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Title: A Role for Plasminogen in Rabbit Embryo Development

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Prior to implantation, mammalian embryos divest themselves of extraembryonic coverings to make intimate contact with the uterine epithelium. In the rabbit, although these coverings remain, they undergo significant modification and are eroded at the points of attachment. Because the uterus is a source of plasminogen (PGN), rabbit embryos may be able to utilize the plasmin (PL) system in restructuring the extraembryonic coverings and implantation. The objectives of this research were to identify plasminogen activator (PA) production by cultured rabbit embryos and determine the effects of PGN and PL on in vitro development.

In the first experiment, development of one to two-cell rabbit embryos in medium supplemented with various levels of PGN of rabbit or porcine origin were evaluated. More embryos developed to the expanded blastocyst, initiating hatching blastocyst and hatched blastocyst stage as PGN increased and development to the morula stage was

significantly accelerated. One- to two-cell embryos cultured for 96 h to the blastocyst stage had more cells when cultured in 60 or 120 ug/ml (88.8 $\pm$ 9.2 and 109.9 $\pm$ 10.9, respectively) compared to 0 or 30 ug/ml PGN (69.4 $\pm$ 14.6 and 73.3 $\pm$ 12.2, respectively).

Embryos cultured from the one- to two-cell stage in the level of PL equivalent to total available PL in 120 ug/ml PGN (45 ug/ml PL) developed similarly to embryos cultured in 120 ug/ml PGN. Embryos cultured in PL at a level equivalent to the PL contaminant in 120 ug/ml PGN (13 ug/ml PL) development similarly to embryos cultured in 0 ug/ml PGN.

Using a caseinolytic agar gel assay with human plasminogen as the substrate, protease activity was low in media recovered from embryos of d 4 and d 5 equivalent gestational age and increased markedly from d 6 through d 7. The protease activity was plasminogen dependent and amiloride-sensitive, suggesting that it was due to an urokinase-type PA.

These results suggest that PGN and PL enhance the development of one to two-cell rabbit embryos to later preimplantation stages. Rabbit embryos convert PGN to PL using an urokinase-type PA that is produced in high levels during the peri-implantation period.

A Role for Plasminogen in Rabbit Embryo Development

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Typed by Mark A. Grobner for Mark A. Grobner

Dedicated to
Robert and Pat Grobner

as near to perfect parents
as God could create

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Introduction

During early preimplantation development, the mammalian embryo is surrounded by an acellular glycoprotein matrix referred to as the zona pellucida. This covering, produced early in oogenesis, has several functions important to preimplantation embryo development such as providing sperm receptors, blocking polyspermy and maintaining embryo integrity during tubal transport. Before implantation, the embryo must divest itself of the zona pellucida in order to make direct contact with the uterine epithelium.

Shedding of the zona pellucida, or hatching, is postulated to occur by one of three mechanisms. The first mechanism is of a physical nature, attributed to blastocoelic expansion causing a rupture in the zona pellucida. The hydrostatic pressure in the blastocoel thins the zona pellucida and by sheer force produces a fracture through which the blastocyst escapes. The second mechanism involves the action of a zonalytic agent that dissolves the zona pellucida allowing the blastocyst to escape. This zonalytic agent or zona lysin could be a protease of either embryonic or uterine origin. The third model proposes a combination of the two mechanisms, that

is, a protease weakens the zona pellucida facilitating rupture by the expanding blastocyst.

In the rabbit, typical hatching, where the developing embryo is free of the zona pellucida for some time prior to implantation, does not occur. However, the extracellular coverings undergo extensive structural and biochemical changes during the preimplantation period. At no time during development is the rabbit embryo directly exposed to the uterine environment. The embryo is surrounded by not only the zona pellucida, but by several other extracellular coverings which remain, although modified, until implantation. At implantation, a unique point in the surrounding coverings is dissolved so that embryonic and uterine tissues can make direct contact.

Remodeling of the zona pellucida and extracellular coverings and uterine attachment are thought to be mediated by a protease of either uterine or embryonic origin. One possible protease involved in these events could be the serine protease plasmin (PL), formed by cleavage of the zymogen plasminogen (PGN) by plasminogen activator (PA).

Several reasons merit evaluating preimplantation rabbit embryo development and the role of PGN in both embryo development and hatching as it relates to implantation. First, it is of interest that rabbits never completely shed their extracellular coverings, but still implant. Unlike other mammals hatching is not a

prerequisite to implantation. The study of these events may provide insights into uterine-embryonic interactions. Secondly, rabbit embryos exhibit maximal expansion during in vivo preimplantation development; increasing in volume by several magnitudes prior to implantation. This expansion is not observed in vitro, possibly due to the lack of either a maternal signal at a critical stage or exposure to some uterine constituent. This signal or constituent may be a protease involved with the remodeling of the extraembryonic coverings and a prerequisite for expansion. Since rabbit embryos are maximally expanding, they serve as a model in which to study factors affecting blastocoel formation and expansion. Finally, the proposed model of PGN of uterine origin, being activated by PA, of embryonic origin, would provide a readily available source of protease for embryo induced proteolysis. This model would provide further insight into uterine-embryonic interactions during preimplantation development.

The objectives of these experiments were to evaluate the development of rabbit embryos in media supplemented with PGN and to determine if rabbit embryos produce PA.

Literature Review

During rabbit embryo preimplantation development, the extracellular embryonic coverings undergo considerable structural and biochemical changes (Denker and Gerdes, 1979). Zygotes are initially surrounded by a zona pellucida to which a mucoprotein layer (mucolemma) of tubal origin is added during oviductal transport (Boving, 1963). After entering into the uterus and development to the blastocyst stage, the zona pellucida becomes indiscernible, and a new layer, the neozona, is formed. The neozona immediately surrounds the embryo and is composed of material from both trophoblastic and maternal origins (Denker and Gerdes, 1979). By day 6 post-coitus (pc), a mucous layer of uterine origin, the goliolemma, is added to the neozona and mucolemma (Boving, 1963). These changes are thought to be important in the initiation of implantation (Denker, 1977; Denker and Hafez, 1975) and mediated by proteases of uterine or blastocyst origin (Kirchner, 1972; Denker, 1974; Denker, 1977; Denker and Hafez, 1975; Denker and Gerdes, 1979).

This review will be divided into two sections; the first will deal with early rabbit embryo development, emphasizing changes occurring in the extracellular coverings, and the second will review the PGN system and it's role in preimplantation development.

Rabbit preimplantation embryo development

Rabbits are induced ovulators, requiring mating to stimulate a neuro endocrine reflex arc that causes lutienizing hormone (LH) release thereby inducing ovulation. Levels of LH rise 30 min pc and peak 1-2 h pc (Scaramuzzi, et al., 1972). Ovulation follows at 9.75-13.5 h pc (Harper, 1961) at which time the ova are shed in a cumulus clot. The cumulus clot is quickly moved into the ampulla of the oviduct via the fimbria. Within 10 min of ovulation, the ova move along the ampulla to the ampullary-isthmic junction (Harper, 1965) where they remain for 48 h before entering the isthmic portion of the oviduct (Greenwald, 1961). Fertilization occurs at the ampullary-isthmic junction as characterized by sperm penetrating the ova within 1-2 h after ovulation (Chang and Adams, 1967). At this time, the zygote formed is surrounded by the zona pellucida. In rabbits, the zona pellucida appears not to act as a block to polyspermy; the vitelline membrane serves this function.

The first cleavage is completed 21-25 h pc (Table 1), approximately 12 h after fertilization, while the embryo is still in the ampullary-isthmic junction. By 48 h pc, the rabbit embryo has undergone further cleavages to the 32-cell or morula stage. The morula continues its journey thru the isthmus of the oviduct and arrives at the uterus

70-80 h pc.

During tubal transport, a mucin coat of considerable thickness, the mucolemma, is deposited around the developing embryo as well as any foreign bodies present in the rabbit oviduct (Denker and Gerdes, 1979). Greenwald (1958) found the secretion of the mucolemma to be positively influenced by exogenous progesterone while exogenous estrogen reduced mucin coat deposition. Mucolemma thickness increased with time in the oviduct from 11.9 u at 24 h pc to 33.2 u at 72 h pc (Alliston and Pardee, 1973). Hafez (1962) found that ova retained in the oviduct demonstrated an increase in mucolemma thickness over time. The mucolemma surrounding the morula and early blastocyst is characterized by concentric stratification, unique histochemical properties, and highly sulfated mucopolysaccharides, and unlike most epithelial mucins, is devoid of bound sialic acid (Denker and Gerdes, 1979). The zona pellucida at this time is rich in protein components, periodate-accessible vicinal hydroxyl groups and sulfate ester groups (Denker and Gerdes, 1979).

By 72 h pc the morula has traversed the isthmus and undergone compaction with the eventual appearance of an early blastocoel as the embryo enters the uterus (Lewis and Gregory, 1929). At compaction the visual identity of the individual cells (blastomeres) comprising the morula is lost. Prior to compaction, the blastomeres are in loose

contact and held together primarily by the zona pellucida.

Cavitation of the embryo begins at the late morula stage with early blastocysts entering the uterus at 70-80 h pc. Rabbit trophoblast is first visually differentiated at this time as a result of the swelling of the spherical mass serving to separate cells into those that will form trophoblast and those that will form inner cell mass (Daniel, 1964). Although rabbit morulae rarely exhibit specialized intercellular junctions, blastulation is marked by the appearance of tight junctions with fused membranes along the lateral surfaces of adjoining trophoblast cells (Van Blerkom et al., 1973). Prior to this time, intercellular contact is maintained through microvilli. Van Blerkom et al. (1973) found no significant difference in the development of junctional complexes in embryos grown in vivo to d 4 pc with those cultured in media supplemented with either serum or bovine serum albumin (BSA). They concluded that the oviduct does not supply any essential factors required for the developing embryo other than nutritional.

The formation of tight junctions produces a permeability seal at the outer surface of the embryo that becomes less leaky as the embryo advances in development (Benos and Biggers, 1981). The blastocoel develops due to the action of the Na^+/K^+ pump that transports ions across the membranes into the intercellular spaces between

compacted cells. Osmosis causes water to move to the interior of the morula, with hydrostatic pressures responsible for the expansion of the blastocyst. During blastocoel formation and expansion in the rabbit, there is an increase in Na^+ uptake and in the numbers of Na^+/K^+ pumps (Benos and Biggers, 1981). Expansion of the blastocyst involves both cellular hyperplasia and fluid accumulation of the blastocoel.

Formation of the blastocoel appears to not be dependent on cell number, number of DNA replicative cycles or cellular divisions (Prather and First, 1988). Although the exact mechanism responsible is not yet known, it has been hypothesized that the timing of blastocoel formation in the mouse is dependent on the nucleo-cytoplasmic ratio (Smith and Mclaren, 1977). Krishnan and Daniel (1967) claimed that the specific uterine protein "blastokinin" or "uteroglobin" induces, as well as regulates, blastocoel formation and expansion of rabbit embryos. However, Maurer, Onuma and Foote (1970) cultured 2- and 4-cell rabbit embryos to hatching blastocysts in the presence of BSA suggesting blastokinin was not necessary for blastocoel formation. The culture of 1-cell (Kane, 1972), 2- to 4-cell (Naglee et al., 1969) and morula-stage (Kane, 1975a, 1983a) embryos to expanded blastocysts has also been accomplished in media without uterine components but with BSA. Blastokinin may instead be involved in protecting the

embryo from possible deleterious effects of free progesterone because physiological levels of progesterone were found to retard embryonic development to the expanded and hatched blastocyst stages (Maurer and Beier, 1976). Blastokinin binds progesterone with high affinity (Beato, 1977) and low amounts of blastokinin allow blastocoel formation, but limit blastocyst expansion and prevent implantation (Beier, 1974).

Uterine development of rabbit embryos from blastocoel expansion to implantation is accompanied by the production and secretion of blastokinin. The earliest time that blastokinin can be detected in uterine secretions is 16 h pc or approximately 4 h after ovulation (Kirchner, 1972), although substantial levels are not detected until day 3 of pregnancy (Arthur and Daniel, 1972). Blastokinin peaks at d 5 pc and declines through d 10 pc. During preimplantation rabbit embryo development, blastokinin represents the predominant protein in rabbit uterine secretions (Beier, 1967, 1968; Krishnan and Daniel, 1967). Blastokinin has also been identified in blastocoel fluid from day 6 blastocysts in vivo (Beier, 1967; Kirchner, 1969; Hamana and Hafez, 1970), while no blastokinin could be detected in blastocysts cultured from 2- to 8-cell embryos (Beier and Maurer, 1975).

From their entrance into the uterus until approximately d 5.5, blastocysts usually reside in a

cluster at the ovarian end of the uterus. During this time, overall embryo diameter increases 5-fold due to fluid accumulation in the blastocoele and cell number increases 65-fold to 9000 cells (Table 1). Daniel (1964) found that by 4 d pc, the volume of a single trophoblast cell ceases to be reduced. Prior to this time, cleavage resulted in the halving of cell volume while doubling the number of cells with no significant effect on overall embryonic volume.

As embryos enter the uterus, the extraembryonic coverings begin structural and chemical transformations, initially characterized by thinning, due to expansion of the blastocyst (Denker and Gerdes, 1979). This thinning led Boving (1963) to believe the zona pellucida was lost at this time since it was no longer visible with the light microscope. According to Denker and Gerdes (1979), who used both electron microscopy and histochemical methods, by d 4.5, complete dissolution of the zona pellucida occurs, however the mucolemma persists. Erosion of the zona pellucida, morphologically, can be observed at d 3.5 pc where vicinal hydroxyl groups and protein are lost. Loss of the zona pellucida is followed by deposition of new material on the inner surface of the mucolemma, forming the neozona (Denker and Gerdes, 1979). This layer was not previously reported, probably due to confusion of the neozona with the mucolemma (Boving, 1957, 1963) or the zona

pellucida (Kirchner, 1973, 1975; Enders, 1971). Denker and Gerdes (1979) made the distinction through the use of a complete series of developmental stages characterized by electron microscopy and histochemistry. Neozona composition was found to be similar to that of the zona pellucida (Denker and Gerdes, 1979). The neozona is a protein rich, mucospolysaccharide with a high content of periodate-accessible vicinal hydroxyl groups, containing sulfate esters as well as bound sialic acid. Sialic acid residues impart a certain resistance to protease attack of the blastocyst coverings since their removal by neuraminidase facilitated trypsin proteolysis (Denker, 1970a).

The origin of the neozona is still in question, although trophoblast appears to contribute an essential component of it (Denker and Gerdes, 1979). At 4.5 d pc, granules appear between the mucolemma and the trophoblast coincident with the appearance of membrane-encased granules within the trophoblastic cytoplasm. Denker (1977) found that when proteases blocked implantation, deposition of material in the neozona continued after 7 d pc. Large secretory granule-like structures with flocculent contents were observed in the trophoblasts and large quantities of granular or flocculent material were between the neozona and trophoblast. Uterine participation in neozona formation has not been clearly defined. The finding by

Kirchner (1972) that uteroglobin diffuses into the blastocyst coverings allowed for the hypothesis that at least some of the material present in the neozona could be of uterine origin.

Blastocysts begin to be evenly distributed by peristaltic-like movements of the myometrium on day 5.5 pc (Boving, 1957, 1963). By d 6, the blastocyst has expanded to a diameter of 3 mm and is comprised of 80,000 cells. Cell number doubling time remains fairly constant at eight h through to d 6 pc (Daniel, 1964). Doubling time increases by 5 h each day through d 10 pc. The decrease in the doubling rate is attributed to some change in mitotic duration (Daniel, 1964), the formation of trophoblastic knobs on day 6 and the onset of cell death (Adams et al. 1961).

Trophoblastic knobs represent the fusion of individual trophoblast cells to form a syncytiotrophoblast consisting from a few to 20 cells (Enders and Schlafke, 1971). Trophoblastic knobs play a role in dissolution of the blastocyst coverings and establishment of the first cellular contact with the endometrium. Along with the formation of trophoblastic knobs, d 6 embryos exhibit the onset of primitive streak formation and the outgrowth of endoderm in a posterior direction with the elongation of the embryonic disc (Alliston and Pardee, 1973).

Blastocyst coverings early on d 6 show no

morphological changes except that the first traces of uterine material can be found on the outer surfaces of the mucolemma (Denker and Gerdes, 1979). By mid d 6, the neozona has increased in thickness representing from 40-50% the thickness of the blastocyst coverings. By this time the blastocyst has expanded to fill the lumen of the uterus allowing the position of the individual blastocyst to be detected from outside the uterus. This expansion causes stretching of the uterus including the myometrium, even before implantation begins.

From mid to late day 6, the deposition of material of uterine origin occurs on the outside of the mucolemma. Its periodic acid-Schiff positive material and protein content are relatively low. This layer of uterine origin (gloiolemma) is assumed to play a role in primary adhesion of the blastocyst to the uterine epithelium (Boving, 1957, 1959 and 1963). The thickness of the gloiolemma varies from one blastocyst to another and even in different areas within the same blastocyst. The thickness of the gloiolemma is similar to that of the mucolemma (Denker and Gerdes, 1979).

By d 7 pc the blastocyst has expanded to 6 mm and contains 250,000 cells. Blastocyst coverings are quite different from those found around morulae and consist of the three layers: neozona, mucolemma and gloiolemma. Embryonic disc has formed and mesoderm is beginning to grow

out at the posterior end of the disc (Alliston and Pardee, 1973). Gargus et al. (1989) reported that cells of the inner cell mass may have begun to differentiate by d 7 forming the embryonic disc. Their findings that discrete areas of cells in the blastocyst exhibited hyperexcitability and bound myosin-specific antibody suggest that clusters of precardiac cells were present.

Prior to implantation, the blastocyst orients itself within the uterine lumen with the embryonic disc mesometrially and the abembryonic pole antimesometrially. Implantation in the rabbit is invasive with the blastocyst penetrating the uterine mucosa (Perry, 1981). During implantation in the rabbit, two distinct processes occur; formation of the yolk sac placenta, or obplacenta, at the abembryonic-antimesometrial region followed by formation of the chorioallantoic placentae, at the embryonic-mesometrial pole (Denker, 1977). Both placentae result in hemochorial contact, with the yolk sac placenta persisting from d 7 through d 11 and the chorioallantoic placenta persisting from d 8 to term.

Blastocyst coverings begin to dissolve in the abembryonic-lateral region near the trophoblastic knobs and dissolution progresses between the knobs (Boving, 1963; Denker, 1970a, 1970b, 1974 and 1975). Dissolution of the extraembryonic coverings is observed as swollen remnants of the coverings and is almost completed abembryonically by d

7.5 (Denker, 1977).

Starting on d 8, trophoblastic cells adjacent to the embryonic disc penetrate the endometrial epithelium (Denker, 1977). The trophoblast is multilayered and transformed symplasmatically (cells have fused to form multinucleate structures) at the surface while uterine stromal cells opposed to the embryonic disc decidualize. Cellular contact between the embryo and uterus occurs with fusion of trophoblast and uterine epithelium of placental folds occurs (Larsen, 1961). Trophoblast cells grow deeper into the endometrium and the hemochorial placenta is formed.

During uterine development of the blastocyst, remodeling of the extraembryonic coverings and implantation, several enzymes have been detected in the uterus and in the coverings of rabbit blastocysts. Several glycosidases have been detected in the endometrium, trophoblast and uterine secretions including β-galactosidase, β-N-acetylglucosaminidase, β-glucuronidase and α-amylase, with some increasing in concentration at implantation Denker (1977). Denker (1977) speculated that the glycosidases function in a supportive or preparative role in the dissolution of the blastocyst coverings by removing sugars to allow for protease degradation of the glycoproteins.

Denker (1977) found that neuraminidase, although not

capable of lysing the blastocyst coverings alone, facilitated proteolysis by trypsin. Glycoproteins of the blastocyst coverings are rich in sialic acid which lend resistance to degradation by proteases. Although unable to localize neuraminidase at the site of lysis of the blastocyst coverings, Denker (1977) found the content of sialic acid at the abembryonic pole to be reduced at the time of implantation.

Exopeptidases have also been detected during preimplantation development of rabbit embryos and appear to play a supportive role in dissolution of the blastocyst coverings (Denker, 1977). Of interest are the aminopeptidases, amino acid arylamidases, particularly arylamidase I. Arylamidase I activity can first be detected at the time the blastocyst enters the uterus and its secretion is dependent on maternal progesterone (Denker, 1976; van Hoorn and Denker, 1975). The greatest activity can be found at d 5 pc with a sharp decline in activity occurring just prior to implantation (van Hoorn and Denker, 1975). It has also been shown that the blastocyst, through some unknown mechanism, stimulates the release of arylamidase I from the uterine epithelium. The role of arylamidases in preimplantation development is unknown although a decline in secretion at d 7 precludes any role in implantation (Denker, 1977). It may play a role in providing amino acids to developing blastocysts.

Endopeptidases such as papain and trypsin have been shown to be effective in the lysis of rabbit blastocyst coverings (Denker, 1977). Of physiological importance is the detection of elevated uterine proteolytic activity between 5 and 7 d pc, the time dissolution of the blastocyst coverings and implantation occurs (Kirchner, 1972a). Denker (1977) referred to the endopeptidase as blastolemmase based on its apparent role in the dissolution of the blastocyst coverings. He found blastolemmase to be virtually non-detectable in early blastocysts and sporadically detectable in d 5-6 blastocysts with a dramatic rise in protease activity between d 6.5 and 7. This activity has been localized to the blastocyst coverings and trophoblast (Kirchner, 1972a; Denker, 1974a; Denker, 1975). Denker (1977), using cryostat sections and a gel substrate test, found a latent high activity of blastolemmase in the trophoblast restricted to the abembryonic-antimesometrial region at d 7-7.5. At d 8, with the formation of the obplacenta largely complete, blastolemmase activity in this region declined. Blastocyst fluid remained free of protease activity at all stages (Denker, 1977).

Blastolemmase production is suggested to occur either by the trophoblast and localized on the surface (Denker, 1969, 1971c, 1972) or in the endometrium and extruded into the uterine secretions (Kirchner, 1972, 1975). Using

unfertilized ova, morulae and agarose beads, Denker (1975) and Denker and Hafez (1975) attempted to differentiate the origin of blastolemmase as either trophoblast or endometrium. When unfertilized ova, morulae or agarose beads were placed into the uterus of pregnant and pseudopregnant rabbits, no proteolysis was detected. Because blastolemmase activity was localized to the abembryonic pole of the blastocyst, Denker (1974) used the finding that blastocysts inversely oriented in the uterus retained proteolytic activity in the abembryonic pole as further evidence for trophoblast as the origin of blastolemmase. Denker (1977), again used agarose beads to simulate preimplantation embryos lacking a trophoblast, was unable to detect the presence of protease activity in beads removed from pregnant or pseudopregnant uteri. Blastolemmase is speculated to be either directly synthesized by the trophoblast or synthesized by the endometrium as a proenzyme and then activated by the trophoblast (Denker, 1977).

Denker (1977) characterized blastolemmase through the use of protease inhibitors and only gel films as substrates have proved useful in identifying blastolemmase activity. No activity towards synthetic trypsin and chymotrypsin substrates has been detected. Most important is the use of specific inhibitors, especially those with a high specificity for trypsin (Denker, 1977). Based on inhibitor

assays, blastolemmase is classified in the trypsin family.

Although the zona pellucida and other extraembryonic coverings are never completely shed during uterine development, rabbit embryos developing in vitro have been found to hatch. Kane (1975a) attributed this difference to the addition of the mucolemma during the in vivo development of the rabbit embryo. Using one-cell embryos collected lacking a mucolemma and morulae collected after the mucolemma has been added, Kane (1975b) found that by day 6, more embryos lacking the mucolemma had hatched. In vitro hatching is probably due to a rupture of the zona pellucida caused by blastocoel expansion and not to protease action (Kane, 1983b). Hatching is also associated with an increase in blastocyst cell number (Kane, 1983a). Kane (1987) speculated that in vivo, the blastocyst coverings are softened, allowing for expansion and further development of the blastocyst while in vitro, growth is limited unless hatching occurs.

Plasminogen Activators and Plasmin

Plasmin (PL) is a trypsin-like serine protease derived from the zymogen, plasminogen (PGN) through the action by another serine protease, plasminogen activator (PA). Historically, the PGN/PL system has been associated with blood clot lysis by the conversion of fibrinogen and fibrin

to a form which will no longer clot (Christman et al., 1977). In the last two decades, further roles for both PL and PA have been discovered.

Plasminogen activators are ubiquitous in their distribution, having been found in serum, vascular endothelium, urine and in almost all tissues (Christman et al., 1977). There are two immunologically distinct types of PA that also differ in molecular weights. Urokinase-type PA (uPA) are produced by the kidney and isolated from urine and have molecular weights of 30-55 kD whereas tissue-type PA (tPA) have a molecular weight of 72 kD (Dano et al., 1985; Degen et al., 1986). Tissue-type PA are localized in vessel walls (Larsson and Astedt, 1985) and appear to be important in intravascular fibrinolysis. Urokinase-type PA are thought to be more important in tissue remodeling and cell migration (Dano et al., 1985).

Plasmin is produced by a two step cleavage of the zymogen PGN with the resultant active enzyme being composed of two chains. One step involves the cleavage by PA of an arginine-valine peptide bond resulting in the formation of the two chains. The other step involves the release of an activation peptide from the amino terminus, which is catalyzed by PL leaving a form of PGN that is more readily converted to PL (Lijnen and Collen, 1988). Christman et al. (1977) has reviewed the controversy as to the sequence of proteolysis. Regardless of the actual sequence, the PL

resulting from activation consists of a heavy chain of approximately 60 kD, a light chain of approximately 25 kD containing the active site and an activation peptide of 7 kD. The two chains are held together by disulfide bridges.

Besides being active in clot lysis, PL has been given a role in several other tissues. During ovulation, PL participates in the breakdown of the follicle wall (Beers et al., 1975; Reich et al., 1985). High levels of PA are produced during the preovulatory period by granulosa cells in response to gonadotropin stimulation (Shimada et al., 1983; Canipari and Strickland, 1986; Knecht, 1986). Plasminogen activator has in itself been given a role in ovarian function and development including cumulus cell detachment and granulosa cell proliferation (Shimada et al., 1983; Espey et al., 1985; Liu et al., 1986).

Plasminogen activator production has also been detected in Sertoli cells of the testes which can produce either uPA or tPA (Lacroix et al., 1977; Marzowski et al., 1985; Hettle et al., 1986). Lacroix and coworkers (1977) postulated that PA facilitate the movement of germ cells through intercellular junctions and possibly the release of mature spermatids. Hettle and coworkers (1986) instead feel that PA are involved in the movement of cytoplasmic extensions of Sertoli cells between spermatocytes and the basal lamina. The uterus has also been found to produce PA (Kwaan and Albrechtsen, 1966; Harpel et al., 1966, 1967;

Mullins et al., 1980). Uterine PA production may be involved in the remodeling of the uterine epithelium during both estrus and implantation.

The first report of PA activity by embryonic tissue was by Liedholm and Astedt (1975) using rat embryos. They found that PA activity was highest in oviductal embryos and decreased to a level below detection once the embryos were near implantation in the uterus. This fibrinolytic activity was thought to be necessary to prevent adhesion in the oviduct, while implantation would require an eventual loss of activity. Plasminogen activator production was characterized in preimplantation mouse embryos by Strickland et al. (1976). They found a biphasic pattern of PA production by mouse blastocysts. The first phase began at d 6, peaked at d 8 and corresponded to the invasive phase of trophoblast cells in utero (Strickland et al., 1976; Sherman et al., 1976). Plasminogen activator production during this phase was associated with the trophoblast as demonstrated by the production of PA by inner cell mass-free trophoblastic outgrowths (Sherman et al., 1976). The second phase peaked at d 12.5, continuing through d 15 and was characterized by higher levels of enzyme production (Strickland et al., 1976). This phase of PA production was associated with parietal endoderm cells and began as these cells differentiated from the inner cell mass (Strickland et al., 1976). Enzyme activity associated

with parietal endoderm may facilitate migration of endoderm cells along the trophoblast during yolk sac enlargement (Sherman et al., 1976) and may be involved in the metabolism of Reichert's membrane that accompanies embryo growth (Strickland et al., 1976).

Bode and Dziadek (1979) demonstrated PA production not only by parietal endoderm, but also by visceral yolk sac endoderm and mesoderm and amnion of mouse embryos. In contrast to the results of Strickland et al. (1976), they suggest that culture of dissociated d 10 tissues may show different growth and migratory properties than intact embryos leading to the restricted detection of PA activity in trophoblast and parietal endoderm. However, Strickland (1980) pointed out that the experimental procedures employed by Bode and Dziadek (1979) cannot distinguish between enzyme synthesis by tissues and enzyme adherence to cells or extracellular matrix.

Sherman (1980) in further characterizing the relationship between PA production and mouse embryo development concluded that PA is unlikely to be involved in either hatching or acquisition of adhesiveness by the trophoblast. Embryos that failed to hatch were found to contain fibrinolytic activity and Sherman (1980) proposed that the secretion of PA is involved in trophoblast invasiveness. He also mentioned that PA may act directly upon intercellular proteins to aid in invasion of the

endometrium. The finding by Kubo and Spindle (1980) that trophoblastic attachment in cultured mouse blastocysts is dependent on trypsin-like activity and trophoblastic outgrowth is associated with fibrinolytic activity supports Sherman's proposal.

In evaluating the effects of proteases and PGN supplementation of media on mouse embryo development, Menino and O'Claray (1986) found significant enhancement of hatching, attachment and trophoblastic outgrowth in media containing PGN and PL. Proteolytic activities determined using a caseinolytic agar gel assay for embryos cultured in media containing various levels of PGN indirectly demonstrated the ability of mouse embryos to activate PGN to PL.

Using an electrophoretic technique in combination with a casein agar gel underlay (zymography), as described by Granelli-Piperno and Reich (1978), Marotti et al. (1982) were able to detect the production of both tPA and uPA by mouse embryonic tissues. Plasminogen activator produced by the parietal endoderm was found to be tPA while uPA was found to be produced by visceral endoderm and extraembryonic mesoderm (Marotti et al., 1982).

Plasminogen activator production has also been demonstrated in pig blastocysts (Mullins et al., 1980; Fazleabas et al., 1983). Cultured d 12 blastocysts were found to secrete large quantities of PA into the medium

over 48 h in a time dependent fashion (Mullins et al., 1980). Uterine PA levels corresponding to this time frame were found to be low and subsequent investigations demonstrated the presence of a protease inhibitor. This inhibitor was found to be effective against both PA produced by the d 12 embryo and urokinase, and its secretion was induced by progesterone. Mullins et al. (1980) suggested the inhibitor may be responsible for the non-invasive implantation characterized by pig blastocysts in the uterus and the invasive implantation demonstrated when transplanted to certain ectopic sites.

Fazleabas et al. (1983) found PA production by pig blastocysts to be biphasic, similar to PA production by mouse embryos. Cultured blastocysts released PA into the medium initially between d 10-12 during the time of elongation, and later between d 14-16, corresponding to the marked increase in DNA content. During these times, uterine flushings were found to contain PGN, the zymogen substrate for PA which peaked at d 12 (Fazleabas et al., 1983). The PGN content of the uterine flushings is presumably due as a serum transudate. Secretion of a PL inhibitor by the endometrium was discovered concurrent to the secretion of PA by the blastocysts (Fazleabas et al., 1983). This inhibitor can be released in nonpregnant gilts by the administration of estrogen and it is suggested that the production of estrogens by the elongating blastocyst

may trigger the release of the inhibitor (Fazleabas et al., 1983). This may serve to prevent the invasive implantation by the pig blastocyst and prevent damage to the endometrium by a proteolytic cascade of reactions initiated by the blastocyst.

Bovine embryos produce PA in a stage dependent fashion, with low levels being produced through the blastocyst stage and increasing at initiation of hatching (Menino and Williams, 1987). Levels of PA plateaued during the time that bovine embryos hatched in vitro. The conversion of PGN to PL in media supplemented with PGN followed a similar pattern (Menino and Williams, 1987). Although the numbers of bovine embryos developing to a particular stage were not different for the addition of PGN the time to initiation and completion of hatching were accelerated as PGN concentration increased. A similar pattern was exhibited for ovine embryos collected at the 16-cell to morula stage and cultured in the presence of PGN (Menino et al., 1989). More embryos hatched and development to the initiating hatching stage was accelerated in media with 120 µg/ml PGN compared to media lacking PGN. The production of PA was low for the first 48 h of culture, increased at 72-120 h and plateaued after a peak at 144 h (Menino et al., 1989).

TABLE 1. Timing of Embryonic Development in the Rabbit.

Embryo development stage	Time d (pc)	Time h (pc)	Approximate cell number	Overall embryo diameter (mm)
2-cell	1	21-25	2	.16
4-cell		25-32	4	.16
8-cell		32-40	8	.16
16-cell		40-47	16	.16
32-cell	2	48	32	.16
Blastocoel Formation	3-4	75-96	140	.20
	4	96	1112	.27
Blastocyst expansion	5	120	9000	1.0
	6	144	80000	2.9
Implantation	7	168	250000	6.0

Adapted from Daniel, 1964 and Schultz and Tucker, 1977.

Materials and Methods

Rabbit embryo collection

New Zealand White does were superovulated by one of two methods. The first consisted of subcutaneous injections of 0.5 mg porcine follicle stimulating hormone (pFSH; Sigma Chemical Co., St. Louis, MO) twice daily for 3 d. The second method consisted of a single subcutaneous injection of 150 IU of pregnant mares serum gonadotropin (PMSG; Calbiochem-Behring Corp., San Diego, CA). Ninety six hours from either the initial pFSH injection or single PMSG injection, the does were naturally mated and injected intraperitoneally with 100 IU of human chorionic gonadotropin (hCG; Sigma). Embryos were surgically collected from does 1, 3, 4 and 5 d after mating. For embryo collection, does were anesthetized by an i.m. injection of 60 mg Ketamine (Vetalar; Park-Davis, Morris Plains, NJ) and 10 mg Xylazine (Rompun; Haver, Shawnee, KS) per kg of body weight. A ventral midline laparotomy was performed and the reproductive tract was exteriorized. Collection of d 1 and d 3 embryos was accomplished by a retrograde flush of the oviduct through a catheter inserted in the infundibulum. Collection of d 4 and d 5 embryos was accomplished by inserting a catheter proximal to the cervix of each uterine horn and flushing by inserting a 26 gauge

needle proximal to the utero-tubual junction.

Each individual oviduct and uterine horn was flushed with 5 and 10 ml, respectively, of Ham's F-12 medium (HF-12; Sigma) buffered with 25 mM N-[2-hydroxyethyl] piperazine-N-'-[2-ethanesulfonic acid] (Hepes; Sigma). Flushings were examined using a dissecting microscope and embryos exhibiting normal morphology for their respective age were collected from the flushings by aspiration. The embryos collected were washed once in HF-12 buffered with 25 mM Hepes and supplemented with 15 mg/ml bovine serum albumin (BSA; Sigma) and placed into a test tube held at 37^{o}C in a waterbath until transported back to the laboratory for culture.

Embryo culture

Before culture, embryos were washed three times in microdrops of medium HF-12 supplemented with 15 mg/ml BSA. Embryos were cultured in 50 ul microdrops of HF-12 with 15 mg/ml BSA under paraffin oil (Fisher Scientific Co., Tustin, CA) at 37^{o} C in a humidified atmosphere of 5% CO_2 in air. The number of embryos per microdrop varied from 4 to 10 depending on the developmental age at collection. Media supplements to test the effects of PGN and PL on rabbit embryo development were made to the HF-12 with 15 mg/ml BSA.

Cultures lasted from 24 to 192 h during which embryos were morphologically evaluated at 24-h intervals at 100-200 X using an inverted stage phase contrast microscope and stage of development recorded. Where appropriate, after evaluation, embryos were transferred to fresh media and media were recovered and stored at -20^{o}C until assayed for proteolytic activity. Medium containing no embryos and incubated under identical conditions was collected to serve as a reference for estimating protease release by the embryos and to detect plasmin contamination in the plasminogen containing media.

Proteolytic activity determinations

Plasminogen activator levels were measured in culture media containing 0 µg/ml PGN using a caseinolytic agar gel assay (See appendix A) as described by Menino and Williams (1987) with skim milk as the substrate (Bjerrum et al., 1975). Plasminogen activator content in embryos recovered at the end of culture were determined after the embryos were frozen and thawed three times to facilitate cracking of the zona pellucida and disruption of the cell mass. Plasmin activities in culture media with 30, 60 and 120 µg/ml PGN were also determined by this procedure.

Plasminogen activator activities were determined with urokinase (E.C.3.4.21.31; Sigma) as the standard in

concentrations of 0, 0.1, 0.5, 1.0, 5.0 and 10.0 milliunits/ml, where 1 unit of urokinase activated that amount of PGN that produced a change in A_{275} of $1.0 \cdot ml^{-1} \cdot min^{-1}$ when measured as perchloric acid-soluble products from α-casein. Media from cultures of 0 µg/ml PGN were assayed for PA with media from drops containing no embryos cultured under the same conditions. Media drops lacking embryos were used to detect non-specific protease production by the embryos and the level of PL contamination of the PGN substrate. Fifteen microliters of either media or urokinase standards were incubated with 15 µl of PGN at 37°C for 15 min. Twenty-five microliter aliquots of the incubated mixture were then placed into wells cut in an agar gel plate containing skim milk and incubated for 45 h at room temperature. At the end of incubation, plates were fixed for 10 min with 3% acetic acid and rinsed with tap water. The diameters of the lytic zones were measured using a vernier caliper.

Kaaekuahiwi and Menino (In Press) reported a 10-fold increase in sensitivity of the caseinolytic agar gel assay using human plasminogen (hPGN) as the substrate. The caseinolytic agar gel assay was modified by incubating the media and standards with hPGN at 37°C for 15 min and decreasing the plate incubation time to 24 h. The urokinase standards were reduced in concentration to 0, 0.01, 0.05, 0.1, 0.5 and 1.0 milliunits/ml.

Plasminogen activator concentrations in the media were determined from the equation of the line calculated for the ring diameters for the urokinase standards and the standard log concentrations. The quantity of PA produced by the embryo (PA_E) was determined by deducting the amount in the media without any embryos for each time. Plasminogen activator production per 24 h per viable embryo ($milliunits \cdot ml^{-1} \cdot e^{-1}$) was calculated by dividing PA_E by the number of viable embryos, as evaluated morphologically at 200X magnification, observed at each interval.

Plasmin activity in the culture media was determined with PL (E.C.3.4.21.7; Sigma) as the standard in concentrations of 0, 5, 10, 50, 100 and 500 µg/ml. Fifteen microliter of either media or PL standard were aliquoted to wells cut in the agar gel plates and incubated at 64 h at room temperature. Media from microdrops lacking embryos and exposed to the same culture conditions were assayed to correct for plasmin contamination of both the porcine and rabbit plasminogen. At the end of incubation these plates were fixed and ring diameters of the lytic zones were measured using a vernier caliper.

To examine for non-specific protease activity in media and embryos cultured in HF-12, 15 µl of either media or embryos in media were aliquoted to wells cut in agar gel plates. The plates were incubated at room temperature for 64 h and the lytic zone ring diameters for media from

microdrops containing embryos were compared to media from microdrops without embryos and PL standards.

Experiment Ia. Development of rabbit embryos in HF-12 supplemented with porcine plasminogen

Three hundred one- to two-cell embryos (Figure 1) were cultured in microdrops of HF-12 with 15 mg/ml BSA containing 0 (n=82), 30 (n=70), 60 (n=69) or 120 (n=79) µg/ml porcine plasminogen (pPGN; Sigma) for 192 h. At 24-h intervals, embryos were morphologically evaluated and transferred to fresh microdrops and media were collected and stored at -20°C until assayed for levels of PL and PA.

Experiment Ib. Development of rabbit embryos in HF-12 supplemented with rabbit plasminogen

One hundred ninety five, one- to two-cell embryos were cultured in microdrops of Ham's F-12 with 15 mg/ml BSA containing 0 (n=40), 75 (n=40), 150 (n=39) or 300 (n=40) µg/ml rabbit plasminogen (rPGN; Sigma) and 60 µg/ml pPGN. Levels of rPGN used were chosen based on activities assayed for 60 µg/ml pPGN. Low levels of rPGN (75 and 150 µg/ml) were chosen since the level of total available PL (20 and 40 µg/ml) present in these concentrations encompassed the level of total activatable Pl in 60 µg/ml pPGN (25.1

ug/ml). The high level of rPGN (300 µg/ml) was found to approximate the PL contaminant of the 60 µg/ml pPGN (5.7 and 7.6 µg/ml, respectively). At 24-h intervals, embryos were morphologically evaluated.

Experiment II. Evaluation of a mitogenic effect of plasminogen on rabbit embryos developing in vitro

Two hundred sixteen, one- to two-cell embryos were cultured in HF-12 with 15 mg/ml BSA containing 0(n=53), 30(n=54), 60(n=54) and 120(n=55) µg/ml pPGN for 96 h. At 24-h intervals, embryos were evaluated morphologically and at the end of culture, embryos were fixed and stained with hemotoxylin and eosin (see appendix B) to determine cell numbers (Figure 2).

Experiment III. Development of rabbit embryos in medium supplemented with plasmin or plasminogen

One hundred forty four, one- to two-cell embryos were cultured in HF-12 with 15 mg/ml BSA containing 0 (n=39) or 120 µg/ml pPGN (n=31) and 13 (n=37) or 45 (n=37) µg/ml porcine PL. Plasmin levels were chosen to represent the assayed levels of PL contaminant and total (activatable and contaminant) PL in 120 µg/ml pPGN (13 and 45 µg/ml PL, respectively) . Embryos were cultured for 168 h and morphological evaluations were made at 24-h intervals.

Experiment IVa. Evaluation of plasminogen activator production in rabbit embryos in vitro cultured to d 6 equivalent gestational age

Five hundred fifteen rabbit embryos were collected at d 3 (238), d 4 (160) and d 5 (16) and cultured in 50 µl drops of HF-12 with 15 mg/ml BSA (Figure 3 and 4). Embryos collected on d 3, 4 and 5 were cultured for 72, 48 and 24 h, respectively. Media were recovered at 24-h intervals for determination of PA content. At the termination of culture, embryos were recovered and stored at -20°C until assayed for PA. Media containing no embryos and incubated under identical conditions were collected to detect non-specific protease production by the embryos.

Experiment IVb. Evaluation of plasminogen activator production in rabbit embryos cultured in vitro to d 7 equivalent gestational age

Finding that the use of human PGN (hPGN; Sigma) as a substrate in the PA assay offered greater sensitivity (Kaaekuahiwi and Menino, In Press), a second group of embryos, collected on corresponding days, were cultured and assayed for PA production. Embryos collected on d 3 (n=37), 4 (n=24) and 5 (n=46) were cultured for 96, 72 and 48 h, respectively. At 24-h intervals, morphological

evaluations were made and media was recovered and stored at -20°C until assayed for PA activity. Embryos were recovered at the end of culture and stored at -20°C until assayed for PA activity. Plasminogen activator production was assayed as previously described using the caseinolytic agar gel assay with hPGN as the substrate.

Experiment IVc. Identification of plasminogen activator type produced by d 5 rabbit embryos cultured to d 7 equivalent gestational age

To identify the type of PA producedo by rabbit embryos, amiloride, a competitive inhibitor of urokinase (Lenich et al., 1989) was incubated with media before evaluation of PA activity. Forty-five microliters aliquots of pooled media recovered at 24 or 48 h of culture from d 5 embryos were incubated with 22.5 µl of either 100 mM amiloride (Sigma) or HF-12 for 90 min at room temperature. Media were assayed for PA by adding 22.5 µl of hPGN (240 µg/ml), incubating for 15 min at 37°C and analyzing for PA with urokinase as the standard.

Statistical analysis

Developmental observations were tabulated as total number of embryos per treatment which developed to the morula, blastocyst, expanded blastocyst, initiating

hatching blastocyst and hatched blastocyst stages. Differences in the number of embryos reaching a certain cell stage due to treatment were determined using Chi-square procedures (Steel and Torrie, 1980). Differences due to treatment in the timing of cell stage formation, nuclei number and ring diameter for media containing or lacking embryos in the PA and PL assays were detected using analysis of variance and least significant differences procedures. Difference in PGN production over time were detected using analysis of variance and least significant difference procedures.

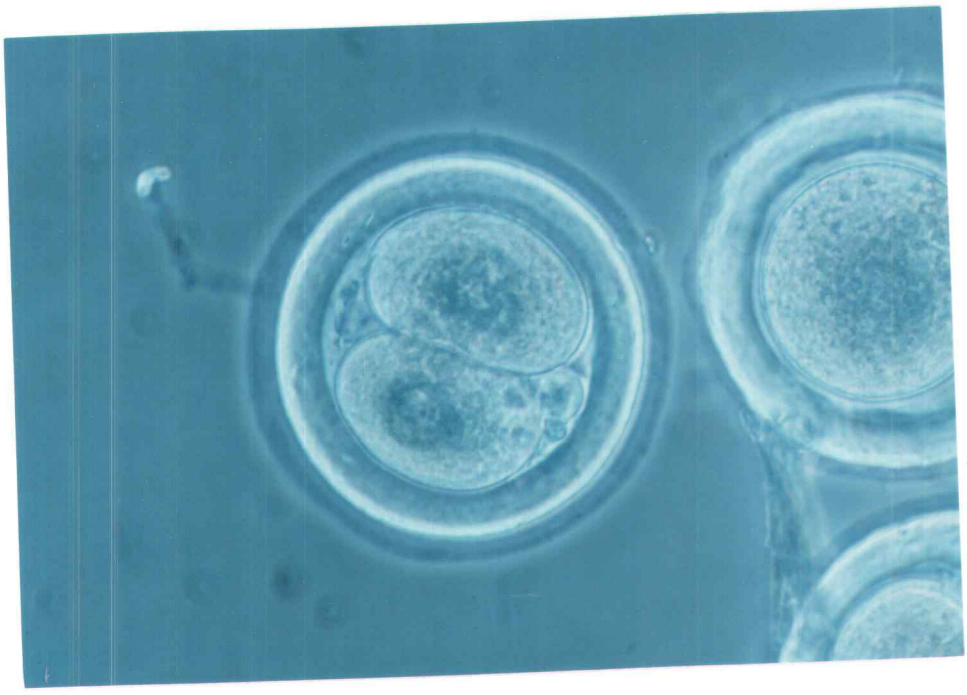

Figure 1. One- to two-cell rabbit embryos collected 24 h after mating.

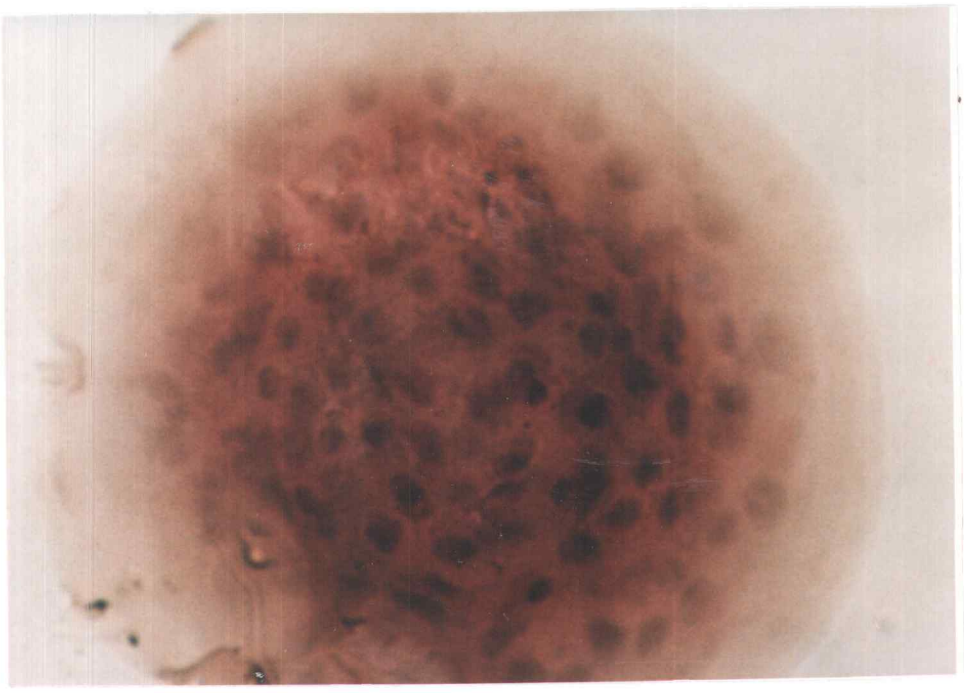

Figure 2. Hematoxylin and eosin stained blastocyst after 96 h in medium with 120 ug/ml plasminogen.

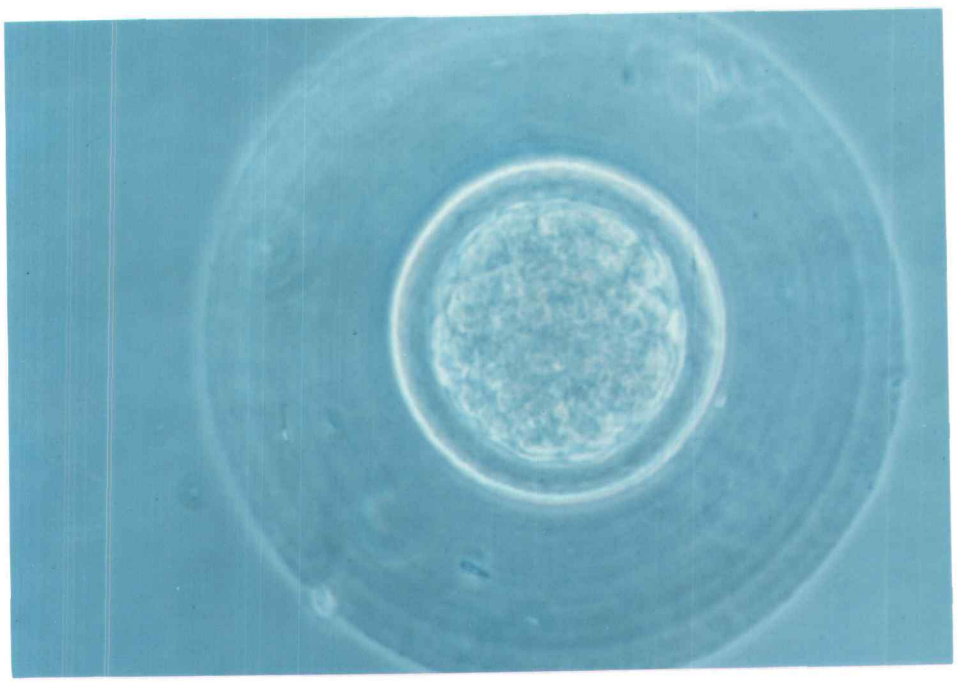

Figure 3. Morulae and early blastocysts collected 72 h after mating.

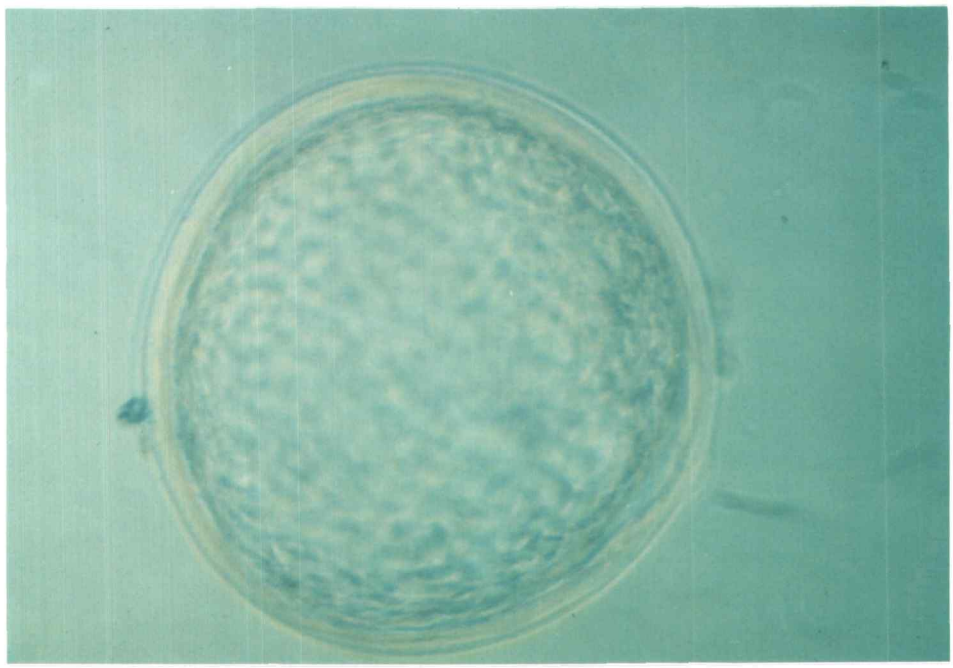

Figure 4. Blastocysts collected from the uterus 120 h after mating.

Results

Rabbit embryo collection

A total of 178 does were superovulated and collected to obtain embryos for the various experiments. Of these, 90 does (51%) provided embryos while flushes from the remaining 88 (49%) were barren. Embryo recovery from individual does varied from none to a maximum of 192 viable embryos. Overall, 1552 embryos were recovered (average 17.2 embryos per recovery) of which 1370 (average 15.2 embryos per recovery) were cultured.

Experiment Ia. Development of rabbit embryos in HF-12 supplemented with porcine plasminogen

The number of rabbit embryos developing in vitro to the morula and blastocyst stages did not differ ($P>.10$) among the four levels of pPGN (Table 2). More embryos developed to the expanded blastocyst ($P<.01$), initiating hatching blastocyst ($P<.05$) and hatched blastocyst ($P<.05$) stages in media with 60 and 120 µg/ml pPGN than in media with 0 or 30 µg/ml pPGN (Table 2; Figure 5 and 6). Hatching (complete shedding of the zona pellucida) was only observed for embryos cultured in either 60 or 120 µg/ml pPGN (Figure 7). Timing of several developmental events

were significantly different although inconsistent across the four pPGN levels (Table 3). Porcine plasminogen at 120 µg/ml decreased the time to morula formation ($P<.01$) by an average of 7.4 h compared to the other levels. Blastocyst formation was accelerated in embryos cultured in 60 µg/ml pPGN ($P<.05$) and tended to occur sooner in the high levels of pPGN (60 and 120 µg/ml) than in the low levels (0 and 30 µg/ml).

Proteolytic activities, expressed as ring diameters of the caseinolytic zones for media with 0 µg/ml pPGN (PA assay) and 30, 60 and 120 µg/ml pPGN (PL assays) are reported in table 4. Proteolytic activities in media with embryos did not differ from media without embryos for the PA and PL assays (Table 5). Time was a significant effect for the PA assay as well as for the PL assays for media containing either 60 or 120 µg/ml pPGN. There was a significant interaction between the main effects of embryo presence or absence and time in culture for medium with 30 µg/ml pPGN.

Experiment Ib. Development of rabbit embryos in HF-12 supplemented with rabbit plasminogen

In vitro development of rabbit embryos to the morula and blastocyst stages was similar for embryos cultured in media with no supplemental PGN and media supplemented with

either 60 µg/ml pPGN or 75, 150 or 300 µg/ml rPGN (Table 6). A greater number of embryos that developed to blastocysts expanded and initiated hatching when cultured in the presence of PGN of either porcine or rabbit origin ($p<.05$). The greatest number of embryos initiating hatching (50%) occurred in the presence of 300 µg/ml rPGN. No embryos hatched during the 192 h of culture.

Experiment II. Evaluation of a mitogenic effect of plasminogen on rabbit embryos developing in vitro

The number of blastocysts initiating hatching by 96 h of culture were greater ($p<.05$) for embryos cultured in media containing pPGN (Table 7). No embryos were found to initiate hatching in media lacking pPGN. Development to the morula, blastocyst and expanded blastocyst did not differ ($p>.10$) among the levels of added pPGN, although twice the number of blastocysts were found to exhibit expansion at 96 h for embryos cultured in the presence of pPGN versus those cultured in media lacking pPGN. Time to the blastocyst, expanded blastocyst and initiating hatching blastocyst did not differ ($p>.10$) for embryos cultured in the various levels of pPGN (Table 8). Morula formation was observed an average of 12 h earlier for embryos cultured in 120 µg/ml pPGN compared to embryos cultured in 0, 30 or 60 µg/ml pPGN ($p<.05$).

Cell numbers of morulae at 96 h (average 28.9 nuclei per morula) were not different (p>.10) for embryos cultured in the various levels of pPGN (Table 9). Cell numbers of blastocysts were greater (p<.10) for embryos cultured in 60 and 120 µg/ml pPGN (88.8 and 109.9, respectively) than for embryos cultured in 0 and 30 µg/ml pPGN (69.4 and 73.3, respectively).

Experiment III. Development of rabbit embryos in medium supplemented with plasmin or plasminogen

Similar numbers of embryos developed to the morula stage in all media (Table 10). The number of embryos developing to the blastocyst stage were similar for culture in control media and media containing either 120 µg/ml pPGN or 45 µg/ml PL. A greater number of embryos (P<.05) developed to the blastocyst stage in media supplemented with 45 µg/ml PL (46%) than in media supplemented with 13 µg/ml PL (19%). More embryos initiated hatching (P<.05) in media supplemented with 120 µg/ml pPGN and 45 µg/ml PL (19% and 32%, respectively) than embryos cultured in media lacking supplementation or supplemented with 13 µg/ml PL (5% and 3%, respectively). Only rabbit embryos cultured in media supplemented with 120 µg/ml pPGN and 45 µg/ml PL exhibited hatching.

Time to morula formation was accelerated (P<.05) for

embryos cultured in media supplemented with either pPGN or PL over the control media (Table 11). Plasmin supplementation at 45 µg/ml accelerated ($P<.05$) time to blastocyst development over other media.

Experiment IVa. Evaluation of plasminogen activator production in rabbit embryos in vitro cultured to d 6 equivalent gestational age

The development of rabbit embryos collected on d 3, 4 and 5 pc and cultured for 72, 48 and 24 h respectively are presented in table 12. Embryos collected on d 4 and 5 were at least blastocyst stage embryos; thus the numbers of morula are not reported. Rabbit embryos collected on d 3 and d 4 exhibited signs of hatching by the end of culture while those collected on d 5 did not. Embryos from d 3 and d 4 collections that exhibited hatching by the end of the culture were found to have left the zona pellucida while remaining within the confines of the mucolemma (Figure 8). Day 4 embryos that initiated hatching or were expanded at 48 h of culture appeared to have their zona pellucida eroded while morulae within the same drops had intact zonae pellucidae. Several blastocysts that were collapsed at the end of the culture also were found to have eroded zonae pellucidae.

Proteolytic activities, expressed as ring diameters of

the caseinolytic zones, are reported in table 13. Lytic zone ring diameters were greater ($p<.05$) for media in which embryos were cultured than the no embryo media controls for d 3 collection. This effect was pronounced when the analysis of variance was blocked for replication effect (difference in date of embryo collection). There was no interaction between embryo presence or absence and replication. Time of culture was not a significant effect for d 3 or 4 collected and cultured embryos. Day 5 embryos were only cultured for 24 h and hence had no time factor. Although non-significant, ring diameters for media from culture drops containing embryos tended to be numerically higher than no embryo controls for d 4 and 5 collected embryos ($P>.14$ and $P>.13$, respectively; Table 13).

Experiment IVb. Evaluation of plasminogen activator production in rabbit embryos cultured in vitro to d 7 equivalent gestational age.

A second set of embryos was collected for evaluation of PA production in a more sensitive assay utilizing human plasminogen as the substrate. Developmental data for embryos collected on d 3, 4 and 5 pc and cultured for 96, 72 and 48 h, respectively, are presented in table 14. Embryos that were collected on d 4 pc were of generally poor morphology with few developing to the blastocyst stage and none

exhibiting hatching as in the previous collection.

Non-specific proteolytic activities, expressed as ring diameters of the caseinolytic zones, for media in which embryos were cultured did not differ from the no embryo controls (average ring diameters of .48 + .03 and .48 ± .02, respectively). Plasminogen activator production by rabbit embryos measured using hPGN as a substrate are reported in table 15. Correlation coefficients were 0.85-0.92 for standard curves of ring diameters of the lytic zones by the log urokinase concentration that were used to calculate PA production by rabbit embryos. Embryos collected at d 3 show little PA activity through 48 h of culture with a significant 10-fold increase at 72 h and a 20-fold increase at 96 h (table 15). Plasminogen activator activity detected for embryos collected on d 4 show a similar trend to increase over time although non-significant. Embryos collected on d 5 and cultured for 24 or 48 h have the numerically greatest levels of PA production. The level of PA production at 24 h is double the highest level of production of either d 3 or 4 collected embryos. At 48 h there is a significant 10-fold increase in PA production.

Experiment IVc. Identification of plasminogen activator type produced by d 5 rabbit embryos cultured to d 7 equivalent gestational age

Addition of amiloride to the media reduced the ring diameters of the caseinolytic zones ($P<.05$). Mean lytic zone ring diameters were 0.59 ± 0.07 and 0.65 ± 0.04 cm (24 and 48 h, respectively) for media with amiloride compared to 0.96 ± 0.05 and 1.11 ± 0.09 cm (24 and 48 h, respectively) for media incubated with HF-12.

TABLE 2. Rabbit Embryo Development in Ham's F-12 with 15 mg/ml Bovine Serum Albumin Containing 0, 30, 60 or 120 ug/ml Plasminogen.

Plasminogen level (ug/ml)	Number of embryos	Number of embryos developing to the:[a] Morula	Blastocyst	Expanded blastocyst	Initiating hatching blastocyst	Hatched blastocyst
0	82	65[b] (79)	19[b] (23)	3[b] (4)	1[b] (4)	0[b] (0)
30	70	53[b] (76)	25[b] (36)	9[b] (13)	2[b] (3)	0[b] (0)
60	69	54[b] (78)	25[b] (36)	18[c] (26)	9[c] (13)	3[c] (4)
120	79	63[b] (80)	22[b] (28)	19[c] (24)	9[c] (11)	6[c] (8)

[a]Values presented are the number (percent) of embryos.

[b,c]Numbers in the same column without common superscripts are different ($P<.05$).

TABLE 3. Time (h) to the Morula, Blastocyst, Expanded Blastocyst, Initiating Hatching Blastocyst and Hatched Blastocyst Stages for Rabbit Embryos Cultured in Media Containing 0, 30, 60 or 120 µg/ml Plasminogen.

	Development to the:									
Plasminogen	Morula		Blastocyst		Expanded blastocyst		Initiating hatching blastocyst		Hatched blastocyst	
(µg/ml)	n	$\bar{x} \pm$ SEM[a]	n	$\bar{x} \pm$ SEM	n	$\bar{x} \pm$ SEM	n	$\bar{x} \pm$ SEM	n	$\bar{x} \pm$ SEM
0	65	63.9 ± 1.4[b]	17	107.3 ± 5.9[b]	2	120.0 ± 0	3	120.0 ± 0[b]	0	--
30	53	65.7 ± 1.6[b]	24	115.0 ± 4.8[b]	7	116.6 ± 9.7	1	72.0 ± 0[c]	0	--
60	52	61.8 ± 1.7[b]	17	94.6 ± 4.3[c]	11	109.1 ± 5.0	8	123.0 ± 5.4[b]	3	152.0 ± 8.0
120	57	56.4 ± 1.6[c]	11	104.7 ± 4.9[b]	11	124.4 ± 5.4	7	133.7 ± 7.1[b]	6	148.0 ± 9.6

[a]Mean ± standard error of the mean.

[b,c]Values in the same column without common superscripts are different ($P<.05$).

TABLE 4. *Proteolytic Activities in Ham's F-12 Medium Supplemented with 0, 30, 60 or 120 µg/ml Plasminogen.*

	Ring diameter (cm)[a]							
Plasminogen level:	0 µg/ml[b]		30 µg/ml[c]		60 µg/ml[c]		120 µg/ml[c]	
Time (h)	Embryo	No embryo	Embryo	No embryo	Embryo	No embryo	Embryo	No embryo
24	.46 ± .01[d]	.50 ± .01	.50 ± .03	.48 ± .01	.50 ± .02	.54 ± .01	.74 ± .08	.87 ± .07
48	.44 ± .01	.47 ± .02	.48 ± .02	.47 ± .01	.49 ± .01	.48 ± .01	.84 ± .17	.68 ± .05
72	.48 ± .03	.52 ± .01	.46 ± .01	.47 ± .01	.50 ± .01	.51 ± .01	.75 ± .10	.70 ± .05
96	.50 ± .00	.51 ± .01	.50 ± .03	.46 ± .01	.48 ± .01	.49 ± .01	.66 ± .11	.68 ± .04
120	.45 ± .01	.46 ± .02	.45 ± .01	.47 ± .01	.48 ± .02	.50 ± .01	.77 ± .09	.79 ± .03
144	.50 ± .06	.48 ± .03	.49 ± .02	.45 ± .01	.47 ± .04	.49 ± .01	.62 ± .04	.75 ± .04
168	.54 ± .03	.54 ± .05	.46 ± .03	.47 ± .01	.45 ± .03	.45 ± .01	.53 ± .07	.62 ± .04
192	.48 ± .02	.51 ± .02	.46 ± .01	.50 ± .01	.47 ± .01	.47 ± .01	.66 ± .09	.69 ± .05

[a]Diameter of lytic zone on the casein-agar gel plate.

[b]Plasminogen activator assay ring diameters.

[c]Plasmin assay ring diameters.

[d]Means ± standard error of the means.

TABLE 5. Summary of Analysis of Variance for Ring Diameters Measured for Media Supplemented with 0, 30, 60 or 120 µg/ml Plasminogen.

0 ug/ml Plasminogen

Source	df	MS	F	Probability
Embryo (A)	1	.0045	2.30	.139
Time (B)	7	.0047	2.39	.044
Embryo X Time	7	.0009	.44	.867
Error	32	.0020		

30 ug/ml Plasminogen

Source	df	MS	F	Probability
Embryo (A)	1	.0073	.92	.340
Time (B)	7	.0008	1.03	.419
Embryo X Time	7	.0017	2.16	.046
Error	80	.0008		

60 ug/ml Plasminogen

Source	df	MS	F	Probability
Embryo (A)	1	.0027	2.98	.088
Time (B)	7	.0044	4.90	.000
Embryo X Time	7	.0005	.55	.795
Error	80	.0009		

120 ug/ml Plasminogen

Source	df	MS	F	Probability
Embryo (A)	1	.01	.45	.505
Time (B)	7	.05	2.15	.047
Embryo X Time	7	.02	.88	.524
Error	80	.02		

TABLE 6. Rabbit Embryo Development in Ham's F-12 with 15 mg/ml Bovine Serum Albumin Containing 0 or 60 ug/ml Porcine Plasminogen and 75, 150 or 300 ug/ml Rabbit Plasminogen.

Plasminogen level (ug/ml)	Number of embryos	Number of embryos developing to the:[a] Morula	Blastocyst	Expanded blastocyst	Initiating hatching blastocyst
0	40	35 (88)	11 (28)	10[b] (25)	4[b] (10)
Porcine					
60	36	33 (92)	18 (50)	13[b,c] (36)	11[c] (30)
Rabbit					
75	40	28 (70)	19 (48)	19[c,d] (48)	15[c,d] (38)
150	39	32 (82)	22 (56)	22[d] (56)	16[c,d] (41)
300	40	35 (88)	26 (65)	26[d] (65)	20[d] (50)

[a]Values presented are the number (percent) of embryos.

[b,c,d]Numbers in the same column without common superscripts are different ($P<.05$).

TABLE 7. Rabbit Embryo Development at 96 h in Ham's F-12 with 15 mg/ml Bovine Serum Albumin Containing 0, 30, 60 or 120 ug/ml Plasminogen.

Plasminogen level (ug/ml)	Number of embryos	Number of embryos developing to the:[a] Morula	Blastocyst	Expanded blastocyst	Initiating hatching blastocyst
0	53	44 (83)	16 (30)	4 (8)	0 (0)[b]
30	54	42 (78)	15 (38)	9 (17)	6 (11)[c]
60	54	48 (89)	13 (24)	9 (17)	7 (13)[c]
120	55	45 (82)	13 (24)	8 (15)	7 (11)[c]

[a]Values presented are the number (percent) of embryos.

[b,c]Numbers in the same column without common superscripts are different ($P<.05$).

Table 8. Time (h) to the morula, blastocyst, expanded blastocyst and initiating hatching blastocyst stages for rabbit embryos cultured for 96 h in HF-12 containing 0, 30, 60 or 120 ug/ml plasminogen.

Plasminogen	Development to the: Morula		Blastocyst		Expanded blastocyst		Initiating hatching blastocyst	
(ug/ml)	n	$\bar{x} \pm$ SEM[a]	n	$\bar{x} \pm$ SEM	n	$\bar{x} \pm$ SEM	n	$\bar{x} \pm$ SEM
0	42	58.3 ± 0.2[b]	7	88.8 ± 4.4	4	96.0 ± 0	0	--
30	37	50.6 ± 1.2[b]	11	80.7 ± 3.7	9	96.0 ± 0	6	96.0 ± 0
60	46	51.7 ± 2.4[b]	11	74.2 ± 2.2	9	96.0 ± 0	7	96.0 ± 0
120	37	41.5 ± 2.7[c]	9	72.0 ± 0	8	93.3 ± 3	7	96.0 ± 0

[a]Mean ± standard error of the mean.

[b,c]Values in the same column without common superscripts are different ($P<.05$).

TABLE 9. Number of Nuclei in Rabbit Morulae and Blastocysts Cultured for 96 h in Plasminogen.

Plasminogen	Morulae		Blastocysts	
(ug/ml)	n	$\bar{X} \pm$ SEM[a]	n	$\bar{X} \pm$ SEM
0	10	29.6 ± 3.2	6	69.4 ± 14.6[b]
30	15	29.5 ± 2.6	8	73.3 ± 12.2[b]
60	13	29.3 ± 2.8	14	88.8 ± 9.2[b,c]
120	13	27.3 ± 2.8	11	109.9 ± 10.4[c]

[a]Mean ± standard error of the mean.

[b,c]Values in the same column without common superscripts are different ($P<.05$).

TABLE 10. Rabbit Embryo Development in Ham's F-12 with 15 mg/ml Bovine Serum Albumin Containing 0 or 120 µg/ml Plasminogen and 13 or 45 µg/ml Plasmin.

Plasmin or plasminogen level (ug/ml)	Number of embryos	Number of embryos developing to the:a			
		Morula	Blastocyst	Initiating hatching blastocyst	Hatched blastocyst
0	39	26 (66)	13 (33)b,c	2 (5)b	0 (0)b
Plasmin					
13	37	21 (57)	7 (19)b	1 (3)b	0 (0)b
45	37	30 (81)	17 (46)c	12 (32)c	10 (27)c
Plasminogen					
120	31	26 (84)	13 (42)b,c	6 (19)c	4 (13)c

[a]Values presented are the number (percent) of embryos.

[b,c]Numbers in the same column without common superscripts are different (P<.05).

TABLE 11. Time (h) to the Morula, Blastocyst, Expanded Blastocyst and Initiatiog Hatching Blastocyst and Hatched Blastocyst Stages for Rabbit Embryos Cultured in Ham's F-12 Containing 13 or 45 µg/ml Plasmin and 0 or 120 µg/ml Plasminogen.

Plasminogen or plasmin level (ug/ml)	Development to the: Morula n	Morula $\bar{x} \pm$ SEM	Blastocyst n	Blastocyst $\bar{x} \pm$ SEM	Expanded blastocyst n	Expanded blastocyst $\bar{x} \pm$ SEM	Initiating hatching blastocyst n	Initiating hatching blastocyst $\bar{x} \pm$ SEM	Hatched blastocyst n	Hatched blastocyst $\bar{x} \pm$ SEM
0	26	68.0 ± 11.1[b]	12	108.0 ± 12.5[b]	1	120.0 ± 0	2	120.0 ± 0	0	—
Plasmin 13	21	59.0 ± 5.9[c]	6	104.0 ± 19.6[b]	2	108.0 ± 17.0	1	120.0 ± 0	0	—
45	29	57.0 ± 5.7[c]	7	92.0 ± 9.1[c]	4	102.0 ± 12.0	7	113.0 ± 18.1	10	134.0 ± 16.7
Plasminogen 120	26	55.0 ± 16.3[c]	6	120.0 ± 26.3[b]	3	104.0 ± 13.9	3	128.0 ± 13.8	3	136.0 ± 13.8

[a]Mean ± standard error of the mean.

[b,c]Values in the same column without common superscripts are different ($P<.05$).

TABLE 12. Development of Rabbit Embryos in Ham's F-12 with 15 mg/ml Bovine Serum Albumin.

Day of embryo collection	Number of embryos	Number of embryos developing to the:[a]				
		Morula	Blastocyst	Expanding blastocyst	Initiating hatching blastocyst	Hatched blastocyst
3	238	122 (51)	122 (51)	58 (24)	50 (21)	2 (8)
4	160	NA[b]	160 (100)	92 (58)	67 (42)	21 (13)
5	16	NA	16 (100)	6 (38)	-	-

[a]Values presented are the number (percent) of embryos.

[b]Embryos collected on d 4 and 5 were blastocysts.

TABLE 13. Proteolytic Activities in Ham's F-12 with 15 mg/ml Bovine Serum Albumin.

Time in culture (h)	Day of embryo collection: d 3 Embryo	d 3 No embryo	d 4 Embryo	d 4 No embryo	d 5 Embryo	d 5 No embryo
24	.86 ± .02[a]	.88 ± .04	.90 ± .01	.88 ± .01	.92 ± .03	.85 ± .02
48	.87 ± .02	.80 ± .06	.91 ± .01	.88 ± .04	--	--
72	.89 ± .01	.81 ± .04	--	--	--	--
X	.87 ± .01	.83 ± .03	.91 ± .01	.88 ± .02	.92 ± .03	.85 ± .02

[a]Values represent the mean ring diameters and standard errors.

TABLE 14. Development of Rabbit Embryos in Ham's F-12 with 15 mg/ml Bovine Serum Albumin.

Day of embryo collection	Number of embryos	Number of embryos developing to the:[a] Blastocyst	Expanding blastocyst	Initiating hatching blastocyst	Hatched blastocyst
3	37	18 (49)	9 (24)	9 (24)	3 (8)
4	24	5 (21)	3 (13)	3 (13)	0 (0)
5	40	40 (100)	40 (100)	2 (5)	2 (5)

[a]Values presented are the number (percent) of embryos.

TABLE 15. Plasminogen Activator Production by Rabbit Embryos Cultured in Ham's F-12 with 15 mg/ml Bovine Serum Albumin.

	Day of embryo collection:[a]		
Time in culture (h)	d 3	d 4	d 5
Media			
24	1.07[b]	5.48	51.13[b]
48	1.28[b]	10.37	544.71[c]
72	15.85c	38.69	--
96	25.62[c]	--	--
Embryos	5.29[b,c]	6.70	273.98[b,c]
SEM[d]	4.67	11.91	133.18

[a]Values presented represent plasminogen activator production in milliunits X $10^{-4} \cdot ml^{-1} \cdot d^{-1} \cdot$viable embryo^{-1}.

[b,c]Means without common superscripts are different ($P<.05$).

[d]Pooled standard error of the means.

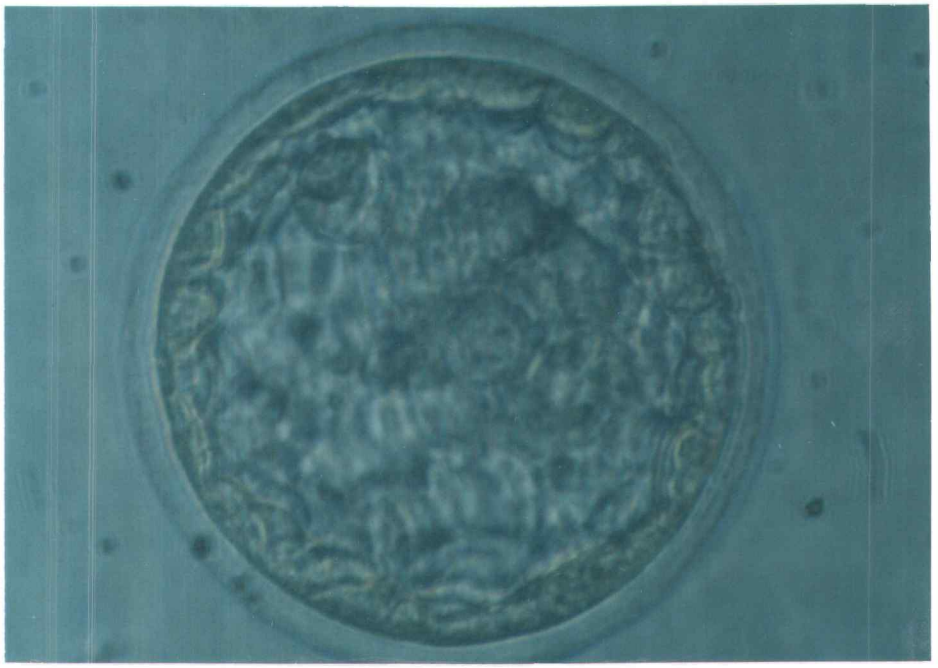

Figure 5. Expanded blastocyst after 96 h culture in 60 µg/ml plasminogen.

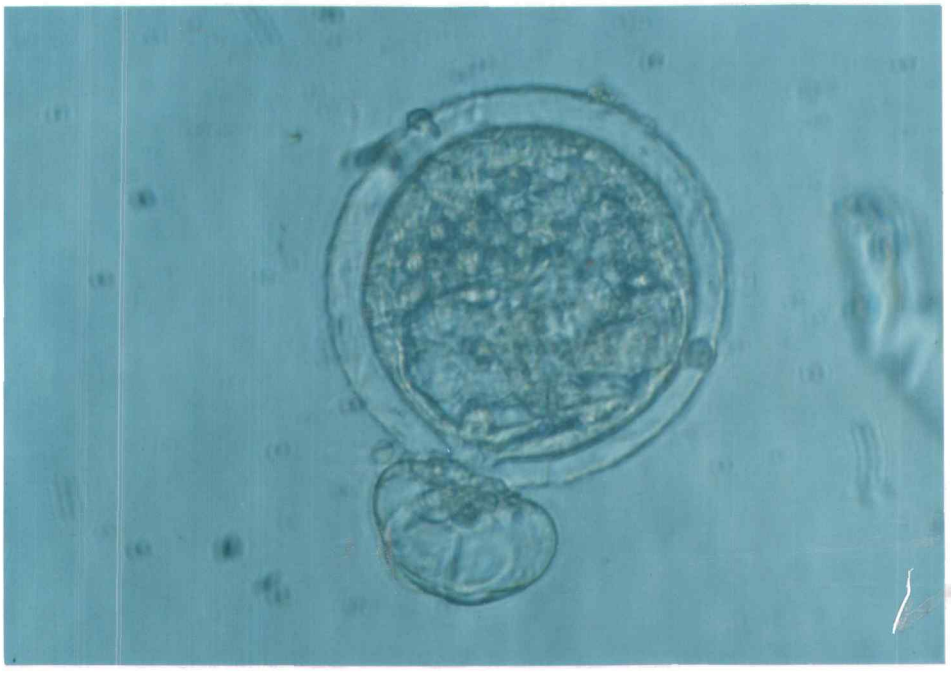

Figure 6. Initiating hatching blastocyst after 120 h culture in 120 ug/ml plasminogen.

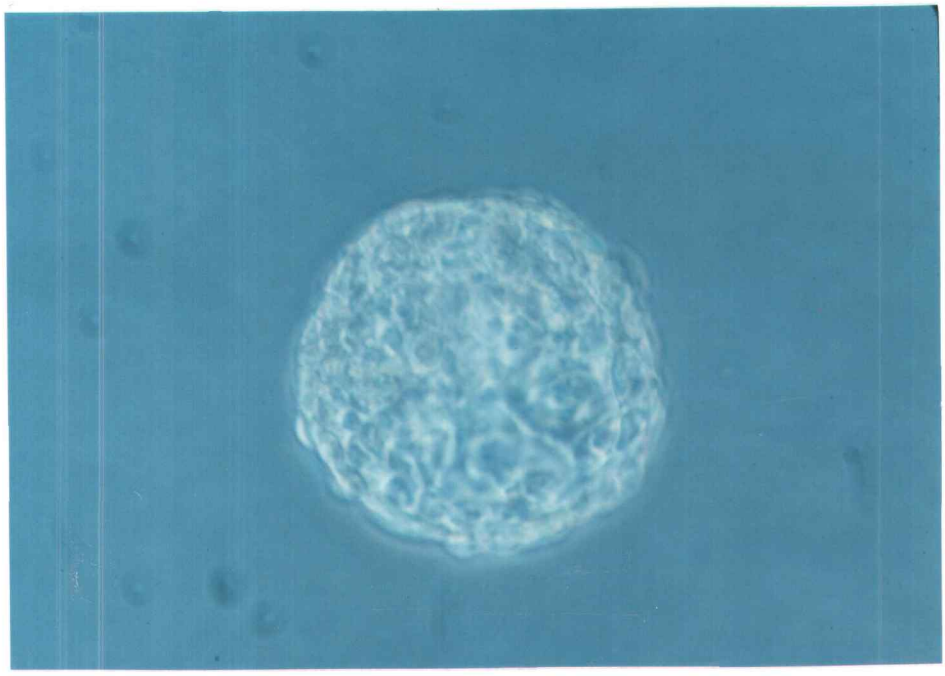

Figure 7. Hatched blastocyst after 144 h culture in 120 ug/ml plasminogen.

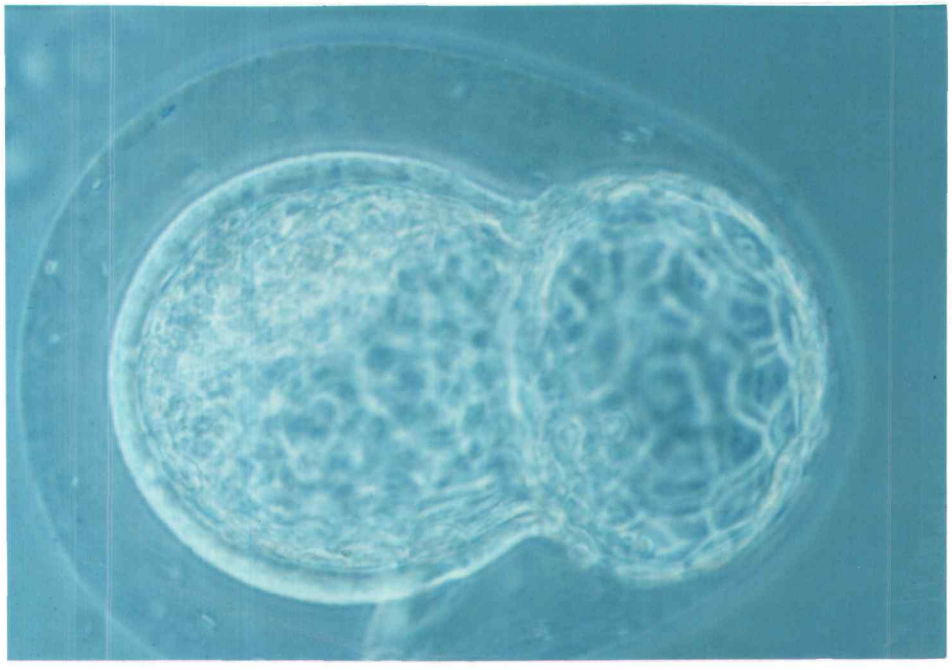

Figure 8. Day 4 collected blastocyst hatching from the zona pellucida into the mucolemma after 48 h culture.

Discussion

The results suggest that PGN stimulates in vitro development of preimplantation rabbit embryos. This effect is readily detected by the increase in the percentage of one to two-cell embryos developing to the expanded blastocyst, initiating hatching blastocyst and hatched blastocyst stages in media containing either 60 or 120 µg/ml pPGN and to the expanded and initiating hatching blastocyst stages in media containing 75, 150 or 300 µg/ml rPGN. The only embryos collected at the one to two-cell stage that hatched were those cultured in media containing PGN. These results compare favorably to the development of ovine embryos in similar levels of pPGN (Menino et al., 1989).

Proteases appear to facilitate in vitro hatching of rabbit blastocysts because pronase and trypsin both caused hatching (Onuma, Maurer and Foote, 1968; Kane, 1983; Kane 1986). Kane (1985) observed one to two-cell rabbit embryos to hatch when cultured in media containing BSA and determined that the factor in BSA responsible for initiating hatching was not a protease. Kane (1987) proposed that in vitro hatching was the result of several unique conditions associated with culture. Furthermore, Kane (1987) suggests that in vitro hatching was necessary to prevent inhibition of development by the strong zona

pellucida-mucolemma complex. Lack of a thick mucolemma, thought to be responsible for preventing in utero hatching (Kane, 1975a) in embryos collected from the oviducts, and the hydrostatic pressure caused by blastocyst expansion (Kane, 1983b) have been offered as mechanisms for in vitro hatching.

Development of rabbit embryos in rPGN was used to validate that the enhanced development attributed to pPGN was physiological. Although there are no reports of PGN levels in the pregnant rabbit uterus, Fazleabas et al. (1983) found levels of PGN to peak at 95 µg/uterine horn at d 12 pc in swine, the time of elongation of the blastocyst. Rabbit PGN levels were chosen to approximate swine uterine levels as well as the level of PL contaminant in 60 µg/ml pPGN (300 µg/ml rPGN) and total PL (both activatable and contaminant) in 60 µg/ml pPGN (75 and 150 µg/ml rPGN). All levels of rPGN examined produced development as good as or better than pPGN at 60 µg/ml. Several factors could explain these results. First, rabbit embryos may respond with greater development to rPGN, assuming that it is PGN producing the developmental enhancement. This would be confirmed by the dose response to rPGN, where development tended to improve as rPGN increased. Secondly, although all levels of detectable PL contaminant of rPGN were below levels detected in pPGN at 60 µg/ml, rabbit embryos may respond better to PL of rabbit origin. The site of action

for PL may have a higher affinity for PL of rabbit origin compared to porcine. Cell surface receptors for both PL and PGN have been identified on human carcinoma cells (Burtin and Fondandeche, 1988). Finally, these results could be the result of a non-specific protein contaminant of rPGN, again explaining the dose response with increasing levels of rPGN showing enhanced development. Kane (1985) identified a contaminant of <10 kD to be responsible for enhanced development of rabbit embryos cultured in media supplemented with BSA.

Rate of development to the morula stage was accelerated by the addition of 120 µg/ml pPGN compared to lower levels of pPGN or a lack of pPGN. Acceleration of development to later stages was inconsistent. A pattern of accelerated development similar to that reported for the bovine embryo was seen with rabbit embryos, although development was more pronounced in the bovine system (Menino and Williams, 1987). Likewise, a non-significant trend of accelerated development by ovine embryos cultured in the presence of pPGN has been reported (Menino et al., 1989).

Kane (1983a) found that in vitro hatching was associated with an increase in cell numbers of blastocysts. One to two-cell embryos developed to the hatched blastocyst stage only when cultured in the presence of PGN. An increase in nuclei numbers of blastocysts cultured for 96 h

in the presence of pPGN compares favorably with the proposal of Kane (1983a). The increase in cell numbers coupled with the accelerated development observed suggest that the added PGN may have a mitogenic effect on the development of one to two-cell cultured rabbit embryos. Proteases have been shown to induce growth factor-like effects on various cell types in vitro (Cunningham, 1981) and in particular to have a mitogenic effect on cultured mouse embryos (Pienkowski et al., 1974; Konwinski et al, 1978).

The mitogenic effect detected for rabbit embryos cultured in vitro could be the result of the direct effect of the protease on the embryo or an indirect effect through the weakening of the zona pellucida. Blumberg and Robbins (1975) found proteases, of particular interest PL, to stimulate deoxyglucose uptake and induce cell division in chick embryo fibroblasts. The effect of proteases appear to be mediated through the breakdown of a cell surface protein. More recently, Burtin and Fondaneche (1988) demonstrated receptors for both PGN and PL on human carcinoma cells and bound PL retained its proteolytic activity. Kane (1986) suggested a more likely role for proteases in the softening of the zona pellucida through a partial digestion, allowing the blastocyst to grow and stimulate growth by removing a block to expansion. Blastocysts expanding but not hatching have been found to

collapse and the trophoblast has been found to consist of more than one layer of cells (Kane, 1983a, 1983b).

The increase in hatching and accelerated development in media containing PGN could be a result of PL from plasminogen activation by rabbit embryos, PL contamination in media supplemented with PGN or an unkown role for PGN. Initially, no differences in ring diameters between media from cultures with and without embryos indicated that no PA or PL production by rabbit embryos was detected in any of the media. This could be due to either failure of the caseinolytic gel assay to detect PA and PL in the media or lack of PA in preimplantation rabbit embryos. However, using the more sensitive assay, PA was detected only in d 5-7 embryos, suggesting that it is only expressed by d 5 and increases to d 7. Denker and Gerdes (1979) suggested that the proteolytic attack on the zona pellucida occurs from within, rather than exterior to the zona pellucida. If PA was present within the embryonic coverings and exhibited its effects internally, it is possible that PL produced would not be detected in the media. In characterizing blastolemmase activity, Denker (1977) found the highest activity in the blastocyst coverings while low to moderate protease activity has been detected in the trophoblast (Kirchner, 1972a, Denker, 1974a, 1975). In these studies, no attempt was made to measure protease activity released from the embryo.

The significant effect of time for proteolytic activities detected in the PL assays could be explained by PL contamination in the media. The time effect follows a general decrease in activity over time. Since all media were prepared at the start of the experiment, a natural decrease in PL activity in time would be expected as PL degradation occurred. A significant time effect in proteclytic activity was not observed in media with 30 µg/ml pPGN owing to an embryo by time interaction.

Rabbit embryos developing in vitro lag behind their in vivo counterparts, suggesting the lack of PA detection may be developmentally related. By ultrastructural comparison, Van Blerkom et al. (1973) found no differences between in vivo and in vitro development over the first four days. However, embryos cultured from the one- to two-cell stage in vitro lag behind comparable aged in vivo embryos in both cell number and blastocyst diameter beginning at d 3 and embryos developing in vitro, usually do not have more than 500 cells (Kane, 1987). In light of this, embryos were allowed to develop for 3, 4 or 5 days pc before being removed and cultured for PA detection. Embryos collected on days 3 and 4 were found to hatch as characterized by escape from the zona pellucida. The mucolemma prevented escape of the blastocyst from the extracellular surroundings. Day 5 embryos were not observed to hatch. Kane (1987) suggested that exposure to uterine enzymes

softens the zona pellucida-mucolemma complex allowing for expansion without rupture of the zona.

Proteolytic activities, as measured by ring diameters of the caseinolytic zones, were numerically greater for media from cultures with embryos compared to media without embryos. The only significant difference was observed for day 3 embryos, most likely a result of the large number of embryos cultured for this time period. Of interest was the significant effect of replication during day 3 on proteolytic activities. Five collections of embryos on separate days over an 8 week period were involved in obtaining all the embryos placed in culture. There was great variation among collections in embryo development as well as proteolytic activity. This effect showed no interaction with the main effect of lytic zone ring diameters of media from embryo containing drops compared to media from drops lacking embryos.

With the finding that hPGN as a substrate in the caseinolytic gel assay provided a 10-fold increase in sensitivity (Kaaekuahiwi and Menino, In Press), a second set of embryos were collected, cultured and assayed for PA. Embryos were cultured for 24 h longer than the previous culture, thereby ending on an equivalent gestational age of d 7. This was to coincide with the time of implantation in the rabbit and the detection of maximum blastolemmase activity in the blastocyst coverings (Denker, 1977).

Embryo development was similar for embryos collected on d 3 of both collections. Day four embryos were of poor morphology and limited numbers. Day five embryos were observed to hatch, probably the result of allowing the embryos to develop for 24 h longer.

Plasminogen activator production was low through embryonic age corresponding to d 5 pc and showed a sharp rise at day 6 and 7. Although similar patterns of production of PA were observed for embryos collected at different equivelent gestational ages, the greatest levels of PA production were detected for embryos collected at d 5 and cultured for 48 h. This difference is most likely due to the developmental retardation of cultured embryos reported past d 4 (Kane, 1987). Up to day 4, there is little difference in in vitro and in vivo rabbit embryo development (Van Blerkem et al., 1973). It appears that once rabbit embryos enter the uterus, factors of uterine origin are required for normal expansion and cell division. Blastokinin would serve as an example of a uterine factor necessary to promote normal blastocyst development, showing peak uterine levels at d 5 pc (Krishnan and Daniel, 1967). Jung (1989) reported a 10-fold increase in protein synthesis between day 4 and 5 blastocysts developing in vivo. Rabbit blastocysts cultured in vitro exhibit reduced amino acid incorporation that was dependent on the length of culture (Jung et al., 1987). Cultured d 3 and d

4 rabbit embryos have also been shown to have an increase in secondary lysosomes (Fischer, 1988; Hegele-Hartung et al., 1988) with a corresponding enhancement in protein degradation. Hegele-Hartung and co-workers (1988) concluded that development in vitro was slowed to a rate where a period of 24 h matches only a few h of in vivo development. Embryos exposed to the d 5 uterine environment may be assumed to be more vigorous and have the necessary mechanisms for protein production, here being able to produce greater concentrations of PA.

If we assume that the PA detected is the same enzyme as blastolemmase reported by Denker (1977), the differences in PA production between d 3 and d 4 recovered embryos and d 5 recovered embryos may be related to an increase in trophoblast cells present in the later embryos. On d 7 pc, Daniel (1967) estimates that an expanded blastocyst recovered from the uterus is composed of 250,000 cells while Kane (1987) suggests that in vitro cultured morulae do not develop beyond 2000 cells. Biochemically, Denker (1977) demonstrated blastolemmase activity in the trophoblast, which peaked by d 7-7.5. By cell numbers alone, a 125-fold increase in PA production would be expected between embryos collected on d 3 and 4 compared to d 5. The 21-fold difference between d 3 and 5 embryo PA production on the equivalent gestational age of d 7 may be explained by cell death and degeneration reducing the

actual number of trophoblast cells (Daniel, 1967; Streffer et al., 1980; Fischer, 1987, 1988). The lower level of PA production may also be a result of developmental retardation of the embryos (Fischer, 1987, 1988; Hegele-Hartung et al., 1988) or the production of a PA inhibitor by the trophoblast (Feinberg et al., 1989).

The amount of PA associated with the embryos tended to fall in the middle range for PA detection in the media for the respective days cultured. This would suggest that much of the PA produced is actually secreted by the blastocyst in a similar fashion as bovine (Menino and Williams, 1987) and ovine (Menino et al., 1989) embryos.

Amiloride inhibition of the PGN dependent proteolytic activity detected from media and cultured embryos, suggests that the PA secreted is an uPA. Mouse trophoblasts have been reported to produce uPA in culture coinciding to the time of early attachment at the onset of implantation (Strickland et al., 1976; Strickland and Mahdavi, 1978). Marotti et al. (1982) found both tPA and uPA secretion by mouse embryonic cells. The parietal endoderm secretes predominantly tPA and the visceral endoderm and extraembryonic mesoderm secretes uPA. The tPA produced by the parietal endoderm is speculated to play a role in the breakdown of Reichert's membrane (the basement membrane secreted by these cells) while the uPA is involved in the movement of mesoderm cells between the ectoderm and

endoderm (Marotti et al., 1982).

Development of one- to two-cell rabbit embryos in the presence of PL suggests that PL is the embryo development enhancer. The level of PL equal to the contaminant present in 120 µg/ml pPGN (13 µg/ml) gave developmental results similar to the control medium. The observation that 45 µg/ml PL, equivalent to the total available PL in 120 µg/ml pPGN, supported similar development as 120 µg/ml pPGN, suggests early staged rabbit embryos were activating PGN. However, two observations do not support this conclusion. First, the assumption that the developmental effect in 120 µg/ml pPGN is due to embryonic conversion of PGN to PL is not supported because PL concentrations significantly greater than the contaminant were not detected. Plasminogen activator production was not detected before an equivalent gestational age of d 4. This might be due to not having used the more sensitive caseinolytic assay for earlier staged embryos. However, differences in development were not apparent until the blastocyst stages (d 4 or more in development), when low levels of PA could be detected. Also, although not statistically different, embryo development to the hatching blastocyst stages was two-fold greater in 45 µg/ml PL compared to 120 µg/ml pPGN. The slight amount of PA production by cultured embryos at the blastocyst stage may have produced sufficient PL to enhance development, but not to a level comparable to 45

µg/ml PL. Secondly, Kane (1986) has reported a lack of a PL effect on rabbit embryo development and Denker (1977) has shown that epsilon amino caproic acid (EACA), an inhibitor of PL, is unable to inhibit in vivo implantation. The highest level of PL (25 µg/ml) used by Kane (1986), however, was below the level of PL (45 ug/ml) found to produce the best developmental enhancement in these data. Plasmin supplemented media has been shown to enhance development to the blastocyst and hatched blastocyst stages of mouse embryos where PL levels were similar to those currently used (Menino and O'Claray, 1986). The level of EACA (12 mg/uterine horn) used by Denker (1977) would provide a significantly lower molar concentration of inhibitor (1M) than was found necessary to inhibit the contaminant level of PL (10-13%) in 120 µg/ml pPGN (see appendix C). This level of inhibitor proved deleterious to embryo development with no embryos cultured in its presence developing beyond the stage at which EACA was introduced. Further caution must also be used in interpreting Denker's results, because it is not known how well the inhibitor was distributed in the uterine horn and whether it reached the appropriate site in concentrations that would be effective.

Conclusions

These results suggest that PGN enhanced in vitro rabbit embryo development, possibly through the activation of PGN to PL. This would provide a role for plasminogen of uterine origin as a zymogen for activation by the embryo to provide a source of protease. This protease could function in the remodeling of the extraembryonic coverings and implantation. The finding that rabbit embryos produced low levels of PA prior to d 5 and increased their production on d 6 through d 7 suggests that PA production may be involved in implantation.

The production of PA determined from these data strongly suggest that "blastolemmase" is in fact uPA. Electrophoretic characterization with the use of caseinolytic agar gel overlays are needed to confirm this suspicion.

Another area of further exploration is the potential role of blastokinin as a regulator of PA activity. Blastokinin is a major component of uterine secretions from d 3 to d 7 and peaks in concentration at d 5. Interestingly, blastokinin has been reported to inhibit trypsin, hence it may be involved in regulating PA or PL activity in the uterus during the peri-implantation period.

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APPENDIX

Appendix A

Caseinolytic agar gel assay - Plate preparation

1. Buffer preperation:

Ingredient	Molarity	g/liter
$CaCl_2 \cdot 2H_2O$	.0013	.195
Glycine	.10	7.51
Tris	.038	4.6
Na Azide	.005	.325

2. Dissolve 2 g of nonfat dry milk (Carnation Co., Los Angles, CA.) in 100 ml of buffer (2%).

3. Dissolve 2 g agarose (Sigma Chemical Co., St. Louis, MO) in 100 ml of distilled H_2O (2%) and autoclave.

4. Combine equal volumes of warmed solutions.

   Final volumes required per plate:

   Plasmin assay - 10 ml
   Plasminogen activator assay - 15 ml

   Final concentrations: 1% nonfat milk
   1% agarose

Appendix B

Procedure for H & E staining of embryos

Embryo fixation

1. Wash embryos in 2.0% citrate solution
2. Transfer embryos to 0.7% citrate solution; 3 min.
3. Apply embryos in a small drop of 0.7% citrate solution to a clean and scribed microscope slide.
4. Carefully aliquot 1 to 5 drops of 25% acetic acid in ethanol over the embryos on the slide; air-dry, then apply a few more drops of 25% acetic acid in ethanol over the embryos, then air-dry again.
5. Fix slides with embryos in 25% acetic acid in ethanol for one hour.

Embryo staining

Solution	Time required
1. 100% ETOH	2 min
2. 95% ETOH	2 min
3. 75% ETOH	1 min
4. Running H_2O	2 min
5. Hematoxylin	20 min
6. Rinse/running H_2O	3 min
7. 1% acid	Dip in/out for 15 sec.
8. Rinse/running H_2O	3 min
9. Scotts tap water	30 sec
10. Rinse/running H_2O	5 min
11. Eosin	1 min
12. 70% ETOH	Dip 1 min
13. 95% ETOH	2 min
14. 95% ETOH	2 min
15. 100% ETOH	1 min
16. Xylene	2 min
17. Toluene	2 min
18. Coverslip	

Appendix C

Development of rabbit embryos in media with epsilon amino caproic acid blocked plasmin.

In an effort to differentiate between embryo enhancement effects due to activatable PGN and the PL contaminant in PGN, one to two-cell rabbit embryos were culture in media with no PGN, PGN and with PGN in which the PL contaminant was inactivated by epsilon amino caproic acid (EACA). To determine the level of EACA to be used, media containing either 120 µg/ml pPGN or 300 µg/ml rPGN were titrated with EACA and assayed for plasmin activity using the caseinolytic agar gel assay. Levels chosen were the minimum levels of EACA required to eliminate all PL activity in media containing PGN. These levels were found to be 1 M EACA and .04 M EACA for 120 µg/ml pPGN and 300 µg/ml rPGN respectively. The culture treatments consisted of the following additions to HF-12 medium supplemented with 15 µg/ml BSA:

0 ug/ml PGN

- 0 M EACA
- .04 M EACA
- 1 M EACA

120 ug/ml pPGN

- 0 M EACA
- 1 M EACA

300 ug/ml rPGN

- 0 M EACA
- .04 M EACA

Five hundred fifty-five one to two-cell embryos were collected, pooled and randomly assigned to one of the above treatments. Embryos were placed in 50 ul drops of media under paraffin oil and cultured for 168 h at 37^{o}C in 5% CO_2 in humidified air. At 24 h intervals, embryos were evaluated morphologically.

All embryos cultured in the presence of EACA were inhibited from developing beyond the stage that the culture was started. Embryos cultured in PGN exhibited similar development to previous cultures through 144 h. Cultures of embryos in 0 ug/ml PGN - 0 M EACA became contaminated with yeast and were terminated at 120 h.