daily injections of pFSH i.m. at dosages of 5, 4, 2 and 1 mg on d 10, 11, 12 and 13, respectively. Detection of estrus was started 12 h after the second  $PGF_{2\alpha}$  injection and at 12-h intervals thereafter. Cows observed in estrus were naturally mated, using one of two bulls at the onset of estrus and at 12-h intervals until the cow would no longer accept the bull. Cows were slaughtered 12 to 14 d after estrus and the reproductive tracts were recovered and flushed with Minimum Essential Medium Alpha<sup>3</sup> containing L-glutamine, ribonucleosides and deoxyribonucleosides (MEM $\alpha$ ). Uterine flushings were examined with a dissecting microscope and embryos were collected from the flushings by aspiration with Pasteur pipettes. Embryos were either left intact or microdissected into component embryonic disc and trophoblast using a microscalpel and microforceps while visualizing the embryo at 70x with a dissecting microscope. Embryos and embryonic tissues were cultured for 48 h in sterile culture flasks containing 5 ml MEM $\alpha$  with 10% heat inactivated fetal calf serum<sup>3</sup> (HTFCS) in a humidified atmosphere of 5%  $CO_2$  in air at 37°C. Embryonic discs with associated trophoblast (ED), trophoblastic vesicles (TV) and intact embryos (E) were washed twice in

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<sup>1</sup> Lutalyse, The Upjohn Co., Kalamazoo, MI.

<sup>2</sup> Schering Corp., Kenilworth, NJ.

<sup>3</sup> Sigma Chemical Co., St. Louis, MO.

5 ml MEM $\alpha$  and twice in .5 ml drops of MEM $\alpha$  with 1.5% bovine serum albumin<sup>3</sup> (MEM $\alpha$  + BSA) to remove the HTFCS. Intact embryos, TV and ED were cultured in 50- $\mu$ l microdrops of MEM $\alpha$  + BSA under paraffin oil at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air for 120 h. At 24-h intervals, E, TV and ED were observed for morphology and coded as: 1 = collapsed, 2 = partially collapsed, 3 = expanded, and transferred to fresh microdrops and media were recovered and frozen at -20°C until analyzed for PA and nonspecific proteases. Media from empty microdrops were collected to correct for PA activity due to culture conditions. At 120 h E, TV and ED exhibiting expanded blastocoels were transferred to fresh microdrops and the blastocoelic fluid (BF) was withdrawn with a finely drawn pipet. All E, TV and ED were washed three times in 5 ml of MEM $\alpha$  and tissues and BF were frozen at -20°C until analyzed for total protein, PA and nonspecific protease activity.

Prior to assaying for PA and total protein, tissues were thawed and pooled according to TV, ED or E and subjected to four rounds of freezing, thawing and vortexing to facilitate tissue solubilization. After solubilization, the volume was equalized by adding MEM $\alpha$  to all samples to a total volume of 500  $\mu$ l. Solubilized and diluted tissues were refrigerated (4°C) until PA and total protein analysis.

#### Plasminogen Activator Assay

Plasminogen Activator concentrations in media, tissue and BF were determined using the caseinolytic assay described by Menino and Williams (1987) with modifications. The casein-agar solution was prepared after the method described by Bjerrum et al. (1975) where 2% nonfat dry milk<sup>4</sup>

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<sup>4</sup> Carnation Co., Los Angeles, CA.

was dissolved in .038 M tris (hydroxymethyl)-aminomethane-.10 M glycine buffer containing .195 g/l  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and .650 g/l sodium azide, heated to 55°C and mixed with an equivalent volume of 2% agarose.<sup>3</sup> Fifteen milliliters of the casein-agar mixture were poured into 85x65x2 mm plates and ten 4 mm diameter wells were cut in each solidified casein-agar plate. Fifteen microliters of 120  $\mu\text{g/ml}$  human plasminogen<sup>3</sup> (hPgn) was combined with 15  $\mu\text{l}$  of recovered culture media, homogenized tissue, BF or 0, .0009, .0045, .0091, .0454 and .0909 milliunits/ml (mU/ml) urokinase<sup>3</sup> (E.C. 3.4.21.7) as the standard and incubated for 15 min at 37°C. After incubation, 25  $\mu\text{l}$  of the mixture were pipetted into the wells in the casein-agar gel plates and incubated at room temperature for 24 h. Plates were fixed with 3% acetic acid for 15 min and rinsed in tap water. A digital electronic caliper was used to measure the diameter of the lytic zones. Plasminogen activator concentrations in the culture media, tissue and BF were calculated from equation of the line calculations for ring diameter of the lytic zones by log urokinase concentrations. Plasminogen activator levels were calculated by subtracting the amount of PA in medium without any tissues from the amount of PA in BF or medium containing tissues for each 24-h interval.

#### Nonspecific Protease Assay

Nonspecific protease activity in TV, ED and E were evaluated using the caseinolytic assay described by Menino and Williams (1987), with plasmin (E.C. 3.4.21.7) as the standard. Briefly, 15  $\mu\text{l}$  of pooled TV, ED or E samples were pipetted into wells in 10 ml casein-agar gel plates and plates were incubated at room temperature for 64 h. Plates were fixed with 3% acetic acid for 15 min and rinsed with tap water. Protease activity in the samples were determined from equation of the



line calculations for linear regression with 2.5, 5, 10, 50, 100 and 500  $\mu\text{g/ml}$  plasmin as the standard.

#### Identification of the PA Type Using Specific Antibodies

Identification of the type of PA produced by bovine embryonic tissues was conducted using goat immunoglobulins (IgG) against human uPA<sup>5</sup> (anti-uPA) and tPA<sup>5</sup> (anti-tPA) and a nonspecific goat IgG<sup>3</sup>. Validation for the action of goat anti-uPA on urokinase was determined using the modified caseinolytic assay described by Menino and Williams (1987). Forty microliters of .0909 mU/ml urokinase were incubated for 90 min at room temperature with 20- $\mu\text{l}$  of 0, 40, 400 or 4000  $\mu\text{g/ml}$  anti-uPA in MEM $\alpha$  + BSA. Following incubation, 20  $\mu\text{l}$  of 240  $\mu\text{g/ml}$  hPgn was added and samples were incubated for 15 min at 37°C. Three 25- $\mu\text{l}$  aliquots from each sample were pipetted into wells in the casein-agar gel plates. Plates were incubated at room temperature and the resultant ring diameters were measured as described for the PA assay. To identify the optimum concentration of tPA to be used in the antibody experiments, 0, .05, .1, .5, 1 and 5  $\mu\text{g/ml}$  human tPA were analyzed using the caseinolytic-agar gel assay. The optimum concentration of goat anti-tPA was selected by combining 40  $\mu\text{l}$  of .5  $\mu\text{g/ml}$  tPA with 20  $\mu\text{l}$  of 0, 4, 40, 400 or 5000  $\mu\text{g/ml}$  anti-tPA and incubating for 90 min at room temperature. Following incubation, 20  $\mu\text{l}$  of 240  $\mu\text{g/ml}$  hPgn was added and the reaction mixture was incubated for 15 min at 37°C. Three 25- $\mu\text{l}$  aliquots were pipetted into the wells cut in the casein-agar plates and the resultant ring diameters were measured as described for the PA assay.

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<sup>5</sup> American Diagnostic, Inc., New York, NY.

the resultant ring diameters were measured as described for the PA assay.

Antibody cross-reactivity was determined by adding 20  $\mu\text{l}$  of either 5000  $\mu\text{g}/\text{ml}$  anti-tPA or nonspecific IgG to 40  $\mu\text{l}$  of .0909 mU/ml urokinase and 20  $\mu\text{l}$  of either 400  $\mu\text{g}/\text{ml}$  anti-uPA or IgG to 40  $\mu\text{l}$  of .5  $\mu\text{g}/\text{ml}$  tPA. Reaction mixtures were incubated for 90 min at room temperature before adding 20  $\mu\text{l}$  of 240  $\mu\text{g}/\text{ml}$  hPgn and assaying for PA.

Identification of the type of PA produced by bovine embryonic tissues was determined by incubating 40  $\mu\text{l}$  of pooled media recovered from E, TV or ED, or pooled BF with 20  $\mu\text{l}$  of 5000  $\mu\text{g}/\text{ml}$  anti-tPA, 400  $\mu\text{g}/\text{ml}$  anti-uPA or 5000  $\mu\text{g}/\text{ml}$  IgG for 90 min at room temperature, adding 20  $\mu\text{l}$  of 240  $\mu\text{g}/\text{ml}$  hPgn, incubating for 15 min at 37°C and assaying for PA. Assay conditions were similar to those described for determination of the optimum antibody concentration. For the standard curve, 40  $\mu\text{l}$  of urokinase at concentrations of 0, .0009, .0045, .0091, .0454 and .0909 mU/ml, were used in place of the pooled media under identical conditions.

#### Identification of the PA Type Using Amiloride

The optimum concentration of amiloride<sup>3</sup> (AMR), a competitive inhibitor of urokinase, was selected by combining 45  $\mu\text{l}$  of .0909 mU/ml urokinase or Ham's F-12<sup>3</sup> with 22.5  $\mu\text{l}$  of .002, .02, .2, 2, 20 or 100 mM AMR and incubating at room temperature for 90 min. Following incubation, 22.5  $\mu\text{l}$  of 240  $\mu\text{g}/\text{ml}$  hPgn was added to the reaction mixture, incubated for 15 min at 37°C and assayed for PA. The optimum AMR concentration was determined as 100 mM.

Amiloride cross-reactivity was evaluated by combining 45  $\mu\text{l}$  of 0, .05, .1, .5 and 1  $\mu\text{g}/\text{ml}$  tPA with 22.5  $\mu\text{l}$  of either 100 mM AMR or Ham's

F-12 for 90 min at room temperature. Following incubation, 22.5  $\mu$ l of 240  $\mu$ g/ml hPgn was added to the reaction mixture, incubated for 15 min at 37°C and assayed for PA.

To identify the type of PA produced by bovine embryonic tissues, 100 mM AMR or Ham's F-12 was incubated with pooled media recovered from TV, ED or E, BF and pooled tissues and analyzed for PA with urokinase as the standard.

#### Total Protein Assay

Total protein concentrations of embryonic tissue extracts were determined using the Bio-Rad Protein Assay.<sup>6</sup> Three aliquots of each tissue extract were assayed for total protein with 0, 200, 500, 800 and 1100  $\mu$ g/ml BSA as the standard. Optical density of the samples was measured at 595 nm using a Bausch and Lomb spectrophotometer and total protein concentrations were determined from equation of the line calculations for the standard curve.

#### Statistical Analysis

Differences in plasminogen activator levels due to embryonic tissue source, duration in culture and embryonic tissue morphology were evaluated using multi-way analysis of variance (ANOVA). Differences in PA concentration in BF and per  $\mu$ g of total protein due to tissue type were determined using one-way ANOVA and Fisher's least significant difference (LSD). Correlation-regression analysis was used to examine the relationship between BF PA and embryonic tissue morphology. Differences in PA concentration after incubation with anti-uPA, anti-tPA and nonspecific IgG were evaluated by one-way ANOVA and Fisher's LSD.

<sup>6</sup> Bio-Rad Laboratories, Richmond, CA.

Differences in PA concentrations in medium, tissues and BF following incubation with AMR were determined using the pooled t-test.

## Results

Ova were collected from seven cows for a recovery rate of  $17.9 \pm 16.2$ . Embryos with normal morphology were recovered from four cows for an embryo recovery rate of  $24.0 \pm 11.7$ . Multi-way ANOVA using tissue type, time in culture and morphology as main effects revealed no effect in PA secretion due to time in culture ( $P > .05$ ). Tissue type and morphology exerted effects on PA secretion ( $P < .05$ ) and no interactions were observed. Differences in PA production were not observed between TV and E ( $P > .05$ ); however, PA production by ED was reduced ( $P < .05$ ; Table 1). Plasminogen activator concentration in the medium were greatest in tissues having a collapsed morphology and least in tissues with an expanded morphology ( $P < .05$ ). Caseinolytic activity was only observed when samples were combined with plasminogen indicating the absence of nonspecific proteases.

Plasminogen activator concentrations in BF (Table 2) were greater in TV when compared to ED ( $P < .05$ ); however, PA concentrations in BF from E were not different from either TV or ED ( $P > .05$ ). Plasminogen activator concentrations in BF were subject to considerable variation as indicated by the large EMS. Correlation coefficients for morphology code by blastocoelic PA content for TV, ED and E, were .03, .23 and .06, respectively ( $P > .05$ ). No difference was observed in tissue content of PA among the three tissue types ( $P > .05$ ; Table 2), although TV and E were more than two- to three-fold greater than ED.

Optimum concentrations of anti-uPA and anti-tPA which neutralized the respective PA, were 400 and 5000  $\mu\text{g/ml}$ , respectively (Figures 1 and 2). Anti-uPA and anti-tPA were found not to inhibit tPA and urokinase

activity, respectively. Nonspecific IgG had no effect on either tPA or urokinase activity.

Mean PA concentrations in pooled media collected from TV and E and pooled BF were lower ( $P < .05$ ) after incubation with anti-uPA than with either anti-tPA or nonspecific IgG (Table 3). Plasminogen activator levels did not differ significantly ( $P > .05$ ) between ED treated with anti-uPA and anti-tPA despite a two-fold difference. No difference in PA concentration was observed between tissues and BF treated with anti-tPA and nonspecific IgG ( $P > .05$ ).

Amiloride at a concentration of 100 mM was selected as the neutralizing dose for urokinase (Figure 3). In a cross-reactivity test with tPA, 100 mM AMR had no effect ( $P > .05$ ) on PA activity (Figure 4). As shown in Table 4, PA activity in tissue extracts, pooled media and BF was completely eliminated when incubated with amiloride ( $P < .05$ ).

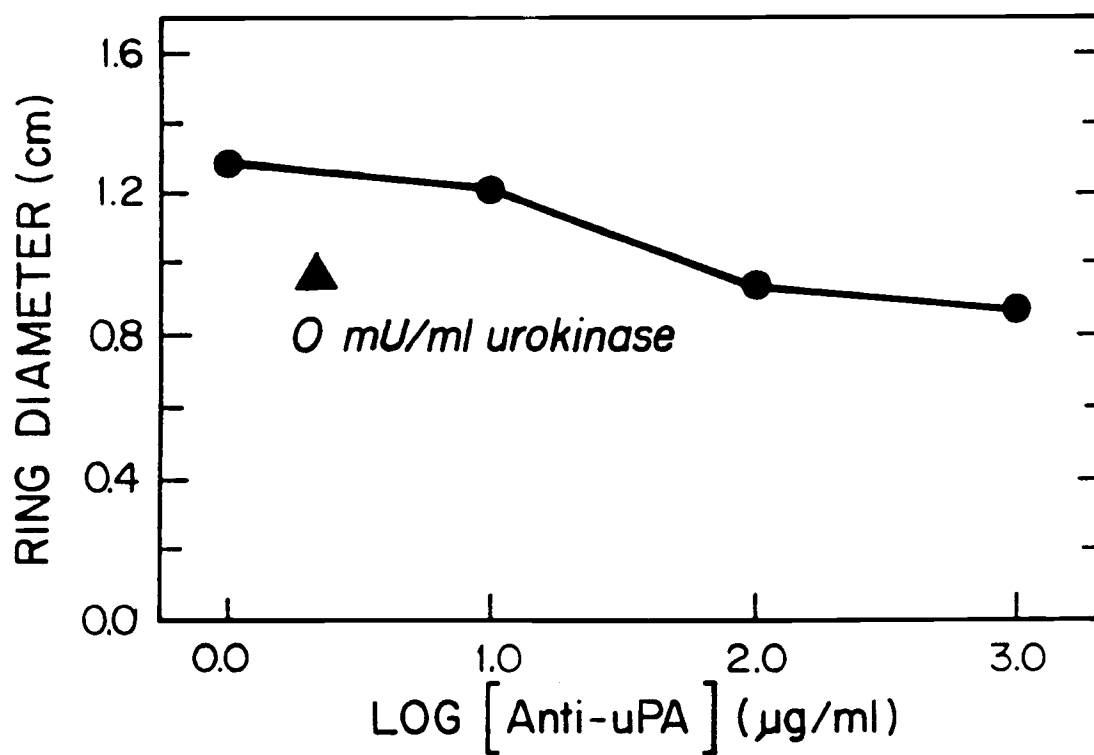


Figure 1. The effect of anti-uPA on urokinase-induced caseinolysis.

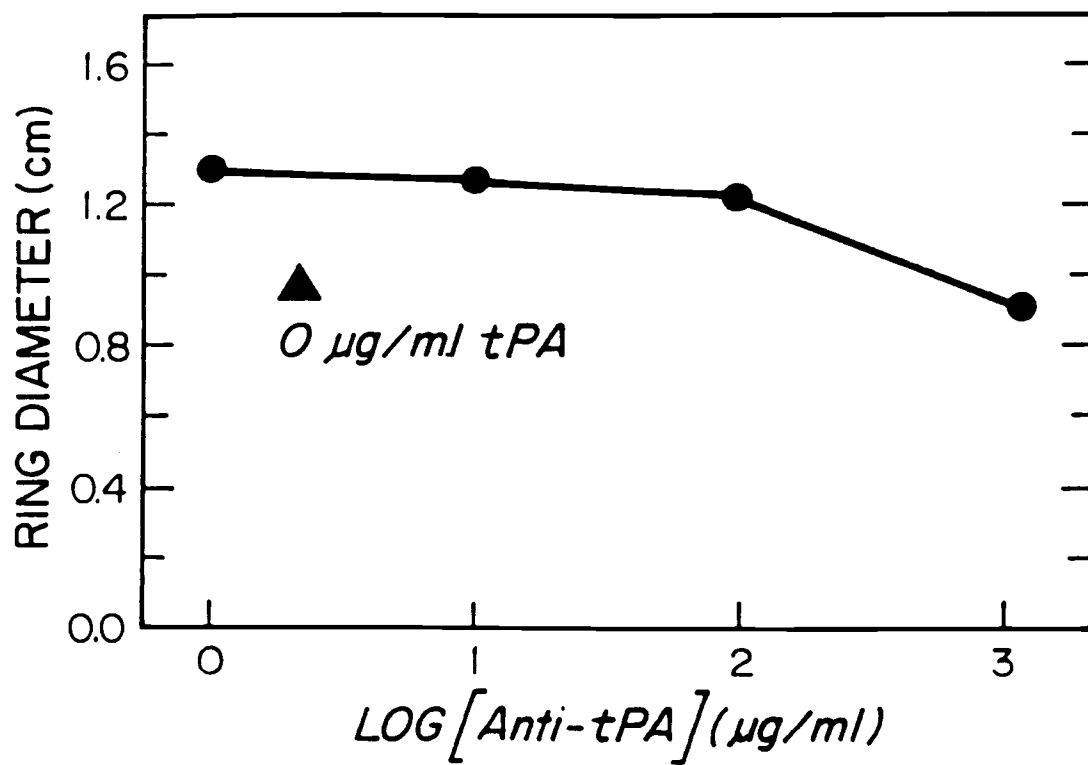


Figure 2. The effect of anti-tPA on tPA-induced caseinolysis.



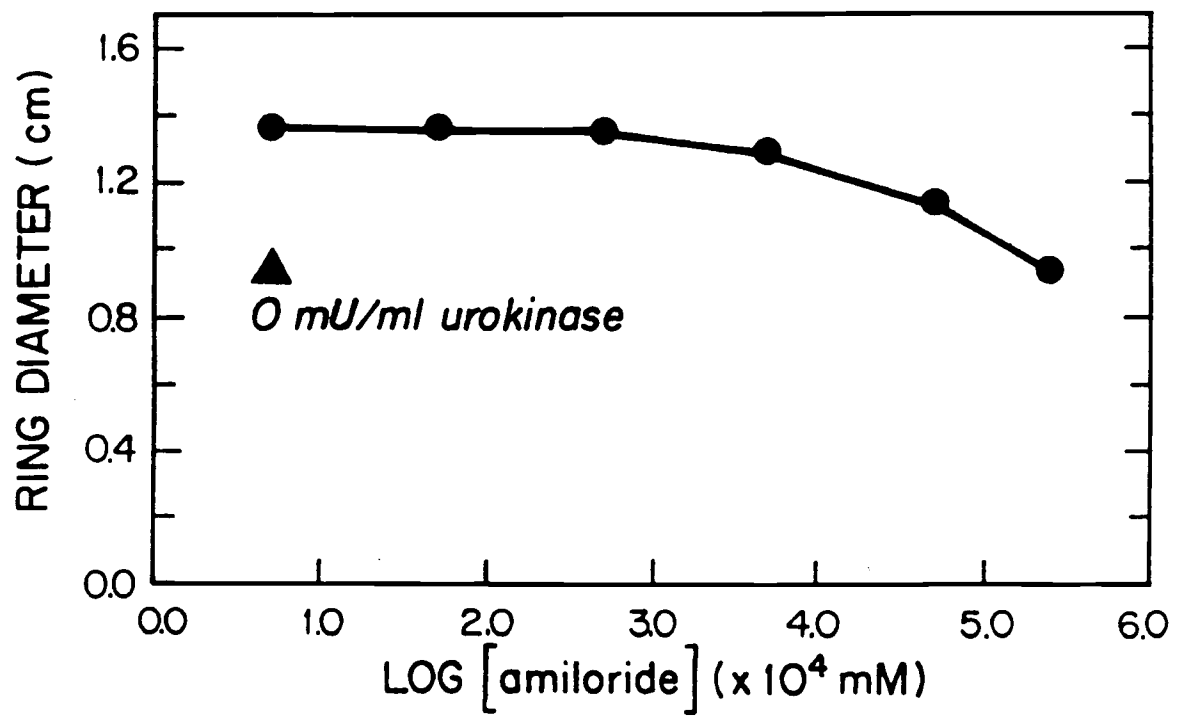


Figure 3. Reduction in caseinolysis by urokinase due to increasing concentrations of amiloride.

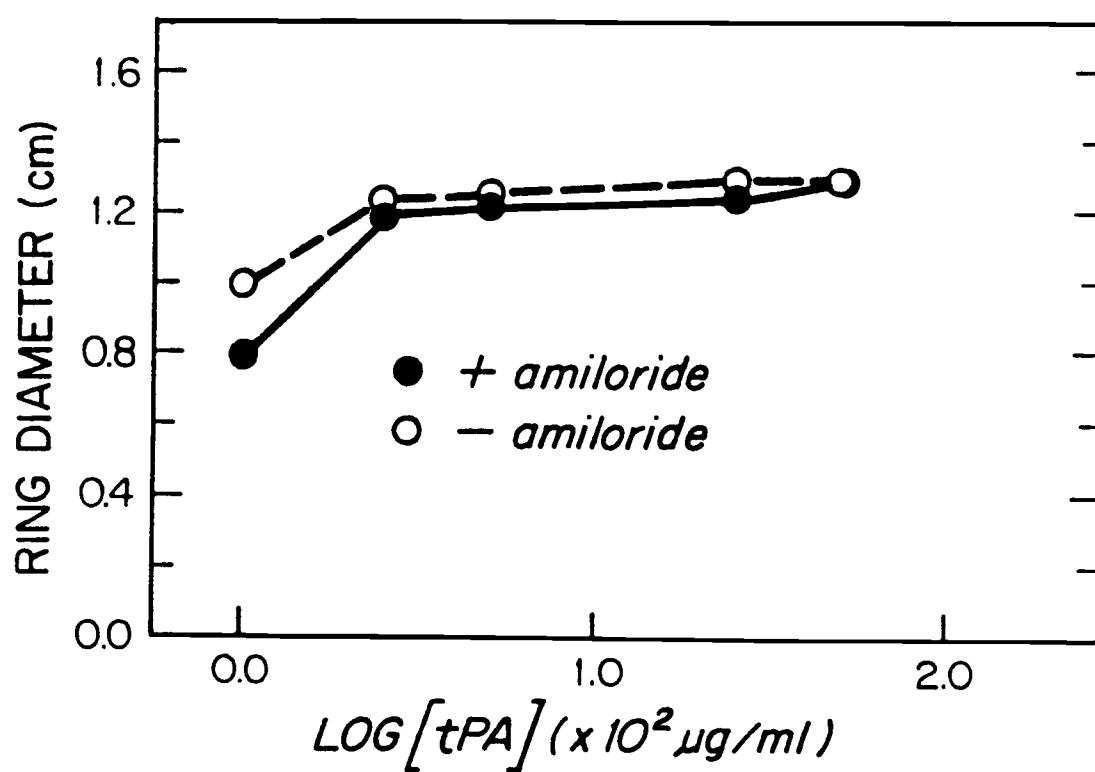


Figure 4. The effect of amiloride on the caseinolytic activity of tPA.

TABLE 1. PLASMINOGEN ACTIVATOR PRODUCTION ( $\text{mU}\cdot\text{ml}^{-1}\cdot 10^{-4}\cdot\text{d}^{-1}$ ) BY CULTURED DAY 12 TO 14 BOVINE EMBRYONIC TISSUES.

Tissue	Morphology at time of media recovery:							
	Collapsed		Partially collapsed		Expanded		Overall	
	n	Mean	n	Mean	n	Mean	n	Mean
Trophoblastic vesicle	25	217.0	43	84.1	128	46.1	196	76.3 <sup>b</sup>
Embryonic disc	9	28.1	16	40.6	64	16.9	89	22.3 <sup>a</sup>
Intact embryo	24	198.0	32	139.8	74	41.8	130	94.7 <sup>b</sup>
Overall	58	179.8 <sup>a</sup>	91	96.0 <sup>b</sup>	266	37.9 <sup>c</sup>	415	27797.6 <sup>d</sup>

a,b,c Overall means within a tissue-type or morphology without common superscripts are different ( $P < .05$ ).

d Error mean square.

TABLE 2. PLASMINOGEN ACTIVATOR CONTENT IN BLASTOCOELIC FLUID AND TISSUE EXTRACTS FROM CULTURED DAY 12 TO 14 BOVINE EMBRYONIC TISSUES.

Tissue	Blastocoelic fluid (mU·ml <sup>-1</sup> ·10 <sup>-4</sup> )		Tissue extract (mU·10 <sup>-4</sup> ·μg <sup>-1</sup> protein)	
	n <sup>a</sup>	Mean	n <sup>b</sup>	Mean
Trophoblastic vesicle	29	212.4 <sup>c</sup>	4	.085 <sup>c</sup>
Embryonic disc	10	5.5 <sup>d</sup>	2	.035 <sup>c</sup>
Intact embryo	16	132.4 <sup>c,d</sup>	4	.109 <sup>c</sup>
EMS <sup>e</sup>		76797.21		.003

<sup>a</sup> Number of individual BF samples.

<sup>b</sup> Number of cows from which samples were pooled.

<sup>c,d</sup> Means in the same column without common superscripts are different (P<.05).

<sup>e</sup> Error mean square.

TABLE 3. MEAN PLASMINOGEN ACTIVATOR CONCENTRATIONS ( $\text{mU}\cdot\text{ml}^{-1}\cdot 10^{-4}$ ) IN POOLED MEDIA AND BLASTOCOELIC FLUID RECOVERED FROM CULTURED DAY 12 TO 14 BOVINE EMBRYONIC TISSUES AND INCUBATED WITH ANTI-UROKINASE PLASMINOGEN ACTIVATOR (ANTI-uPA), ANTI-TISSUE PLASMINOGEN ACTIVATOR (ANTI-tPA) OR NON-SPECIFIC (CONTROL) IMMUNOGLOBULINS.

Source	Immunoglobulin treatment			
	Anti-uPA	Anti-tPA	Control	EMS <sup>c</sup>
Tissue				
Trophoblastic vesicle	9.0 <sup>a</sup>	27.8 <sup>b</sup>	22.9 <sup>b</sup>	312.4
Embryonic disc	3.7 <sup>a</sup>	9.1 <sup>a, b</sup>	15.1 <sup>b</sup>	91.0
Intact embryo	44.5 <sup>a</sup>	81.6 <sup>b</sup>	91.5 <sup>b</sup>	1460.1
Blastocoelic fluid	21.5 <sup>a</sup>	70.4 <sup>b</sup>	65.9 <sup>b</sup>	1746.0

a, b Means in the same row without common superscripts are different ( $P < .05$ ).

<sup>c</sup> Error mean square.

TABLE 4. MEAN PLASMINOGEN ACTIVATOR CONCENTRATIONS ( $\text{mU}\cdot\text{ml}^{-1}\cdot 10^{-4}$ ) IN TISSUE EXTRACTS, POOLED MEDIA AND BLASTOCOELIC FLUID RECOVERED FROM CULTURED DAY 12 TO 14 BOVINE EMBRYONIC TISSUES AND INCUBATED IN THE PRESENCE (+) OR ABSENCE (-) OF 25 mM AMILORIDE.

Source	Amiloride		SE
	+	-	
Tissue			
Trophoblastic vesicle	.0 <sup>a</sup>	7.9 <sup>b</sup>	.6
Embryonic disc	.0 <sup>a</sup>	4.0 <sup>b</sup>	.7
Intact embryo	.0 <sup>a</sup>	10.0 <sup>b</sup>	2.5
Medium			
Trophoblastic vesicle	.0 <sup>a</sup>	27.3 <sup>b</sup>	3.8
Embryonic disc	.0 <sup>a</sup>	7.4 <sup>b</sup>	1.4
Intact embryo	.0 <sup>a</sup>	127.4 <sup>b</sup>	22.7
Blastocoelic fluid	.0 <sup>a</sup>	21.6 <sup>b</sup>	2.1

a, b Means in the same row without common superscripts are different ( $P < .05$ ).

## Discussion

Plasminogen activator secretion by embryonic tissues was not affected by time in culture. This observation is in agreement with the findings of Menino and Williams (1987) and Kaaekuahiwi and Menino (In Press) who reported that PA production plateaus at an equivalent gestational age of d 12 to 14 in the bovine. Menino et al. (1989) also reported that PA production by sheep embryos plateaus once blastulation has occurred. Plasminogen activator production by microdissected tissues was found to be similar to results observed in mice (Strickland et al., 1976; Sherman et al., 1976) and in cattle (Dyk and Menino, 1989) where trophoblast secretes PA. Dyk and Menino (1989) found no PA secretion by ED in the cow, while the present study exhibited low amounts. As indicated in the materials and methods, the ED were not dissected completely free of trophoblast as in the study by Dyk and Menino (1989); consequently it is not surprising that PA was detected in ED in this study. Nevertheless, the concentration of PA associated with ED was considerably lower than the other tissues.

Day 12 to 14 bovine embryos possessed significant quantities of PA in the BF. Embryos of this gestational age possess an endodermal layer lining the blastocoel. Hence, these data suggest that bovine endoderm may be a source of PA similar to reports in the mouse (Bode and Dziadek, 1979; Marotti et al., 1982). Secretion of PA by endoderm into the blastocoel would explain the differences observed due to morphology where collapsed embryos or tissues were associated with higher PA concentrations in the medium. It is likely that blastocoelic PA was liberated into the culture medium when the embryo lost its ability to maintain a patent trophectoderm. Blastocoelic fluid and tissue levels

followed the trend reported for the medium where low levels of PA were observed with ED, presumably due to the remnant of trophoblastic tissue associated with the ED.

Anti-tPA and nonspecific IgG were found to have similar effects on PA activity, whereas treatment with anti-uPA resulted in a 50% reduction in PA activity. These data suggest either the anti-tPA which was raised against human tPA had no cross-reactivity with the bovine embryonic tPA or there was no tPA present. The anti-uPA decreased, but did not eliminate PA activity, suggesting either partial cross-reactivity with bovine embryonic uPA or the presence of tPA or some other protease. However, because no non-specific proteases were detected, the presence of such a component is unlikely. Lenich et al. (1989) found that the addition of AMR, a competitive inhibitor of urokinase, to samples containing plasminogen arrested plasminogen activation by urokinase. Addition of AMR to tissues, media and BF completely eliminated PA activity suggesting that d 12 to 14 bovine embryos produce only uPA. This is contrary to reports for mouse embryos where the trophoblast produces an uPA (Sappino et al., 1989) and parietal endoderm produces a tPA (Marotti et al., 1982). Bovine embryos can apparently secrete uPA into the medium and into the blastocoel. According to Betteridge and Flechon (1988), a basement membrane or extracellular matrix separates the trophectoderm and endoderm. It is conceivable that in bovine embryos, trophectodermal cells secrete PA into the uterine environment, whereas endoderm produces PA for facilitating migration through the extracellular matrix. Why the trophectodermal cells secrete PA is not known, however, this capacity may be an evolutionary remnant of species like the mouse that utilize the plasmin cascade for implantation



(Sherman et al., 1976; Strickland et al., 1976; Sappino et al., 1989).

The ability to identify embryos which will result in pregnancies would be advantageous to the embryo transfer industry. Maintenance of recipients receiving marginal embryos or embryos with limited success in pregnancy establishment is inefficient. An accurate and reliable assay for embryo viability, correlated to the ability to sustain a pregnancy, would improve the overall efficiency of embryo transfer. Several factors are involved in embryonic survival and can be classified as being embryonic, maternal or environmental in origin (Sreenan and Diskin, 1987). The donor exerts a major influence on embryonic survival because of donor-related differences in embryo quality (Sreenan and Diskin, 1987). According to King (1985) these donor-related differences in embryo quality may be due to chromosomal abnormalities and single gene mutations. Therefore, a means of identifying viable embryos and, hence, donors which produce embryos with high survivability would be beneficial. Kaaekuahiwi and Menino (In Press) have indicated that PA production is positively correlated to aspects of in vitro embryo survivability. Although we have characterized the type of PA produced by bovine embryos, further research is needed to determine the physiologic significance of this enzyme in embryo development and evaluate its potential as a means of assessing embryo viability.

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