AN ABSTRACT OF THE THESIS OF

RaeAnne Chamberlin for the degree of Master of Science in Animal Science presented on August 8, 1995

Title: Partial Characterization of Gelatinases Produced by Preimplantation Porcine, Ovine and Bovine Embryos.

Redacted for Privacy

Approved: _____

Alfred R. Menino, Jr.

The amount of extracellular matrix remodeling and cellular migration events occurring during preimplantation development in livestock embryos implicates the activity of matrix degrading enzymes during this period of embryonic growth. Therefore, gelatinases produced by porcine, ovine and bovine embryos during the preimplantation period were partially characterized by SDS-PAGE gelatin zymography. Embryos were collected from crossbred gilts and sows 6 to 13 days after mating, crossbred ewes 7, 9 and 11 days after mating and crossbred cows 12 to 14 days after mating. Embryos were cultured for 24 to 196 h depending on age and species of the embryo in medium containing 0.15% BSA in 100 μl microdrops

under paraffin oil in a humidified atmosphere of 5% CO_2 in air at 39°C. Porcine embryos produced a 69.7 kDa gelatinase during Days 6 to 13 of development. Three additional gelatinases (91.5, 84.1 and 77.9 kDa) were observed during Days 12 to 13 and a low molecular mass gelatinase (42.8 kDa) was detected on Days 11 and 12. Gelatinases were not detected in Days 7 and 9 ovine embryos. Ovine embryos produced 68.7 and 86.1 kDa gelatinases on Day 11. Bovine embryos produced 88.3, 80.9, 72.5 and 43.0 kDa gelatinases on Days 12 to 14 of development. Gelatinolytic activity of porcine and ovine embryos was calcium-dependent and not detected in calcium-free conditions. The calcium and metal chelators, EDTA, EGTA and 1,10-phenanthroline, completely inhibited porcine and ovine embryonic gelatinase activity. Gelatinase activity in porcine and ovine embryos was restored when strontium or barium was substituted for calcium. Gelatinolytic activity in conditioned medium from bovine embryos was not calcium-dependent. EGTA, EDTA and 1,10-phenanthroline did not completely inhibit gelatinase activity in bovine conditioned medium; however, greater inhibition by these reagents was observed with samples of bovine embryonic tissues. The cysteine protease inhibitors, N-ethyl maleimide and leupeptin, the serine protease inhibitors, benzamidine and soybean trypsin inhibitor, and the aspartic acid protease inhibitor, pepstatin A, did not inhibit gelatinase activity by porcine, ovine and bovine embryos. Incubation with the organomercurial reagent, aminophenyl-mercuric acetate (APMA) caused a decrease

in activity of the 77.9 kDa porcine gelatinase and a concomitant increase in activity of the 69.7 kDa porcine gelatinase. Incubation of bovine embryonic tissues with APMA did not change the molecular mass distribution of the gelatinases observed. The optimal pH for gelatinase activity for porcine ovine and bovine embryos was 8.1 to 8.4, 7.9 to 8.5 and 8.1 to 8.4, respectively. The results suggest that the gelatinases produced by porcine, ovine and bovine embryos are matrix metalloproteinases (MMP). The 69.7 and 84.1 kDa porcine gelatinases are likely MMP-2 (gelatinase A) and MMP-9 (gelatinase B), respectively and the 77.9 and 91.5 kDa gelatinases are possibly the proenzyme forms of MMP-2 and -9. The 42.8 kDa porcine gelatinase is possibly a collagenase or stromelysin. The 68.7 and 86.1 kDA gelatinases produced by ovine embryos are also possibly MMP-2 and MMP-9, respectively. The confounding results encountered with the bovine embryos, particularly with the metal chelators, are less supportive but the 72.5 and 88.3 kDa gelatinases may be MMP-2 and -9, respectively. The 80.9 kDa gelatinase is possibly the proenzyme of MMP-2, considering the 8 kDa difference in molecular mass. Similar to porcine embryos, the 43.0 kDa gelatinase observed in bovine embryos may be a collagenase or stromelysin. Although the functional role was not addressed in the present study, production of MMP by porcine, ovine and bovine embryos may be involved in the dramatic changes in morphology that occur during this period of preimplantation development.

PARTIAL CHARACTERIZATION OF GELATINASES PRODUCED BY PREIMPLANTATION PORCINE, OVINE AND BOVINE EMBRYOS

by

RaeAnne Chamberlin

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements of the
degree of

Master of Science

Completed August 8, 1995
Commencement June 1996

Masters of Science thesis of RaeAnne Chamberlin presented August 8, 1995

APPROVED:

Redacted for Privacy

Major Professor Representing Animal Science

Redacted for Privacy

Head of the Department of Animal Sciences

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become a part of the permanent collection of Oregon State University libaries. My signature below authorizes release of my thesis to any reader upon request.

Redacted for Privacy

RaeAnne Chamberlin, Author

ACKNOWLEDGEMENTS

I would like to begin by thanking my committee members, Dr. Theo Dreher, Dr. Frank Moore and Dr. Fred Stormshak. Their interest in my program and time spent reviewing my thesis is greatly appreciated. My major professor and friend, Dr. Fred Menino, I cannot begin to thank. That your wisdom, guidance and encouragement have been instrumental in the development of this thesis project goes without saying but you have also been instrumental in the growth and development of myself. Your influence in my life in the last 6 years has only been a productive and positive experience. I would also like to thank my lab colleagues, Matt Cannon, Tina Clark, and Kelly Schilperoort for the many hours of help with injections, breedings, surgeries and flushes as well as the moral support they have provided me. I would especially like to thank Matt Cannon for everything we have experienced since beginning graduate school together. You are a true friend.

I would like to thank Bob Dickson and Todd Karo for the many last notice kill dates and especially the entertainment and diversions you provided. Many times you put the joy back into being in grad school. All of my friends who kept me in contact with life outside of school and provided many hours of stress relief, especially, Jennifer Duncan, Kristi Vertrees, Mike Bunting, Arwyn and Gerry Larson, and Cara Pascalar.

And most importantly, my family: Dennis Chamberlin for not accepting anything less than my best and truly believing in me, Susan Chamberlin for all the emotional support and giving me the confidence to believe that I can do anything I decide to do and Michael Chamberlin for being the best big brother I could want... and bringing out my stubborn streak. Sometimes not knowing when to quit works to your advantage.

DEDICATION

This thesis is dedicated to the memory of Mike and Sue Laurence, every day I reflect on the wisdom of his words.

TABLE OF CONTENTS

Introduction..... ...1

Review of the Literature..... ...4

Sites for Protease Involvement in Early Embryo Development..... ...4

Evidence for Protease Involvement in Developmental Processes..... ...10

Partial Characterization of Gelatinases Produced by Porcine Embryos..... ...22

Abstract..... ...23

Introduction..... ...24

Materials and Methods..... ...26

Results..... ...30

Discussion..... ...41

Literature Cited..... ...45

Partial Characterization of Gelatinases Produced by Ovine and Bovine Embryos..... ...47

Abstract..... ...48

Introduction..... ...49

Materials and Methods..... ...52

Results..... ...57

Discussion..... ...67

Literature Cited..... ...72

Conclusion..... ...75

Bibliography..... ...76

LIST OF FIGURES

Figure — Page

1.1 Zymograph of conditioned medium recovered from Days 6 (Lane 3), 7 (Lane 4), 8 (Lane 5), 9 (Lane 6), 10 (Lane 7) and 11 (Lane 8) porcine embryos incubated with (a) 5 mM $CaCl_2$ or (b) 0 mM $CaCl_2$.....31

1.2 Zymograph of conditioned medium and embryonic tissues from Day 13 porcine embryos.....32

1.3 Zymographic analysis of pooled conditioned medium recovered from Day 12 porcine embryos.....33

1.4 Zymographic analysis of pooled conditioned medium recovered from Day 12 porcine embryos.....35

1.5 Zymograph of pooled conditioned medium recovered from Day 12 porcine embryos.....36

1.6 Zymograph of pooled conditioned medium recovered from Day 12 porcine embryos.....39

1.7 Zymographic analysis of Day 12 porcine embryonic tissues treated with 0 (lane 2) or 1 mM aminophenyl mercuric acetate (lane 3).....40

2.1 Zymograph of pooled conditioned medium recovered from Day 11 ovine embryos.....59

2.2 Zymographic analysis of pooled conditioned medium recovered from Day 11 ovine embryos.....60

2.3 Zymographic analysis of pooled conditioned medium from Day 13-14 bovine embryos.....61

2.4 Zymographic analysis of pooled conditioned medium recovered from Day 11 ovine embryos.....63

2.5 Zymograph of pooled conditioned medium recovered from Day 11 ovine embryos.....64

2.6 Zymographic analysis of Day 13-14 bovine embryonic tissues.....65

2.7 Zymograph of pooled conditioned medium recovered from Day 13-14 bovine embryos.....66

2.8 Zymograph of Day 13-14 bovine embryonic tissues incubated with 0 (lane 1) or 1 mM aminophenyl-mercuric acetate (lane 2).....68

PARTIAL CHARACTERIZATION OF GELATINASES PRODUCED BY PORCINE, OVINE AND BOVINE EMBRYOS

Introduction

During preimplantation development in mammalian embryos, several processes have been proposed to require the production of extracellular proteinases. These processes include removal of the zona pellucida, endodermal and mesodermal cell migration and expansion and elongation of the blastocyst. The oocyte and early preimplantation embryo are surrounded by an extracellular glycoprotein matrix called the zona pellucida. In order to establish direct contact with the uterus, the embryo must eventually shed the zona pellucida. The mechanism employed by the embryo to effect removal of this matrix is believed to involve proteolytic degradation and mechanical pressure in the form of blastocoelic expansion (Domon et al., 1973). Several proteinases have been implicated in the removal of the zona pellucida, including a trypsin-like protease, termed strypsin (Perona and Wassarman, 1986), the plasminogen activator-plasmin system (Menino and Williams, 1987; Menino et al., 1989) and collagenases (Zuccotti et al., 1991).

Production of extracellular proteinases has also been proposed to facilitate the migration of extraembryonic

endodermal and mesodermal cells. At the blastocyst stage the embryo has formed a fluid-filled cavity called the blastocoel and has differentiated into two cell types, inner cell mass (ICM) and trophectoderm. Trophectodermal cells surround the blastocoel and the ICM which is located at one pole of the embryo. Shortly after blastulation, extraembryonic endodermal cells migrate from the ICM and line the blastocoelic side of the trophectoderm. Several days later, mesodermal cells migrate from the embryonic disc and penetrate the region between the trophectoderm and extraembryonic endoderm. Because endodermal or mesodermal cell migration occurs either over or through an extracellular matrix, it is likely that extracellular proteinases are involved in this tissue remodelling.

The expansion and elongation phase in livestock embryo development is another likely event requiring the participation of extracellular proteinases. The porcine embryo, for example, is transformed from a 10 mm sphere to a 100 cm filament at a rate of 35-40 mm/h on Day 12 of development (Geisert et al., 1982). This rapid elongation is not accompanied by an increase in the rate of cell division (Geisert et al., 1982). Blastocyst elongation is believed to occur through alterations in microfilaments and junctional complexes in trophectodermal cells and formation of filopodia by endodermal cells (Geisert et al., 1982).

These three processes have in common remodelling of an extracellular matrix. The matrix metalloproteinase family of

enzymes is known for its involvement in extracellular matrix remodelling and cellular migration. Therefore, the objective of this thesis was to investigate the production of one class of matrix metalloproteinases, specifically, the gelatinases or matrix metalloproteinases-2 and -9 (MMP-2 and -9), during removal of the zona pellucida, endodermal and mesodermal cell migration, and expansion and elongation in porcine, ovine and bovine embryos. Experiments were designed to detect production of gelatinases in each species and partially characterize the enzymes produced.

Review of the Literature

Sites for Protease Involvement in Early Embryo Development

Removal of the Zona Pellucida

The oocyte and cleavage stage embryo are surrounded by an acellular glycoprotein matrix termed the zona pellucida. The zona pellucida is secreted during the growth phase of the maturing oocyte (Wassarman et al., 1984). The zona pellucida has several important functions, including providing sperm receptors for fertilization, participation in the block to polyspermy and maintenance of embryo integrity as the embryo travels through the oviduct (Wassarman et al., 1984). For most species, the embryo must be devoid of this matrix before it can implant into the uterus. In mammals, two mechanisms have been proposed to work in concert to effect removal of the zona pellucida. They include proteinase degradation from either uterine or embryonic sources and mechanical forces in the form of hydrostatic pressure resulting from blastocoelic expansion (Domon et al., 1973). The process of zona pellucida removal has also been referred to as hatching; however, some investigators will argue that in mouse embryos this term should be reserved for the in vitro event and is not equivalent to in vivo loss of the zona pellucida. Such a

clear distinction between in vitro and in vivo loss of the zona pellucida has not been extended to the livestock species; hence, the term hatching is synonymous with in vitro or in vivo removal of the zona pellucida. Hatching in the livestock species occurs on days 6, 9 and 10 for porcine, ovine and bovine embryos, respectively. Although a discrete hatching enzyme has not been elucidated, several investigators have proposed different enzymes or enzyme systems. Joshi and Murray (1974) isolated an endopeptidase from rat uterine fluid that was present regardless of stage of the estrous cycle. Rosenfeld and Joshi (1977) further suggested that this endopeptidase may play a role in dissolution of the zona pellucida. Hoversland and Weitlauf (1982) reported that mouse embryos incubated in vitro with uterine fluid from estrogen and progesterone-treated, ovariectomized mice were more soluble in sodium isothiocyanate than those incubated with control buffer alone. Perona and Wassarman (1986) reported that strypsin, a trypsin-like protease, was localized to cells of the mural trophectoderm prior to hatching, whereas post-hatching, strypsin was found associated with the exact site on the murine zona pellucida where the embryo emerged. Sawada et al. (1990) described a similar trypsin-like protease secreted by mouse embryos during hatching.

In lower animals, such as sea urchins (Lepage and Gauche, 1989, 1990; Nomura et al., 1991), the medaka (Yasumasu et al., 1992) and squid, (Paulij et al., 1992) hatching enzymes have been identified that are involved in loss of the vitelline

envelope or chorion, the nonmammalian analogs of the zona pellucida. Interestingly, these enzymes have homology to the mammalian family of matrix metalloproteinases (MMP), in particular gelatinases A and B, which are alternatively known as MMP-2 and -9, respectively. Evidence that collagenases may act as a zonalytic agent was provided by Zuccotti et al. (1991), who reported that preparations of collagenase were effective in completely dissolving zonae pellucidae of mice and hamsters. Another proteolytic system that has been speculated to be involved with hatching in mammals including cattle (Menino and Williams, 1987; Coates and Menino, 1994) and sheep (Menino et al., 1989) is the plasminogen activator-plasmin system. Production of plasminogen activator (PA) is initiated during the morula-blastocyst transition in sheep embryos and during blastocoel expansion in cattle embryos and remains elevated throughout hatching (Menino and Williams, 1987; Menino et al., 1989). Sheep embryos cultured with plasminogen have increased incidences of hatching and solubility of the zona pellucida in acidified PBS is enhanced (Menino et al., 1989).

Endodermal and Mesodermal Cell Migration

Embryo development through the early cleavage stages is typified by the maintenance of totipotency among the blastomeres. At the morula stage, extracellular microenvironments are formed that induce the blastomeres to differentiate into one of two cell lineages at the subsequent cell stage. When the embryo develops into a blastocyst, a cavity or blastocoel is formed, and two cell types have differentiated, trophectodermal cells and inner mass cells. Trophectodermal cells are found completely surrounding the blastocoel and the inner cell mass (ICM) is found as a group of cells at one pole of the embryo. In bovine embryos endodermal cells will leave the ICM on Day 8 and by Day 10 completely line the blastocoelic side of the trophectoderm (Betteridge and Flechon, 1988). The collective layer formed of extraembryonic endoderm and trophectoderm is called the trophoblast. On Day 14 in bovine embryos mesodermal cells from the embryonic disc migrate between the extraembryonic endoderm and trophectoderm to form a contiguous layer of mesoderm by Day 18 (Betteridge and Flechon, 1988). In ovine embryos endodermal cells have completely lined the trophectoderm by Day 10 (Wintenberger-Torres and Flechon, 1974). Collagen fibers are present in the basal lamina between the trophectoderm and extraembryonic endoderm by Day

10 (Wintenberger-Torres and Flechon, 1974). In porcine embryos, fibronectin, an extracellular matrix glycoprotein, first appears on the blastocoelic surface of the inner cell mass on Day 6 of development. By Day 8, fibronectin can be found completely lining the blastocoelic surface of the trophectoderm (Richoux et al., 1989). It is along this surface that porcine endodermal cells migrate to form a continuous layer of endoderm by Day 10 (Geisert et al., 1982). Extracellular laminin appears in the blastocyst when the endoderm is established as a continuous monolayer (Richoux et al., 1989). The layer of fibronectin between the endoderm and the trophectoderm gradually thickens and on Days 12-13 mesodermal cells migrate from the embryonic disc through the fibronectin matrix. At this point, laminin is found at the junction of endoderm and mesoderm and trophectoderm and mesoderm (Richoux et al., 1989). Richoux et al. (1989) have proposed that endodermal and mesodermal cells use fibronectin to migrate and that laminin is secreted when migration is complete to stabilize cell-matrix interactions.

Expansion and Elongation

After shedding the zona pellucida, porcine embryos undergo a period of rapid expansion and elongation, increasing in size from a 150 μm to a 9 mm sphere within five days. During this phase of expansion, cellular hyperplasia is

apparent and the growth rate is 0.25 mm/h (Geisert et al., 1982). After reaching a size of 10 mm on Day 12, the growth rate increases to 35-40 mm/h and cellular hyperplasia is not believed to be a factor (Geisert et al., 1982). Geisert et al. (1982) proposed that blastocyst elongation occurs through alterations in microfilaments and junctional complexes of trophectodermal cells and formation of filopodia by endodermal cells. It is probable that proteinases are involved in the restructuring necessary for these alterations. Although, ovine and bovine embryos undergo a similar expansion and elongation process, it does not occur at the speed observed for porcine embryos. Ovine embryos undergo the transition in morphology beginning on Day 10 at 0.4-0.9 mm in size and reaching 10-22 cm in length on Day 12 (Wintenberger-Torres and Flechon, 1974). Bovine embryos develop from a 3 mm sphere on Day 11 to a 25 cm filament by Day 17 (Betteridge and Flechon, 1988). Blair et al. (1994) suggest that decreased diversity in the size of porcine embryos may be associated with increased embryonic survivability. The mechanism involved in this process may include changes to the uterine environment induced by the onset of embryonic estrogen production by mor advanced embryos.

Evidence for Protease Involvement in Developmental Processes

There are two major enzyme systems suspected to be involved in processes in early embryo development. They are the plasminogen activator-plasmin system and the matrix metalloproteinases . Because of the direction of the thesis project, the literature review will focus primarily on the matrix metalloproteinases.

Plasminogen Activators and Plasmin

Biochemistry

Plasminogen activators (PA) are serine proteinases that are relatively specific for the proenzyme, plasminogen. Plasminogen activators cleave the serum zymogen plasminogen into plasmin. Plasmin is also a serine protease but has a much broader substrate range. Two types of PA have been elucidated based on molecular weight, fibrin affinity and immunological reactivity (Dano et al., 1985). Tissue-type PA (tPA) is deduced to have a molecular mass of 70 kDa while urokinase type PA (uPA) has a molecular mass between 31 and 55 kDa. Tissue-type PA is most commonly involved in fibrinolysis

and is enhanced by the presence of fibrin whereas uPA is not. Urokinase-type PA was originally discovered in high concentrations in human urine and has been found to be involved in inflammation (Laiho and Keski-Oja, 1989) and cell migration (Dano et al., 1985). Conversion to active uPA or tPA involves one proteolytic cleavage resulting in a disulfide-linked two-chain molecule that expresses a high level of enzymatic activity.

Plasminogen Activators in Embryos

Embryonic production of PA has been identified in a variety of species including rats (Leidholm et al., 1975; Zhang et al., 1994), mice (Sherman et al., 1976; Strickland et al., 1976), swine (Mullins et al., 1980; Fazleabas et al., 1983), cattle (Menino and Williams, 1987) and sheep (Menino et al., 1989). Plasminogen activator is expressed by early embryos in a phase specific manner correlating with hatching, endodermal cell migration, implantation and mesodermal cell migration. For example, Fazleabas et al. (1983) observed a biphasic pattern of PA production where the first and second phases corresponded to periods of cell migration (Days 10-12) and cell proliferation (Days 14-16) in porcine embryos. PA is produced during the period of blastocoel expansion and hatching by bovine embryos (Menino and Williams, 1987). In ovine embryos, PA production increases during the transition

from morula to blastocyst and remains elevated through blastocoel expansion and hatching (Menino et al., 1989). In mouse embryos, PA production is associated with trophoblastic penetration of the endometrium and with parietal endodermal cell migration (Sherman et al., 1976; Strickland et al., 1976; Sappino et al., 1989; Strickland and Richards, 1992). In those reports where the type of PA has been determined it has been uPA that has been associated with developmental processes (Laiho and Keski-Oja, 1989). For example, uPA has been localized to the invasive margin of the trophoblast (Sappino et al., 1989), although its limited substrate range rules it out as the enzyme directly catabolizing the extracellular matrix. Also, PA inhibitors had only a partial inhibitory effect (20-40%) on the invasive ability of human cytotrophoblasts (Librach et al., 1991). Carmeliet et al. (1994) demonstrated that PA was not required for normal embryo development in transgenic mice lacking the functional genes for both tPA and uPA. Hence, PA may be a functionally redundant gene. Plasminogen activators are, however, known to activate some latent MMP (Reith and Rucklidge, 1992; Vassalli and Pepper, 1994).

Several investigators suggest the combined action of PA and MMP to be involved in processes of embryo development. For instance, collagenolytic activity of embryoid bodies was activated by treatment with plasmin (Adler et al., 1990). Additionally, murine trophoblast and invasive human

cytotrophoblast cells synthesize both MMP and uPA (Behrendtsen et al., 1992; Librach et al., 1991).

Matrix Metalloproteinases

Biochemistry

Matrix metalloproteinases (MMP) are a family of proteinases speculated to have arisen from a primordial gene (Matrisian, 1992). All MMP share similar structural domains which include a pre-domain, a pro-domain, and a catalytic domain that contains the zinc binding region (Matrisian, 1990, 1992; Woessner, 1991). Additionally, most MMP have a hemopexin domain, named for its similarity to the heme binding protein and possibly a fibronectin and/or collagen domain (Matrisian, 1990, 1992; Woessner, 1991). The fibronectin and collagen domains may be involved in substrate binding (Matrisian, 1990). Matrix metalloproteinases are secreted into the extracellular environment and are believed to be involved in normal matrix degradation as well as some pathological processes (Matrisian, 1992). To satisfy the requirements of MMP the members of this family must degrade at least one component of the extracellular matrix (ECM), contain a zinc ion and be inhibited by chelating agents, be secreted in zymogen form and require activation for proteolytic activity, be inhibited by specific tissue inhibitors of

metalloproteinases and finally, share amino acid similarities (Matrisian, 1990; Woessner, 1991). Matrix metalloproteinases have been classified into three groups of proteinases based loosely on substrate specificities: interstitial collagenases, gelatinases and stromelysins (Matrisian, 1990, 1992; Woessner, 1991). Interstitial collagenases are comprised of MMP-1 (interstitial collagenase) and MMP-5 (neutrophil collagenase; Woessner, 1991; Matrisian, 1992). Matrix metalloproteinase-1 is a 52 kDa enzyme and degrades collagens type I, II, III, VII and X (Woessner, 1991; Matrisian, 1992). Matrix metalloproteinase-5 is a 53 kDa enzyme and is specific for collagens type I, II, III. The gelatinases include MMP-2 (Gelatinase A; 72 kDa) and MMP-9 (Gelatinase B; 92 kDa). Substrates for MMP-2 and MMP-9 include gelatins, collagen IV and V, and elastin. Finally, stromelysins consist of MMP-3 (stromelysin 1; 54 kDa), MMP-10 (stromelysin 2; 54.1 kDa) and MMP-7 (matrilysin; 26.7 kDa). Stromelysins have broad substrate specificity and can degrade extracellular matrix proteoglycans, laminin, fibronectin, gelatin, and the globular portion of basement membrane collagens (Matrisian, 1990).

Gene Regulation

Regulation of MMP occurs at three levels: gene transcription, activation of the secreted latent enzyme and inhibition of the active enzyme. Most MMP are not stored, hence, translation and secretion are closely coupled. Typically, MMP-secreting cells receive a regulatory signal that initiates or represses gene transcription. Initiation signals are as diverse as cell fusion, concanavalin A, urate crystals, heat shock, UV irradiation, epidermal growth factor, interferons, interleukin, autocrine agents and chemical agents such as cAMP, cytochalasin B and D, phorbol diesters and prostaglandin E. Repressive signals include retinoic acid, glucocorticoids, estrogen, and progesterone.

Activation of MMP can be achieved with various proteinases including trypsin and plasmin as well as a varied assortment of chemical agents, such as organomercurials, sodium dodecyl sulfate and HOCl (Woessner, 1991). Springman et al. (1990) proposed a "cysteine switch" mechanism for activation where a highly conserved cysteine at residue 73 is associated with a zinc atom held in the catalytic domain. All modes of activation share the property that they disrupt this bond, allowing a functional active site to form (Springman et al., 1990). Proteinases may cleave the peptide past the cysteine residue, thereby exposing the active site.

Alternatively, the chemical agents may encourage the dissociation of the zinc atom from Cys^{73} which exposes the active site. With the active site exposed by either mechanism the enzyme can then autolytically cleave the peptide on the carboxyl terminal side of the cysteine residue, permanently activating the enzyme (Springman et al., 1990). The autoactivation of MMP-2 is associated with the loss of an 8 kDa segment of the amino terminal domain (Stetler-Stevenson et al., 1989) Cleavage occurs at a single locus with removal of residues 1-80 (Stetler-Stevenson et al., 1989). This results in the removal of three cysteine residues including Cys^{73} (Stetler-Stevenson et al., 1989). The MMP-2 site of the autocatalytic cleavage corresponds to homologous sites for MMP-1 and stromelysin cleavage during their activation by organomercurials (Stetler-Stevenson et al., 1989).

Inhibitors can regulate the amount of ECM degradation by MMP. The tissue inhibitors of MMP (TIMP) gene family is a major component of MMP regulation. It consists of three proteins, TIMP-1, -2 and -3. TIMP-1 and TIMP-2 share a sequence homology of 40% and complete conservation of six disulfide bonds (Matrisian, 1992). TIMP-3 also has six disulfide bonds (Leco et al., 1994). TIMP-1 is the largest player in regulation of MMP. It is a 28 kDa glycoprotein found in many tissues. It reversibly binds to MMP-1 and -3 in a 1:1 molar ratio (Matrisian, 1990). TIMP-1 has also been found to complex with the pro-form of MMP-9 (Matrisian, 1990).

TIMP-2 is an unglycosylated, smaller 20 kDa protein. TIMP-2 has been associated with MMP-2 from cultured tumor cells and secreted in complexes with MMP-9 (Woessner, 1991). It has been found to complex with the pro-form of MMP-2 and inhibit its autoactivation but does not inhibit gelatinolysis. A second molecule of TIMP-1 or -2 is necessary for complete inhibition of ECM catabolism (Matrisian, 1992). TIMP-2 also binds to active MMP in a 1:1 molar ratio and specifically inhibits enzymatic activity (Matrisian, 1990). None of the TIMP found to date have been overly selective in their inhibition of the different MMP (Woessner, 1991). TIMP-3 is a 24 kDa protein that is localized to the ECM unlike TIMP-1 and 2. TIMP-3 is highly expressed in kidney, lung and brain and low levels in bone, in contrast to TIMP-1; suggesting that it may have a specific function (Leco et al., 1994). The mechanism for inhibition by TIMP is unknown. It has been suggested that they may bind to a hydrophobic region close to the scissile bond or interact with the zinc atom rendering the enzyme inactive (Wossener, 1991). Other inhibitors include the broad spectrum serum inhibitor α_2-macroglobulin, as well as α_1-macroglobulin and α_1-inhibitor-3 (Woessner, 1991).

Matrix Metalloproteinases in Embryos

Some evidence already exists for MMP involvement in embryo development at several different stages including,

hatching, endodermal and mesodermal cell migration and implantation. The MMP are potential candidates for involvement in hatching. The hatching enzyme of *Loligo vulgaris* is similar to mammalian MMP because it has a pH optimum of 8.5 and is completely inhibited by EDTA and less sensitive to N-ethyl maleimide and phenylmethyl sulfonyl fluoride (PMSF) inhibition (Paulij et al., 1992). Sea urchin embryos express a collagenase-like hatching enzyme gene (Lepage and Gache, 1990). The gene for this enzyme was sequenced and encodes a pre and pro sequence like mammalian metalloproteinases and has homologies to the activation center and active site of mammalian collagenase, gelatinase and stromelysin (Lepage and Gache, 1990). Additionally, collagenase can completely dissolve mouse and hamster zona pellucida (Zuccotti et al., 1991).

The action of MMP on extracellular matrices makes it a likely candidate for processes involved in endodermal and mesodermal cell migration. Adler et al. (1990) demonstrated that differentiation of embryonal carcinoma cells to embryoid bodies was accompanied by changes in their secretion of metalloproteinases. In addition, increased secretion of TIMP was detected as differentiation increased (Adler et al., 1990). RT-PCR showed increased expression of collagenase, stromelysin and TIMP genes associated with differentiation (Adler et al., 1990). Mouse embryos secrete functional collagenase and stromelysin during peri-implantation

development and endoderm differentiation (Brenner et al., 1989). The mRNA for collagenase, stromelysin and TIMP were detected as maternal transcripts in the unfertilized egg, present at the zygote and cleavage stages and increased at blastulation and endoderm differentiation (Brenner et al., 1989). Finally, fibronectin and laminin, likely substrates for MMP, are found lining the blastocoelic cavity and may be the matrix across which endodermal and mesodermal cells migrate (Richoux et al., 1989)

Again, the ability of MMP to degrade extracellular matrices makes them likely proteinases for invasion of the endometrium by invasive-type embryos. Behrendtsen et al. (1992) identified MMP-9 in cultures from invading trophoblast giant cells. Neutralizing antibodies directed to MMP-9 and TIMP effectively inhibited the degradation of ECM by trophoblasts in vitro (Behrendtsen et al., 1992). Invasive human cytotrophoblast cells synthesize both MMP and uPA (Librach et al., 1991). Metalloproteinase inhibitors and a functionally perturbing antibody specific for MMP-9 completely inhibited cytotrophoblast invasion (Librach et al., 1991). Both ovine trophoblast interferon and human recombinant interferon α inhibited the production of latent MMP-1 and -3 but had no effect on the secretion of latent MMP-2 from ovine trophoblast cells (Salamonsen et al., 1994). Interstitial collagenase has been localized to both the cytotrophoblast and syncytiotrophoblast cells (Moll and Lane, 1990). First

trimester trophoblasts secreted both MMP-2 and -9 in large amounts compared to third trimester trophoblast cells (Shimonovitz et al., 1994). The mRNA expression of these collagenases also correlated with activity of the enzymes (Shimonovitz et al., 1994). TIMP-1 has suppressive action on the invasion of human trophoblast cells (Graham and Lala, 1991).

Finally, Salamonsen et al. (1993) demonstrated in vitro induction of pro-MMP-1 and -2 by ovine endometrial cells. Ovine trophoblast interferon inhibited the production of MMP-1 and -3 by cultured ovine endometrial cells (Salamonsen et al., 1994). Additionally, TIMP-1 was expressed by the ovine endometrium during early pregnancy, increasing after Day 10 and continuing through Day 20 of pregnancy (Hampton et al., 1995). Two transcripts of TIMP-2 were differentially expressed during early pregnancy in the ewe (Hampton et al., 1995). The 1.0-kb transcript increased from Day 4 to Day 12 and remained high until Day 20, whereas the 3.5-kb transcript decreased after Day 14 (Hampton et al., 1995). TIMP-1 and -2 were expressed at high levels on Days 10 through 14 (Hampton et al., 1995). In this study, mRNA for pro-MMP-1 and -3 was not detected in any of the same tissues. The relationship between MMP and TIMP expression during early pregnancy in sheep, particularly, at the time of embryonic attachment, may explain the limited invasive ability of ruminant trophoblast in utero.

There are several processes during the development of the preimplantation stage livestock embryo the require that remodeling of the extracellular matrix and/or cell migration. The objective of this thesis was to examine the production of extracellular proteinases, specifically gelatinases, by porcine, ovine and bovine embryos.

PARTIAL CHARACTERIZATION OF GELATINASES PRODUCED BY PORCINE EMBRYOS

ABSTRACT

Gelatinases produced by porcine embryos during the preimplantation period were identified in conditioned medium by SDS-PAGE gelatin zymography. Embryos were collected from crossbred gilts and sows 6 to 13 days after mating and cultured for 24 to 48 h in αMEM containing 0.15% BSA in a humidified atmosphere of 5% CO_2 in air at 39°C. Porcine embryos produced a 69.7 kDa gelatinase during Days 6 to 13 of development. Three additional gelatinases (91.5, 84.1 and 77.9 kDa) were observed during Days 12 to 13 and a low molecular mass gelatinase (42.8 kDa) was detected on Days 11 and 12. Gelatinolytic activity was calcium-dependent and not detected in calcium-free conditions. The calcium and metal chelators, EDTA, EGTA and 1,10-phenanthroline completely inhibited gelatinase activity. Substituting strontium or barium for calcium restored gelatinolytic activity. The cysteine protease inhibitors, N-ethyl maleimide and leupeptin, the serine protease inhibitors, benzamidine and soybean trypsin inhibitor, and the aspartic acid protease inhibitor, pepstatin A, did not inhibit gelatinase activity. Incubation with aminophenyl-mercuric acetate caused a decrease in activity of the 77.9 kDa gelatinase and a concomitant increase in activity of the 69.7 kDa gelatinase. The optimal pH for gelatinase activity was 8.1 to 8.4. These results suggest

that the gelatinases produced by porcine embryos are matrix metalloproteinases (MMP). The 69.7 and 84.1 kDa gelatinases are likely MMP-2 (gelatinase A) and MMP-9 (gelatinase B), respectively. The 77.9 and 91.5 kDa gelatinases are possibly the proenzymes of gelatinase A and B, respectively. The 42.8 kDa gelatinase is possibly a collagenase or stromelysin. Metalloproteinases produced by porcine embryos may be involved in the dramatic changes in morphology that occur during this period of preimplantation development.

INTRODUCTION

Early embryo development in pigs is typified by a dramatic morphologic transformation from a 150 μm sphere on Day 1 to a 100 cm filament on Day 16 (Anderson, 1978). Several events occur during this period including activation of the embryonic genome, cavitation, differentiation of the inner cell mass and trophectoderm, loss of the zona pellucida, endodermal cell migration from the inner cell mass and mesodermal cell penetration of extraembryonic endoderm and trophectoderm. Loss of the zona pellucida and endodermal and mesodermal cell migration are events that may require protease involvement. In nonmammalian species such as the sea urchin (Roe and Lennarz, 1990), squid (Paulij et. al., 1992) and medaka (Yasumosa et al., 1992), hatching enzymes have been

identified that participate in the loss of the vitelline envelope or chorion, the nonmammalian analog of the zona pellucida. These enzymes share similar sequences with mammalian matrix metalloproteinases (MMP), specifically, gelatinases. Zuccotti et al. (1991) reported that preparations of collagenase were effective in completely dissolving zonae pellucidae of mice and hamsters. A serine protease termed "strypsin" (Perona and Wassarman, 1986) has been suggested as participating in hatching in mice and the plasminogen activator - plasmin system has been speculated to be involved in hatching in cattle and sheep (Menino and Williams, 1987; Menino et al., 1989).

Plasminogen activators have been implicated in endodermal cell migration and trophoblast penetration of the endometrium in mice (Strickland et al., 1976; Sappino et. al. 1989; Herz et al., 1993). Brenner et al. (1989) observed production of several classes of MMP including the 70 and 92 kDa gelatinases, collagenase and stromelysin, during mouse blastocyst outgrowth, implantation and egg cylinder development. Reports evaluating protease production by porcine embryos are limited. Fazleabas et. al. (1983) observed a biphasic pattern of plasminogen activator production in which the primary phase (Day 10-12) occurred during early elongation of the embryo and the secondary phase (Day 14-16) coincided with cellular proliferation. Whether the plasminogen activator system is directly involved or if it

serves to activate another family of enzymes to participate in the cell migration and tissue remodelling events in the embryo is not known. There is a precedence for this model because some latent MMP can be activated by the plasminogen activator system (Reith and Rucklidge, 1992; Vassalli and Pepper, 1994). MMP expression can also be stimulated through cellular interactions with the extracellular matrix (Werb et al., 1989). Hence, a potential function of plasminogen activator in pig embryos may be to activate latent MMP for further involvement in the morphologic changes accompanying early development. However, MMP production by porcine embryos has not been characterized. Therefore, the objective of this research was to evaluate MMP produced by porcine embryos during hatching and the transition from spherical to filamentous morphology.

MATERIALS AND METHODS

Embryo Collection and Culture

Crossbred gilts and sows were checked daily for estrus and handmated to one of three boars. Reproductive tracts were recovered at slaughter 6-13 days after estrus and embryos were collected by flushing the reproductive tracts with the alpha modification of Eagle's Minimum Essential Medium (αMEM; Sigma

Chemical Co., St Louis, MO, U.S.A.) containing 1.5% BSA. Embryos were either immediately frozen in snap-cap vials or washed three times and cultured for 24 to 48 h in 100μl microdrops containing αMEM with 0.15% BSA (Sigma, A-4378) under paraffin oil (Fisher Scientific Co., Tustin, CA, U.S.A.) in a humidified atmosphere of 5% CO_2 in air at 39°C. At 24h intervals, conditioned medium was either recovered and replaced with fresh medium or embryos were transferred to fresh microdrops.

Conditioned medium was stored in snap-cap vials at -20°C until assayed for gelatinase activity. At the end of culture, embryos were also recovered and placed in snap-cap vials and stored at -20°C. In order to test for non-specific protease activity associated with constituents of the culture medium, medium without embryos was recovered and assayed under identical conditions.

SDS-PAGE and Zymography

All reagents for SDS-PAGE and zymography were obtained from Sigma unless otherwise indicated. Electrophoresis was performed at 4°C in 12% SDS-polyacrylamide gels copolymerized with 1% gelatin under non-reducing conditions as described by Heussen and Dowdle (1979). Tissue samples were solubilized with 10% SDS and repeated pipetting. Sample aliquots were treated with double strength sample buffer containing 0.125 M

Tris-HCl (pH 6.8), 20% glycerol, 5% SDS, and 0.025% Bromophenol Blue and loaded onto one dimensional slab gels. Low range molecular mass markers (97.4 to 14.4 kDa; BioRad, Richmond, CA, USA) were used as standards. Electrophoresis through the stacking gel was conducted for 1 h at 20 mA for a single gel and 40 mA for a pair of gels. Electrophoresis through the separating gel was conducted for 3 h at 30 mA for a single gel and 60 mA for a pair of gels. Electrophoresis was concluded when the tracker die reached the bottom of the gel. After electrophoresis, gels were incubated in Triton X-100 for one hour and transferred to an incubation bath containing 50 mM Tris-HCl, 5 mM $CaCl_2$ and 0.15 M NaCl (pH 8.4) and shaken for 20-48 h at room temperature. Gels were fixed and stained with 1 g/L amido black (BioRad) in 10:30:60 acetic acid:methanol:water for 1 h. and destained in 10:30:60 acetic acid:methanol:water. The appearance of clear lytic zones or bands against the darkly stained background indicated gelatinase activity.

Metal Ion Dependence

Metal ion dependence was evaluated by incubating gel slices in incubation baths containing 5 mM $MgCl_2$, $SrCl_2$, $ZnCl_2$, $MnCl_2$, or $BaCl_2$ in place of $CaCl_2$ for 20-48 h.

Determination of Optimum pH

The optimal pH range for gelatinase activity was determined by incubating gel slices in the incubation bath with the pH adjusted to 4.3, 5.9, 7.3, 8.1, 8.5 and 9.8. Three to four times during the incubation, the bath was replaced with fresh buffer and the pH of the used bath recorded. After incubation for 20-48 h the gels were fixed and stained as described and the pH of the bath recorded.

Inhibitor Specificity

For inhibitor specificity studies, sample media and tissue were combined with double strength sample buffer and electrophoresed as described above. Gel slices were incubated in the incubation bath described above with the addition of a single inhibitor. The inhibitors used were 0.3 mM pepstatin A, 16 mM N-ethyl maleimide (NEM), 1 mM leupeptin, 16 mM phenyl methyl sulfonyl fluoride (PMSF), 100 μg/ml soybean trypsin inhibitor, 10 mM benzamidine, 10 mM 1,10-phenanthroline, 10 mM EDTA, and 10 mM EGTA. All slices were incubated for 20-48 h and fixed and stained. Loss of gelatinase activity in gels incubated with inhibitors was evaluated by comparison with gels in the control incubation bath.

Aminophenyl-mercuric Acetate Activation

Activation of latent collagenases by the organomercurial, aminophenyl-mercuric acetate (APMA) was performed as described by Stetler-Stevenson et al. (1989). Conditioned medium was incubated with a final concentration of 0.05 N NaOH with and without 0.01 M APMA for 2 h at 37°C. The sample was then combined with double-strength sample buffer and electrophoresed as described above.

RESULTS

Two hundred and twenty nine embryos were recovered from 21 gilts and sows for a mean recovery rate of 10.33 ± 1.05 embryos per animal.

Temporal Expression

Gelatin zymography revealed several gelatinases in porcine embryo tissue and conditioned medium ranging in gestational age from Day 6 to Day 13 after estrus. The molecular mass of each band is the mean of all the molecular mass measurements for that band. Five gelatinolytic bands were observed in conditioned media: 91.5 ± 0.32, 84.1 ± 0.7, 77.9 ± 1.2, 69.7 ± 0.4 and 42.8 ± 2.4 kDa (Figs. 1.1, 1.2

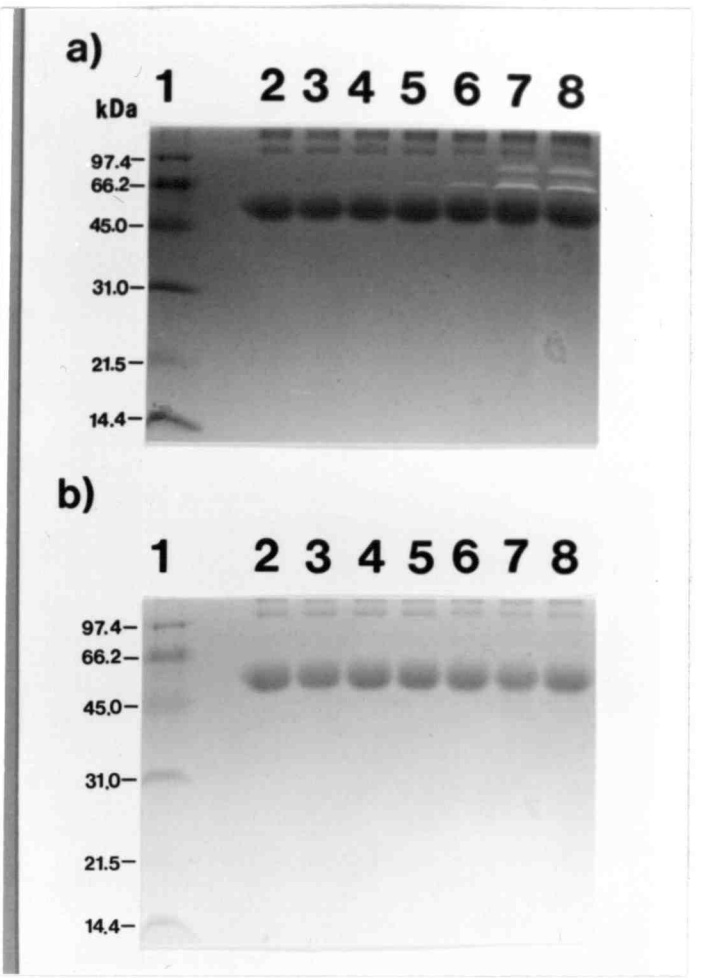

Fig. 1.1 Zymograph of conditioned medium recovered from Days 6 (Lane 3), 7 (Lane 4), 8 (Lane 5), 9 (Lane 6), 10 (Lane 7) and 11 (Lane 8) porcine embryos incubated with (a) 5 mM $CaCl_2$ or (b) 0 mM $CaCl_2$. Molecular mass standards and control medium not containing embryos are in lanes 1 and 2, respectively.

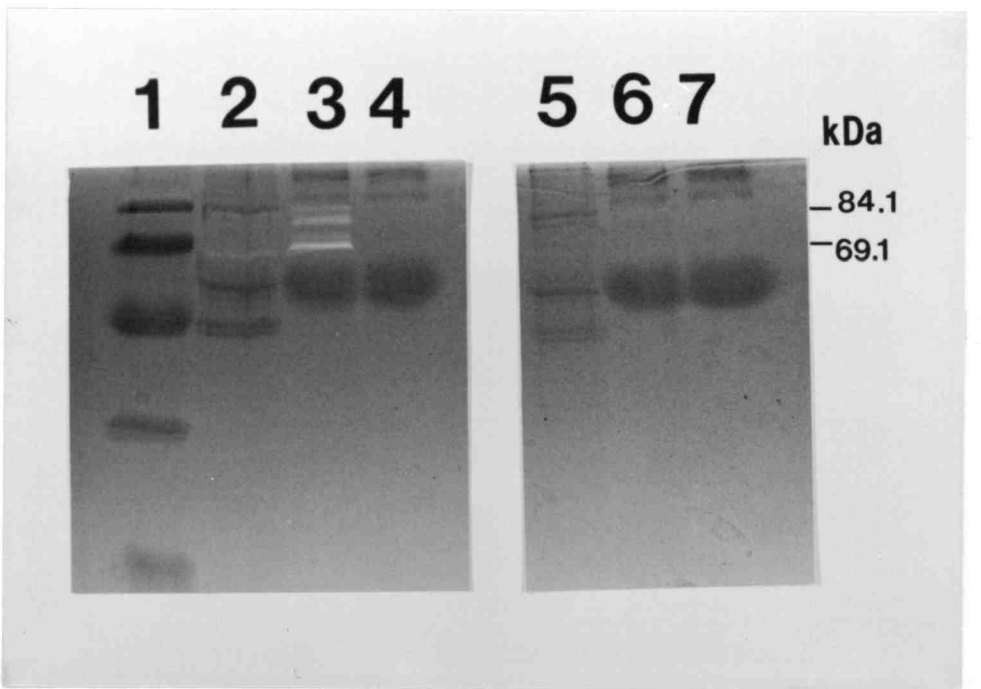

Fig. 1.2 Zymograph of conditioned medium and embryonic tissues from Day 13 porcine embryos. Lanes 1,2,3 and 4 are in the gel incubated with 5 mM $CaCl_2$ and lanes 5,6 and 7 are in the gel incubated with 0 mM $CaCl_2$. Embryonic tissues are in lanes 2 and 5, conditioned media are in lanes 3 and 6 and control media not containing embryos are in lanes 4 and 7. Molecular mass standards are in lane 1.

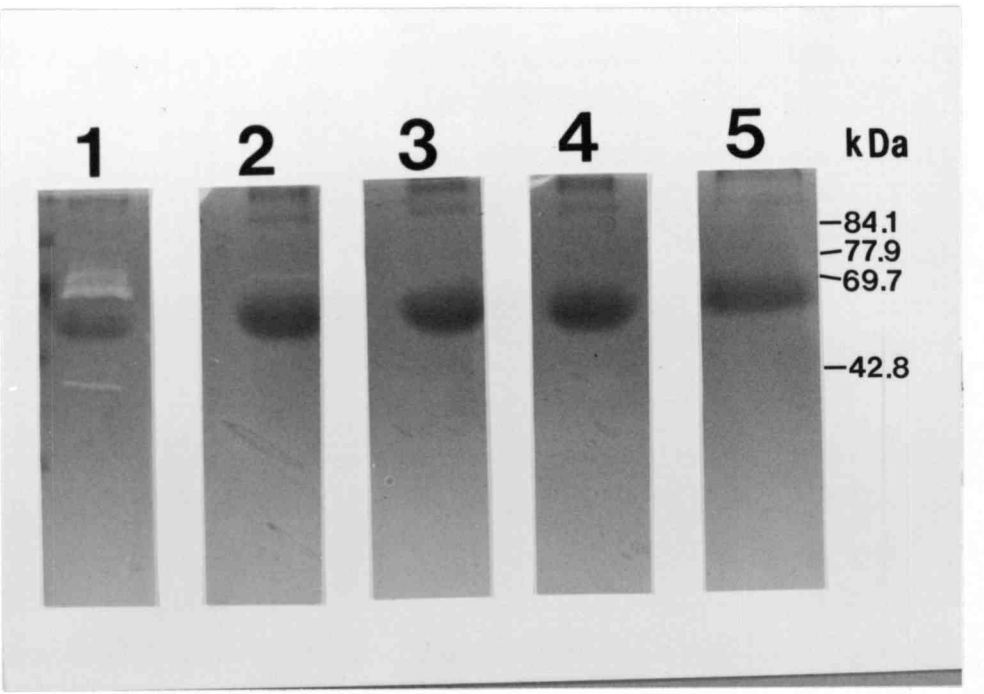

Fig. 1.3 Zymographic analysis of pooled conditioned medium recovered from Day 12 porcine embryos. Gel slices were incubated in Tris-HCl buffer containing 0 M $CaCl_2$ (lane 2) or 5 mM $CaCl_2$ (lane 1) with 10 mM EGTA (lane 3), 10 mM EDTA (lane 4), or 10 mM 1,10 phenanthroline (lane 5).

and/or 1.3). Embryonic tissues also expressed four gelatinases: 83.0 ± 1.4, 74.9 ± 2.5, 67.7 ± 0.7 and 45.1 ± 1.0 kDa. The 69.7 kDa gelatinase was detected in conditioned medium collected from Day 6-13 porcine embryos cultured for 24 to 48 h (Figs. 1.1, 1.2 and 1.3). The 67.7 kDa gelatinase was detected in Day 9-14 embryo tissues collected and cultured for 24 to 48 h or frozen immediately after collection. The 84.1 and 77.9 kDa gelatinases were detected in conditioned medium on Days 9-13 (Fig. 1.1 and 1.2) and the 83.0 kDa gelatinase on Days 10-13 in the embryo tissue. The 91.5 kDa gelatinase was observed in conditioned medium on days 12 and 13 (Figs. 1.3 and 1.4). The 74.9 kDa gelatinase was observed in embryo tissue on Day 13 (Fig. 1.2). The 45.1 kDa gelatinase was detected in the conditioned medium on Days 11 and 12 (Fig. 1.3) and the 42.8 kDa gelatinase was detected on Days 12-14 in embryonic tissue.

Metal Ion Dependence

Gelatinase activity was dependent on the type of metal ion. Incubating gels in the incubation bath containing $CaCl_2$ resulted in the appearance of one or more gelatinases (Fig. 1.5). When gels were incubated in the incubation baths without $CaCl_2$ gelatinase activity either did not appear or was comparably weaker in activity. $SrCl_2$ and $BaCl_2$ restored gelatinolytic activity lost by the removal of calcium from the

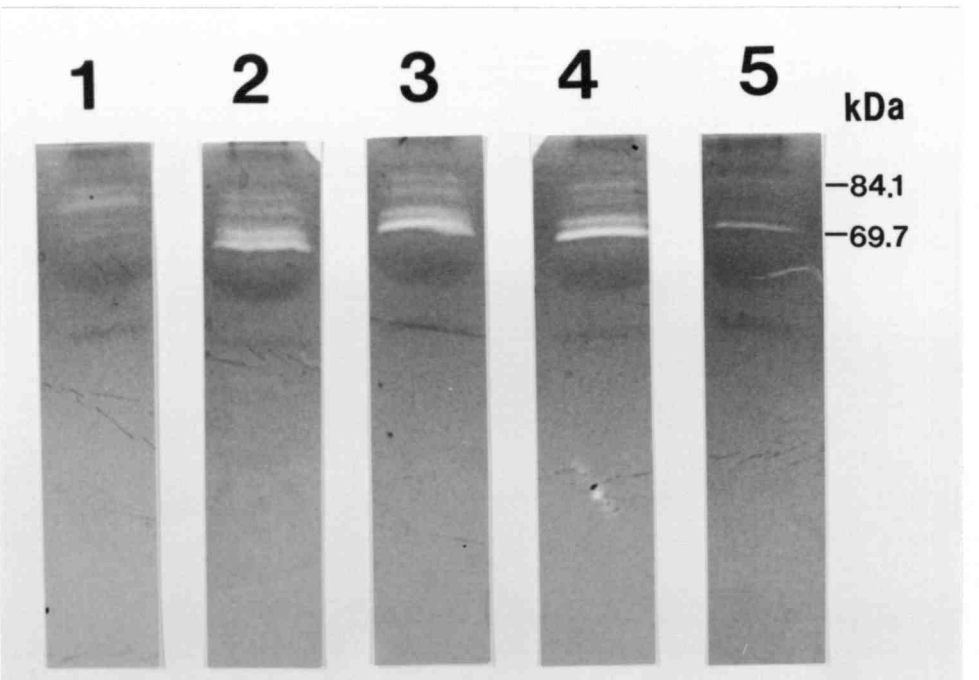

Fig. 1.4 Zymographic analysis of pooled conditioned medium recovered from Day 12 porcine embryos. Gel slices were incubated in Tris-HCl buffer containing 5 mM $CaCl_2$ with the pH adjusted to 9.8 (lane 1), 8.4 (lane 2), 8.1 (lane 3), 7.3 (lane 4) or 5.9 (lane 5).

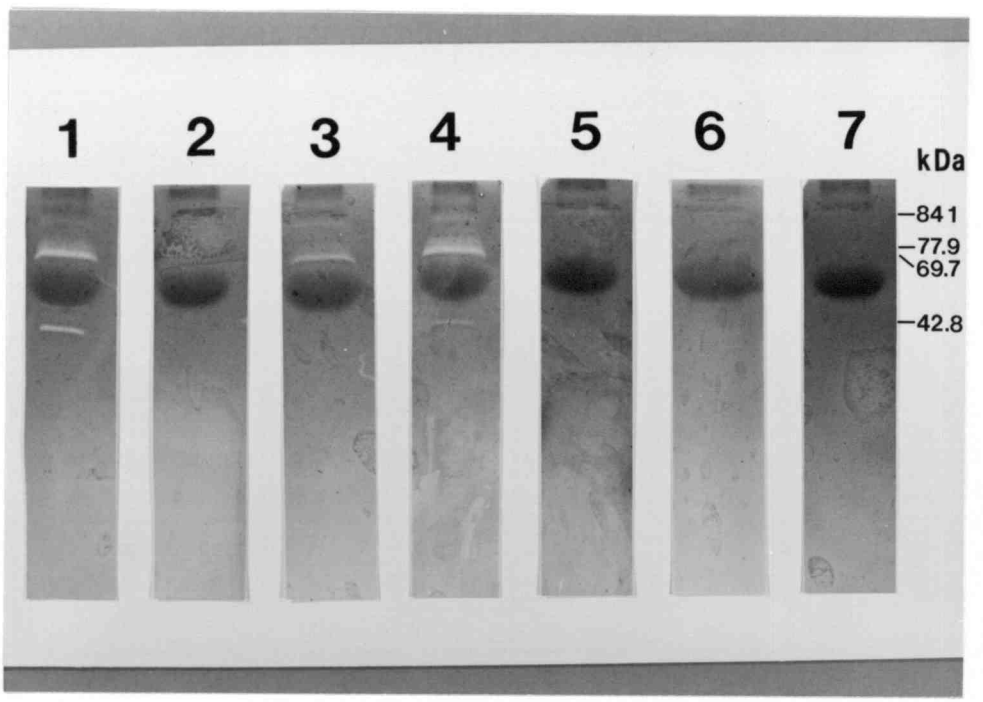

Fig. 1.5 Zymograph of pooled conditioned medium recovered from Day 12 porcine embryos. Gel slices were incubated in Tris-HCl buffer containing 5 mM $CaCl_2$ (lane 1), 0 mM $CaCl_2$ (lane 2), 5 mM $SrCl_2$ (lane 3), $BaCl_2$ (lane 4), $MgCl_2$ (lane 5), $MnCl_2$ (lane 6) or $ZnCl_2$ (lane 7).

incubation bath. $MnCl_2$ and $MgCl_2$ weakly supported some gelatinolytic activity whereas $ZnCl_2$ was not supportive.

Determination of pH Optimum

All four gelatinases were detected in pH 8.1 and 8.5 incubation baths (Fig. 1.4). Gelatinase activity was reduced by raising or lowering the pH. The optimal pH range for these proteinases was approximately 8.1 to 8.5.

Inhibitor Specificity

Gelatinase activity was completely inhibited by the metal ion chelators EDTA, EGTA and 1,10-phenanthroline (Fig. 1.3). Gelatinase activity was not inhibited by the cysteine protease inhibitors, NEM and leupeptin or the serine protease inhibitors soybean trypsin inhibitor (data not shown) and benzamidine or the aspartic acid protease inhibitor, pepstatin A (Fig. 1.6). PMSF, a serine protease inhibitor, partially inhibited gelatinolytic activity. This inhibition was partly due to the isopropanol used in preparing the PMSF (data not shown).

Aminophenyl Mercuric Acetate Activation

Incubating conditioned medium with APMA caused a decrease in the activity of the 77.9 kDa band and an increase in the activity of the 69.7 kDa band (Fig. 1.7).

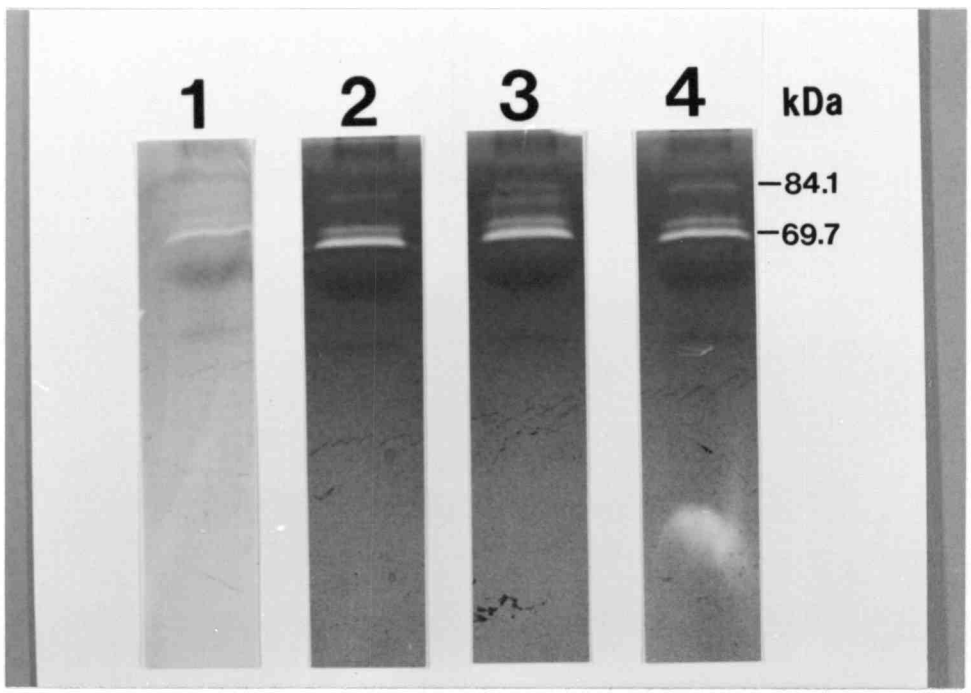

Fig. 1.6 Zymograph of pooled conditioned medium recovered from Day 12 porcine embryos. Gel slices were incubated in 0.3 mM pepstatin A (lane 1), 10 mM benzamidine (lane 2), 16 mM N-ethyl maleimide (lane 3), 1 mM leupeptin (lane 4).

Fig. 1.7 Zymographic analysis of Day 12 porcine embryonic tissues treated with 0 (lane 2) or 1 mM aminophenyl mercuric acetate (lane 3). Molecular mass standards in lane 1.

DISCUSSION

Zymographic analysis has revealed that porcine embryos produce several gelatinases in vitro with molecular masses ranging from 42.8 to 84.1 kDa. These gelatinases demonstrate several properties of MMP. All members of the MMP family have a common ability to degrade at least one component of the extracellular matrix (Matrisian, 1990; Woessner, 1991). The activity of the gelatinases secreted by porcine embryos was completely inhibited by metal ion chelators but not other proteinase inhibitors. The metal ion chelators EDTA, EGTA and 1,10-phenanthroline were the only inhibitors that demonstrated complete inhibition of gelatinase activity. PMSF, a serine protease inhibitor exhibited some inhibition of protease activity that was partly attributed to the isopropanol used to solubilize the PMSF. Other serine proteinase inhibitors, benzamidine and soybean trypsin inhibitor, and the cysteine proteinase inhibitors, NEM and leupeptin, did not have an inhibitory effect on gelatinase activity. The aspartate proteinase inhibitor, pepstatin A, did not have an inhibitory effect on gelatinolytic activity.

Members of the MMP family are secreted in zymogen form (Matrisian, 1990; Woessner, 1991). Addition of organomercurials or certain proteinases to MMP can activate latent enzymes by cleaving a portion of the pro-domain. Removal of the pro-domain allows a conformational change which

destabilizes the association of a cysteine residue with zinc and activates the enzyme (Woessner, 1991). In some members of the MMP family this leads to an autocatalytic reaction that cleaves the remainder of the pro-domain and results in a permanently active enzyme with a molecular mass approximately 10 kDa lower than the pro-enzyme form (Woessner, 1991). When embryo tissues and conditioned medium were incubated with APMA, an increase in gelatinolytic activity was observed at 69.7 kDa band with a concomitant decrease in activity in the 77.9 kDa band. This suggests the loss of an 8 kDa fragment, possibly the portion of the pro-domain.

MMP are active at a pH close to neutrality (Matrisian, 1992). The optimal pH for these gelatinases was approximately 8.1 and 8.5. This indicates that none of the activity was due to lysosomal enzymes which function optimally at pH 5 (Bainton, 1981).

There are three classes of proteinases in the MMP family: collagenases, gelatinases and stromelysins (Woessner, 1991; Matrisian, 1992). Gelatinases are divided into two groups, a 72 kDa enzyme known as MMP-2 and a 92 kDa enzyme called MMP-9 (Matrisian, 1990). The substrates for both MMP-2 and MMP-9 include type IV and V collagen and gelatin. The two major gelatinases observed in the present experiment were the 69.7 and 84.1 kDa enzymes. It is likely that the 69.7 kDa is MMP-2 and the 84.1 kDa is MMP-9. As suggested by incubation with APMA, the 77.9 kDa gelatinase may be the latent or proenzyme

form of the 69.7 kDa gelatinase. Also, the 91.5 kDa gelatinase may be the latent or proenzyme form of the 84.1 kDa gelatinase or MMP-9. Finally, the 42.8 kDa gelatinase could possibly be a stromelysin or a collagenase.

Expression of gelatinolytic activity in porcine embryos can be detected as early as Day 6 of gestation and continues through Day 13. Although the physiologic role of these enzymes was not identified in the present study, it is possible that these enzymes participate in the dramatic morphologic changes occurring in the embryo during this period. Geisert et al. (1982) estimated the initial rate of blastocyst growth to be 0.25 mm/h from the 4 to 9 mm stage and was attributed to cellular hyperplasia. Once past 10 mm in diameter the blastocyst elongates at a rate of 35-40 mm/h (Geisert, 1982) and this process is not attributed to cellular hyperplasia (Geisert et al., 1982). Coincident with these dimensional changes are endodermal and mesodermal cell migration and remodeling of the blastocyst (Richoux, 1989). The extracellular matrix glycoprotein, fibronectin, is detected on the blastocoelic side of the trophectoderm before endodermal cell migration occurs in porcine embryos (Richoux et al., 1989). Endodermal cells leave the inner cell mass on Day 8, migrate over the fibronectin layer adjacent to the trophectoderm and form a continuous layer by Day 10. On Day 12, mesodermal cells migrate through a tridimensional network of fibrillar fibronectin and form a continuous layer by Day 14

(Richoux et al., 1989). Cellular interactions with the ECM can induce MMP production because Werb et al. (1989) demonstrated induction of collagenase and stromelysin gene expression by antibodies to the fibronectin receptor in rabbit synovial fibroblasts. Hence, the production of gelatinases observed in the present study may be involved in the ECM remodeling and cellular migration events that occur at this time.

LITERATURE CITED

Anderson, LL. Growth, protein content and distribution of early pig embryos. Anat Rec 1978; 190:143-154

Bainton, D. 1981. The discovery of lysosomes. J. Cell Biol. 91:66s-76s

Brenner C, Adler R, Rappolee D, Pederson R, Werb Z. Genes for extracellular matrix-degrading metalloproteinases and their inhibitor, TIMP, are expressed during early mammalian development. Genes Devel 1989; 3:848-859

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

Geisert R, Brookbank J, Roberts RM, Bazer F. Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biol Reprod 1982; 27:941-955

Herz J, Clouthier D, Hammer R. LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. Cell 1992; 71:411-421

Heussen C, Dowdle E. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Analy Biochem 1979; 162:196-202

Matrisian L. Metalloproteinases and their inhibitors in matrix remodeling. Trends Genet. 1990; 6:121-125

Matrisian L. The matrix-degrading metalloproteinases. Bioessays 1992; 14:455-462

Menino AR, Dyk AR, Gardner C, Grobner M, Kaaekuahiwi M, Williams J. The effects of plasminogen on In Vitro ovine embryo development. Biol Reprod 1989; 41:899-905

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

Paulij W, Verhoof H, DenucÉ J. Partial purification and characterization of *Loligo Vulgaris* hatching enzyme obtained from hatching medium. Comp Biochem Physiol 1992; 101B:617-622

Perona R, Wassarman P. Mouse blastocysts hatch in vitro by using a trypsin-like proteinase associated with cells of mural trophectoderm. Devl Biol 1986; 114:42-52

Reith A, Rucklidge G. Invasion of brain tissue by primary glioma: evidence for the involvement of urokinase-type plasminogen activator as an activator of type IV collagenase. 1992; 186:348-354

Richoux V, Derribere T, Boucaut J, Flechon J, Thiery J. Distribution of fibronectins and laminin in the early pig embryo. Anatom Record 1989; 223:72-81

Roe J, Lennarz W. Biosynthesis and secretion of the hatching enzyme during sea urchin embryogenesis. J Biol Chem 1990; 265:8704-8711

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

Stetler-Stevenson W, Krutzsch H, Wacher M, Margulies I, Liotta L. The activation of human type IV collagenase proenzyme. J Biol Chem 1989; 264:1353-1356

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

Vassalli J, Pepper M. Membrane proteases in focus. Nature 1994; 370:14-15

Werb Z, Tremble P, Behrendtsen O, Crowey E, Damsky C. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J Cell Biol 1989; 109:877-889

Woessner, Jr. J. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 1991; 5:2145-2154

Yasumasu S, Katow S, Hamazaki T, Iuchi I, Yamagami K. Two constituent proteases of a telostean hatching enzyme: concurrent syntheses and packaging in the same secretory granules in discrete arrangement. Devel Biol 1992; 149:349-356

PARTIAL CHARACTERIZATION OF GELATINASES PRODUCED BY OVINE AND BOVINE EMBRYOS

ABSTRACT

Gelatinases produced by preimplantation ovine and bovine embryos were partially characterized by SDS-PAGE zymography. Embryos were collected from crossbred ewes 7,9 and 11 days after mating and from crossbred cows 12 to 14 days after mating. Embryos were cultured for 24 to 196 h depending on age of the embryo in medium containing 0.15% BSA in a humidified atmosphere of 5% CO_2 in air at 39°C. Ovine embryos produced 68.7 and 86.1 kDa gelatinases on Day 11. Bovine embryos produced 88.3, 80.9, 72.5 and 43.0 kDa gelatinases on Days 12 to 14. Ovine embryonic gelatinases were calcium dependent and were inhibited by the calcium and metal ion chelators, EDTA, EGTA and 1,10-phenanthroline. Substituting strontium or barium for calcium restored ovine embryonic gelatinase activity. Gelatinase activity in bovine conditioned medium was not calcium-dependent and was not completely inhibited by EGTA, EDTA and 1,10-phenanthroline. The serine protease inhibitors, soybean trypsin inhibitor and benzamidine, the aspartic acid protease inhibitor, pepstatin A, and the cysteine protease inhibitor, N-ethyl maleimide, did not inhibit gelatinase activity in either bovine and ovine embryos. Incubation of bovine tissues with aminophenyl-mercuric acetate did not change the apparent molecular mass of the gelatinases produced. The optimal pH for gelatinase

activity was 7.9 to 8.5 for ovine embryos and 8.1 to 8.4 for bovine embryos. These results suggest the gelatinases produced by ovine embryos are matrix metalloproteinases (MMP). The 68.7 and 86.1 kDA gelatinases are likely MMP-2 (gelatinase A) and MMP-9 (gelatinase B), respectively. The confounding results of the bovine study are less suggestive but the 72.5 and 88.3 kDa gelatinases may be MMP-2 and -9 respectively. The 80.9 kDa is possibly the proenzyme of MMP-2 considering the 8 kDa difference in molecular mass. The 43.0 kDa gelatinase is possibly a collagenase or stromelysin. Metalloproteinases produced by preimplantation ovine and bovine embryos may be involved in the cellular migration events and morphologic transformations that occur during this period of development.

INTRODUCTION

Matrix metalloproteinases (MMP) are a family of proteinases involved in extracellular matrix (ECM) degradation and are associated with cell migration and tissue remodelling (Woessner, 1991). MMP share structural homology in at least three domains: a pre-domain that targets the enzyme for secretion, a pro-domain that is lost on activation and a catalytic domain that contains the zinc binding region (Woessner, 1991; Matrisian, 1992). Most MMP also contain a

hemopexin domain and some possess a fibronectin domain similar to the collagen-binding domain of fibronectin. MMP require activation for proteolytic activity that results in the loss of the pro-domain, an approximate 10 kDa fragment. MMP activity is regulated by three inhibitors known as tissue inhibitors of MMP-1, -2 and -3 (TIMP-1,-2 and -3).

Some evidence already exists for MMP involvement in embryo development. Sea urchin and medaka embryos express a hatching enzyme with homology to MMP-2 and -9 (Lepage and Gauche, 1989, 1990; Nomura et al., 1991; Yasumasu et al., 1992; Roe and Lennarz, 1990). Zuccotti et al. (1991) observed that collagenase can completely dissolve mouse and hamster zonae pellucidae. Mouse embryos secrete functional collagenases and stromelysins during peri-implantation development and endodermal differentiation (Brenner et al., 1989). Neutralizing antibodies directed to MMP-9 and TIMP effectively inhibited in vitro the degradation of the ECM by mouse trophoblasts (Behrendtsen et al., 1992). Human cytotrophoblast cells synthesize both MMP and urokinase-type plasminogen activator (Librach et al., 1991).

There are several periods of livestock embryo development where the action of MMP could be involved, including removal of the zona pellucida, endodermal and mesodermal cell migration and embryonic expansion and elongation. Several proteases have been speculated to be involved in hatching including, a trypsin-like protease in the mouse (Perona and

Wassarman, 1986; Sawada et al., 1990), and the plasminogen activator-plasmin system in cattle and sheep (Menino and Williams, 1987; Menino et al., 1989). Hatching occurs on Days 9 and 10 in ovine and bovine embryos, respectively. Another potential site for MMP involvement is endodermal and mesodermal cell migration. Endodermal cells in both species migrate out from the ICM and line the trophectoderm by Day 10. This migration is reported to occur over an ECM adjacent to the trophectoderm (Betteridge and Flechon, 1988). Mesodermal cell migration also occurs between Days 14-18 in bovine and ovine embryos (Betteridge and Flechon, 1988). Collagen is present between the trophectoderm and extraembryonic endoderm in ovine embryos and may serve as the matrix supporting cell migration (Wintenberger-Torres and Flechon, 1974). Hence, MMP may facilitate mesodermal cell migration through this matrix. Unlike embryos that undergo almost immediate implantation such as primates and mice, embryos from the livestock species undergo a period of development prior to implantation that involves rapid expansion and elongation of the embryo. The growth of ovine embryos begins to accelerate on Day 10, measuring 0.4 to 0.9 mm. On Day 14 the ovine embryo can measure 10 cm in length (Wintenberger-Torres and Flechon, 1974). Bovine embryos develop from a 3 mm sphere on Day 13 to a 25 cm filament by Day 17 (Betteridge and Flechon, 1988). In swine, these dimensional changes are believed to occur due to changes in cellular associations and shape and not

proliferation (Geisert et al., 1982). Matrix metalloproteinases may be involved in these cellular association changes.

As the first step in determining the involvement of MMP in early livestock embryo development, the objective of this experiment was to identify and partially characterize gelatinases produced by preimplantation ovine and bovine embryos.

MATERIALS AND METHODS

Embryo Collection and Culture

Ovine embryos

Crossbred ewes were estrous synchronized by one of three protocols. In Experiment 1, two different superovulation regimens were used. The first regimen involved inserting one half of a Synchromate B implant (Sanofi, Overland Park, KS, USA) into the ear for 12 days. On Day 12 (Day 0 = day of implant) the implants were removed and 1000 IU of PMSG (Sigma Chemical Co., St. Louis, MO.) was administered i.m.. Sixteen ewes were synchronized in this manner. In the second regimen, 15 ewes received one half of a Synchromate B implant for 12 days and an injection of 500 IU PMSG on Day 11. In addition, on Days 11, 12 and 13 respectively, 5, 4, and 3 mg of pFSH

(Schering-Plough, Kenilworth, NJ) were administered i.m.. Ewes were observed for estrus at 12h intervals starting at 24 h after implant removal. Ewes were handmated to one of four rams every 12h for as long as they would accept the ram. Mated ewes were randomly scheduled for surgery 7, 9 and 11 days after estrus. In Experiment two, twenty one ewes were administered two injections of 100 μg of Estrumate (Miles, Shawnee Mission, KS) i.m. 9 days apart (Day 0 = Day of first Estrumate injection). Injections of pFSH (Super-Ov; Wyeth-Ayerst Montreal Inc., Canada) were administered i.m. twice daily on Days 8, 9 and 10 for total doses of either 72, 49.5 or 27 U. Ewes were checked for estrus at 12h intervals starting 24 h after the second injection of Estrumate. Ewes were handmated to one of four rams for as long as they would accept the ram. Surgery was performed 11 d after estrus. Anesthesia was induced in ewes by injection of 15 ml of 5% sodium pentothal (Abbott Laboratories, North Chicago, Il, USA) into the jugular vein and was maintained during surgery via inhalation with halothane (Fluothane; Fort Dodge Laboratories, Inc., Fort Dodge, IA) and oxygen. The reproductive tract was exteriorized via ventral midline laparotomy and uteri were flushed in retrograde fashion. Embryos were collected with the alpha modification of Eagle's Minimum Essential Medium (αMEM; Sigma) with 25 mM HEPES and held in αMEM with 25 mM HEPES and 0.15% BSA. After transport to the laboratory, embryos in both experiments were washed three times and

cultured in αMEM with 0.15% BSA to equivalent gestational age Day 15. Conditioned medium was recovered every 24 h until the end of culture.

Bovine embryos

Twenty crossbred beef cows were estrous synchronized by two i.m. injections of 25 mg Lutalyse (Upjohn Co., Kalamazoo, MI) 12 days apart (Day 0 = first Lutalyse injection). Injections of pFSH (Schering-Plough, Kenilworth, NJ) were administered i.m. on Days 10, 11, 12, and 13 at doses of 10, 8, 4 and 2 mg, respectively for a total dose of 24 mg. Cows were observed for estrus beginning 36 h after the last Lutalyse injection. Cows were handmated to one of two bulls. Twelve to 14 days after estrus, embryos were collected by recovering the reproductive tracts at slaughter and flushing the isolated uteri with Ham's F-12 (Sigma) at the laboratory. Embryos were cultured for 24 to 48 h in Ham's F-12 with 0.15% BSA in a humidified atmosphere of 5% CO_2 in air at 39°C. Conditioned medium was recovered at 24 h intervals until the end of culture.

For both ovine and bovine embryos, conditioned medium was stored in snap-cap vials at -20°C until assayed for gelatinase activity. At the end of culture, embryos were also recovered and placed in snap-cap vials and stored at -20°C. To evaluate non-specific protease activity possibly associated with some constituents of the culture medium, medium without embryos was recovered and assayed under identical conditions.

SDS-PAGE and Zymography

All reagents for SDS-PAGE and zymography were obtained from Sigma unless otherwise indicated. Electrophoresis was performed at 4°C in 12% SDS-polyacrylamide gels copolymerized with 1% gelatin under non-reducing conditions as described by Heussen and Dowdle (1979). Electrophoresis of the stacking gel was conducted for 1 h at 20 mA for single gels and 40 mA for double gels. The separating gel was electrophoresed for 3 h at 30 mA for a single gel and 60 mA for two gels. Sample aliquots were treated with double strength sample buffer containing 0.125 M Tris-HCl (pH 6.8), 20% glycerol, 5% SDS, and 0.025% Bromophenol Blue and loaded onto one dimensional slab gels. Low range molecular mass markers (97.4 to 14.4 kDa; BioRad, Richmond, CA, USA) were used as standards. After electrophoresis, gels were incubated in Triton X-100 for one hour and transferred to an incubation bath containing 50 mM Tris-HCl, 5 mM $CaCl_2$ and 0.15 M NaCl (pH 8.4) and shaken for 20-48 h at room temperature. Gels were fixed and stained with 1 g/L amido black (BioRad) in 10:30:60 acetic acid:methanol:water for 1 h and destained in 10:30:60 acetic acid:methanol:water. The appearance of clear lytic zones or bands against the darkly stained background indicated gelatinase activity.

Metal Ion Dependence

Metal ion dependence was evaluated by incubating gel slices in incubation baths containing 5 mM $MgCl_2$, $SrCl_2$, $ZnCl_2$, $MnCl_2$, or $BaCl_2$ in place of $CaCl_2$ for 20-48 h.

Determination of Optimum pH

The optimal pH range was determined by incubating gel slices in the incubation bath with the pH adjusted to 4.3, 5.9, 7.3, 7.9, 8.1, 8.5 or 9.8. Three to four times during the incubation, the bath was replaced with fresh buffer and the pH of the used bath recorded. After incubation for 20-48 h the gels were fixed and stained as described and the pH of the bath recorded.

Inhibitor Specificity

For inhibitor specificity studies, conditioned media or tissue were combined with double strength sample buffer and electrophoresed as described above. Gel slices were incubated in the incubation bath described above with the addition of a single inhibitor. Inhibitors used were 0.3 mM pepstatin A, 16 mM N-ethyl maleimide (NEM), 1 mM leupeptin, 100 μg/ml soybean

trypsin inhibitor, 10 mM benzamidine, 10 mM 1,10-phenanthroline, 10 mM EDTA, and 10 mM EGTA. All slices were incubated for 20-48 h, fixed and stained. Loss of gelatinase activity in gels incubated with inhibitors was evaluated by comparison with gels in the control incubation bath.

Aminophenyl-mercuric Acetate Activation

Activation of latent collagenases by the organomercurial, aminophenyl-mercuric acetate (APMA) was performed as described by Stetler-Stevenson et al. (1989). Conditioned medium was incubated with 0.01 mM APMA dissolved in 0.05 N NaOH or 0.05 N NaOH for 2 h at 37°C. The sample was then combined with double-strength sample buffer and electrophoresed as described.

RESULTS

Sixty-nine embryos were recovered from 17 ewes in Experiment 1 for a mean recovery rate of 4.06 ± 0.66 embryos per ewe and 101 embryos were recovered from 13 ewes in Experiment 2 for a mean recovery rate of 7.77 ± 1.41 embryos per ewe. One hundred thirty-three ova were recovered from 20 cows for a mean recovery rate of 6.68 ± 1.49 embryos per cow.

Temporal Expression

Cultured Day 7 and 9 ovine embryos did not produce detectable gelatinase activity. Conditioned medium from Day 11 ovine embryos produced two gelatinases at 68.7 ± 1.38 (Fig. 2.1) and 86.1 ± 1.39 kDa. Gelatinolytic activity was not detected in ovine embryonic tissues. Four gelatinolytic bands were observed in conditioned media and tissues from Day 12 to 14 bovine embryos: 88.3 ± 1.57, 80.9 ± 1.31, 72.5 ± 0.64 and 43.0 ± 0.47 kDa (Fig. 2.3 and 2.8).

Metal Ion Dependence

Gelatinase activity of ovine embryos was dependent on the type of metal ion present. Incubating gels in the presence of $CaCl_2$ resulted in the appearance of one or two bands (Fig. 2.1). When gels were incubated in the absence of $CaCl_2$ gelatinase activity did not appear. $SrCl_2$ and $BaCl_2$ restored gelatinolytic activity lost by the removal of calcium from the incubation bath. $MnCl_2$, $MgCl_2$ and $ZnCl_2$ were not supportive of gelatinolytic activity.

Determination of Optimum pH

The 68.7 kDa gelatinolytic band of ovine embryos was detected in pH 8.5 and 7.9 incubation baths (Fig. 2.2).

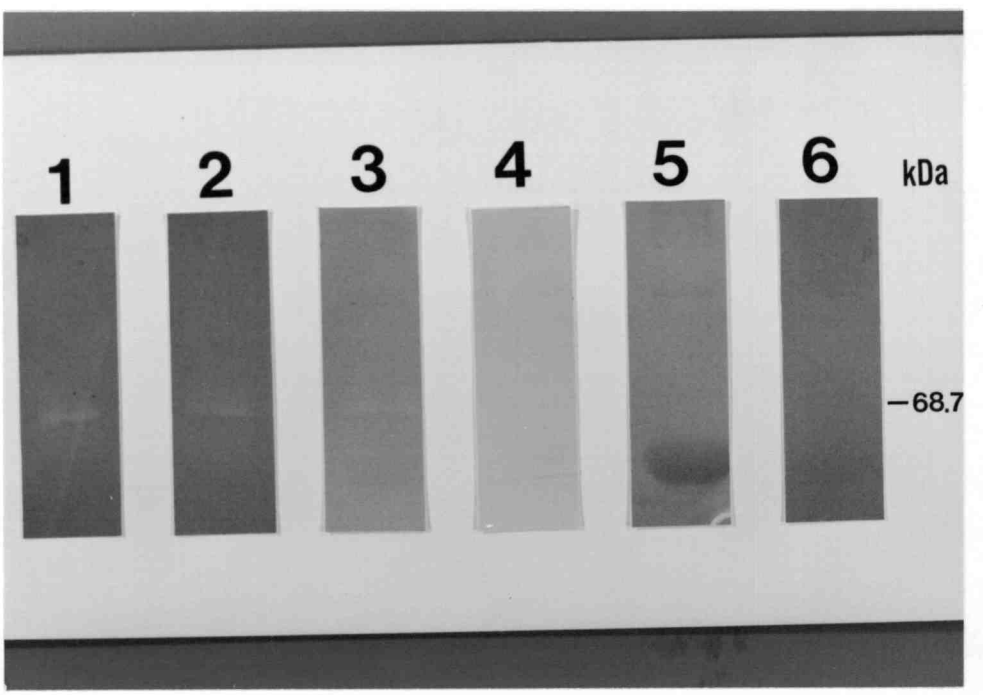

Fig. 2.1 Zymograph of pooled conditioned medium recovered from Day 11 ovine embryos. Gel slices were incubated in Tris-HCl buffer containing 5 mM $CaCl_2$ (lane 1), $SrCl_2$ (lane 2), $BaCl_2$ (lane 3), $MgCl_2$ (lane 4), $MnCl_2$ (lane 5) or $ZnCl_2$ (lane 6).

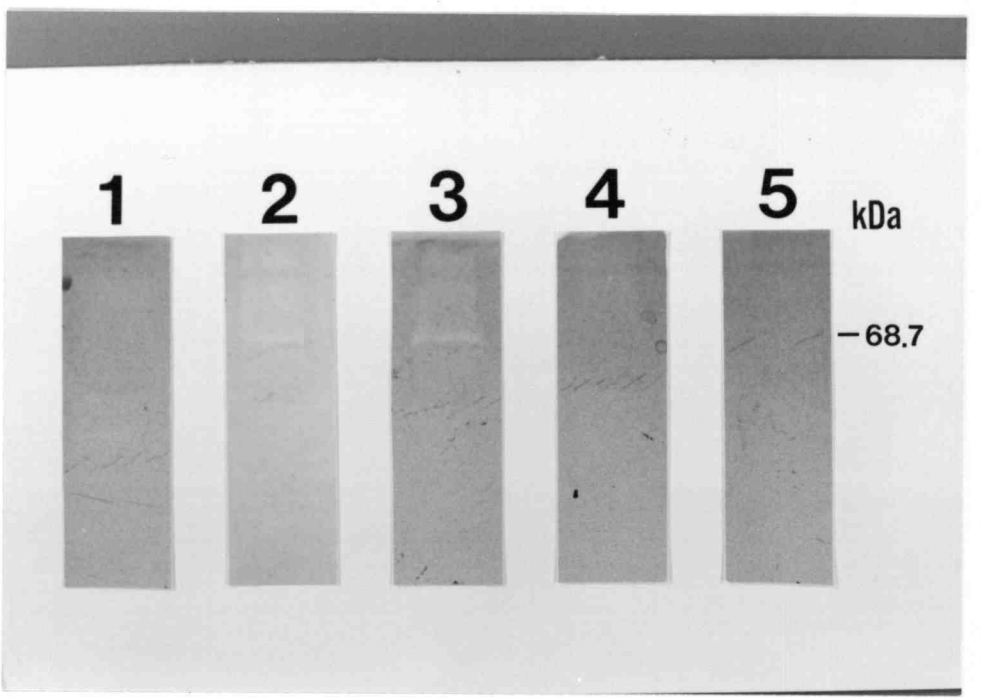

Fig. 2.2 Zymographic analysis of pooled conditioned medium recovered from Day 11 ovine embryos. Gel slices were incubated in Tris-HCl buffer containing 5 mM $CaCl_2$ with the pH adjusted to 9.8 (lane 1), 8.5 (lane 2), 7.9 (lane 3), 5.3 (lane 4) or 4.3 (lane 5).

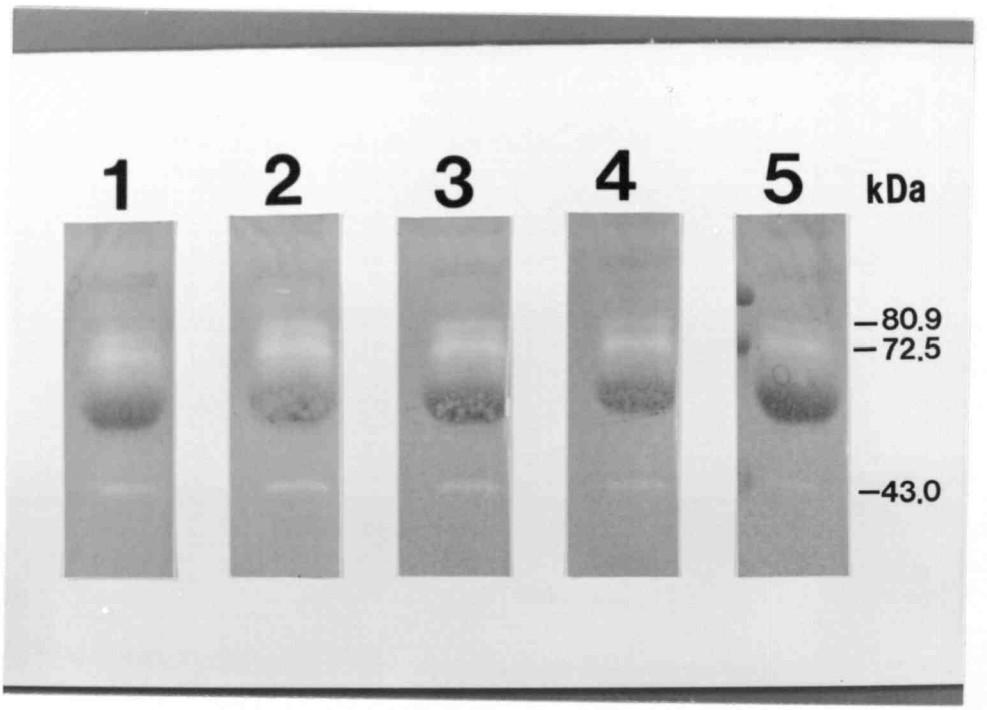

Fig. 2.3 Zymographic analysis of pooled conditioned medium from Day 13-14 bovine embryos. Gel slices were incubated in Tris-HCl buffer containing 5 mM $CaCl_2$ with the pH adjusted to 9.8 (lane 1), 8.4 (lane 2), 8.1 (lane 3), 7.3 (lane 4) or 5.3 (lane 5).

Gelatinase activity was not detected at higher or lower pH. The optimal range for ovine embryo gelatinase activity was approximately 8.5 to 7.9.

All four bovine gelatinases were detected in pH 8.5 and 8.1 incubation baths (Fig. 2.3). As the pH of the incubation baths was raised and lowered the activity of the bands was decreased or lost. The pH optimum for gelatinases produced by bovine embryos was between 8.1 and 8.5.

Inhibitor Specificity

Gelatinase activity of ovine embryos was completely inhibited by the metal ion chelators EDTA, EGTA and 1,10-phenanthroline (Fig. 2.4). Gelatinase activity of ovine embryos was not inhibited by the cysteine protease inhibitors, NEM and leupeptin, the serine protease inhibitors soybean trypsin inhibitor and benzamidine or the aspartic acid protease inhibitor pepstatin A (Fig. 2.5).

Gelatinase activity in bovine embryonic tissues was partially inhibited by EDTA, EGTA and 1,10-phenanthroline (Fig. 2.6), however, conditioned medium from bovine embryos demonstrated reduced inhibition by these chelators (Fig. 2.7). Gelatinase activity in conditioned medium from bovine embryos was not inhibited by pepstatin A, soybean trypsin inhibitor or NEM (Fig. 2.7)

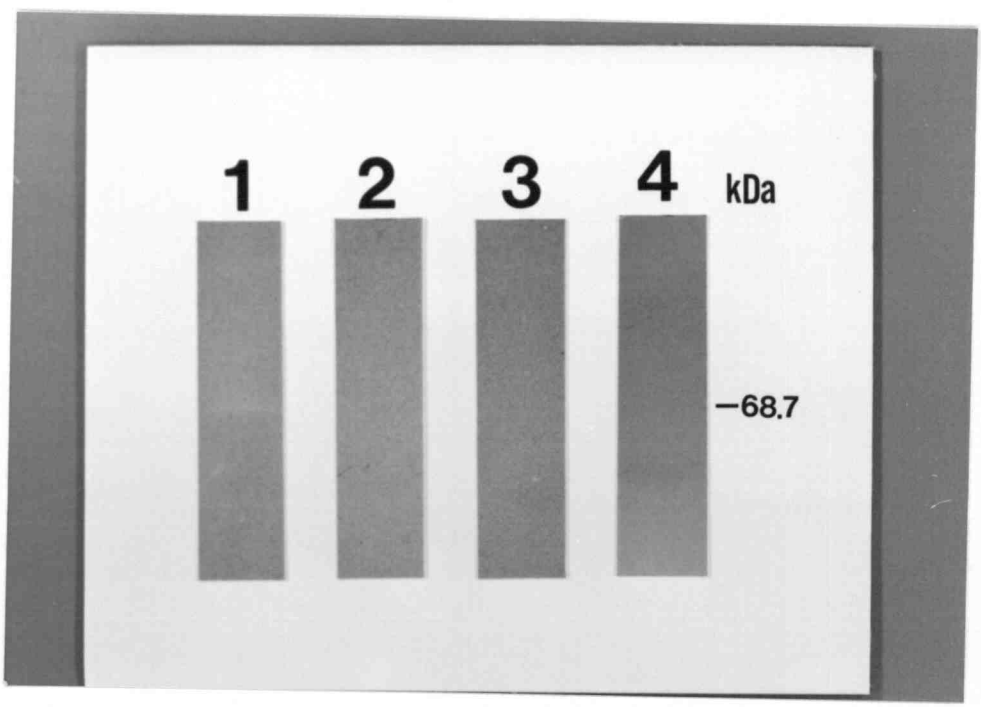

Fig. 2.4 Zymographic analysis of pooled conditioned medium recovered from Day 11 ovine embryos. Gel slices were incubated in Tris-HCl buffer containing 5 mM $CaCl_2$ (lane 1) with 10 mM 1,10-phenanthroline (lane 2), 10 mM EDTA (lane 3) or 10 mM EGTA (lane 4).

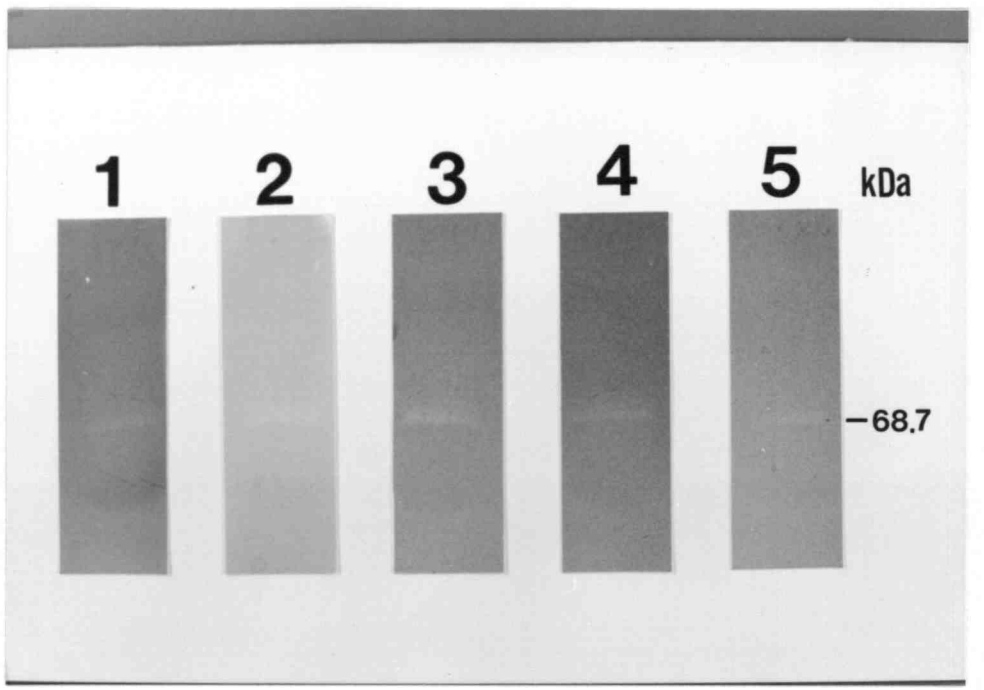

Fig. 2.5 Zymograph of pooled conditioned medium recovered from Day 11 ovine embryos. Gel slices were incubated in Tris-HCl buffer containing 5 mM $CaCl_2$ with 0.3 mM pepstatin A (lane 1), 100 μg/ml soybean trypsin inhibitor (lane 2), 10 mM benzamidine (lane 3), 1 mM leupeptin (lane 4) or 16 mM N-ethyl maleimide (lane 5).

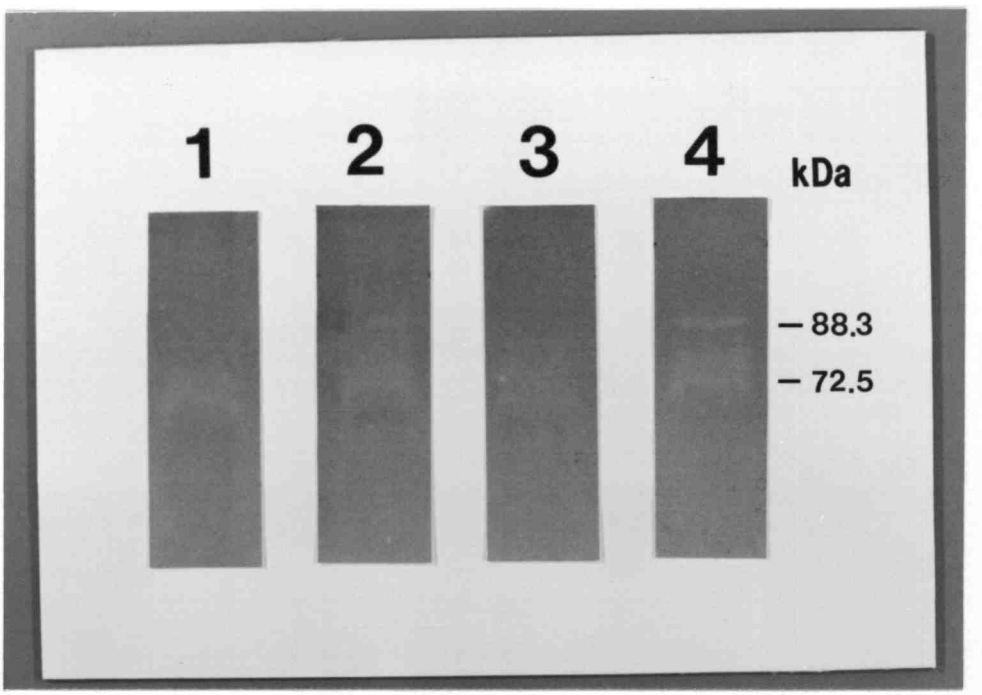

Fig. 2.6 Zymographic analysis of pooled conditioned medium recovered from Day 13-14 bovine embryos. Gel slices were incubated in Tris-HCl buffer containing 5 mM $CaCl_2$ with 10 mM 1,10-phenanthroline (lane 1), 10 mM EDTA (lane 2), 10 mM EGTA (lane 3) or 0 mM $CaCl_2$ (lane 4).

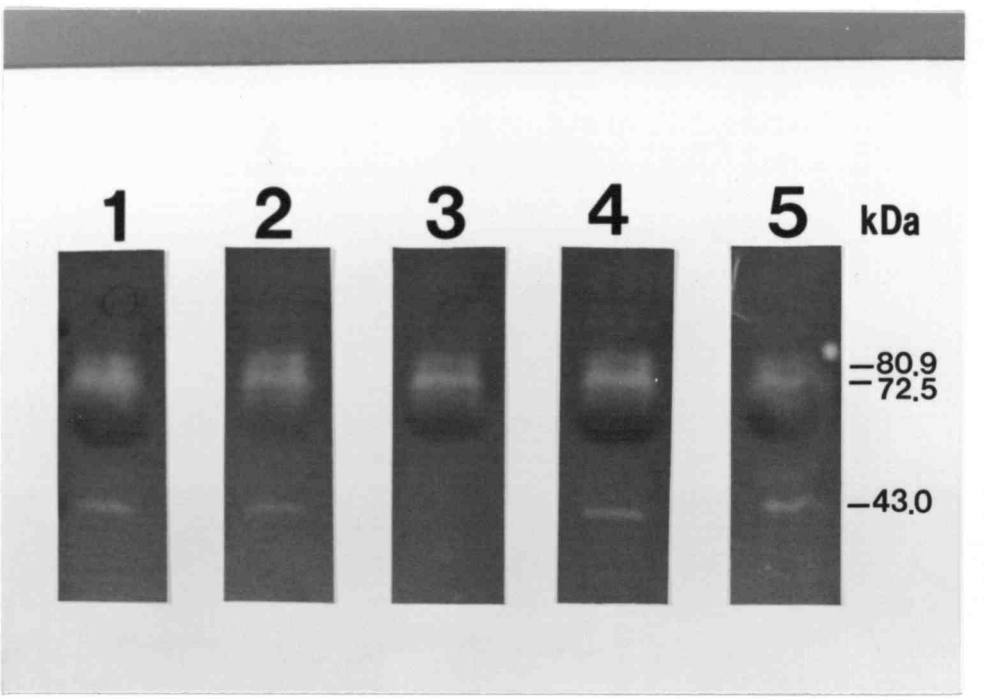

Fig. 2.7 Zymograph of pooled conditioned medium recovered from Day 13-14 bovine embryos. Gel slices were incubated in Tris-HCl buffer containing 5 mM $CaCl_2$ with 0.3 mM pepstatin A (lane 1), 100 μg/ml soybean trypsin inhibitor (lane 2), 5 mM EDTA (lane 3), 5 mM 1,10-phenanthroline (lane 4) or 16 mM N-ethyl maleimide (lane 5).

Aminophenyl Mercuric Acetate Activation

Gelatinolytic activity was not detected in ovine embryonic tissues. No molecular mass changes were observed in bovine gelatinases following incubation with APMA (Fig. 2.8).

DISCUSSION

Zymographic analysis of Day 11 ovine embryos revealed two gelatinases with molecular masses of 68.7 and 86.1 kDa. The latter gelatinase was observed only in limited samples. Cultured Day 7 and 9 ovine embryos did not produce any detectable gelatinases. Embryonic tissues and conditioned medium from Days 12 to 14 bovine embryos expressed four gelatinolytic bands ranging from 88.3 to 43.0 kDa. The activity of the ovine gelatinases was completely inhibited by metal ion chelators but not other proteinase inhibitors. The metal ion chelators EGTA, EDTA and 1,10-phenanthroline demonstrated complete inhibition of gelatinase activity. The serine protease inhibitors benzamidine and soybean trypsin inhibitor, the cysteine protease inhibitors, NEM and leupeptin, and the aspartic acid protease inhibitor pepstatin A did not have an inhibitory effect on gelatinase activity. The activity of gelatinases produced by bovine embryos were only partially inhibited by the metal ion chelators. However,

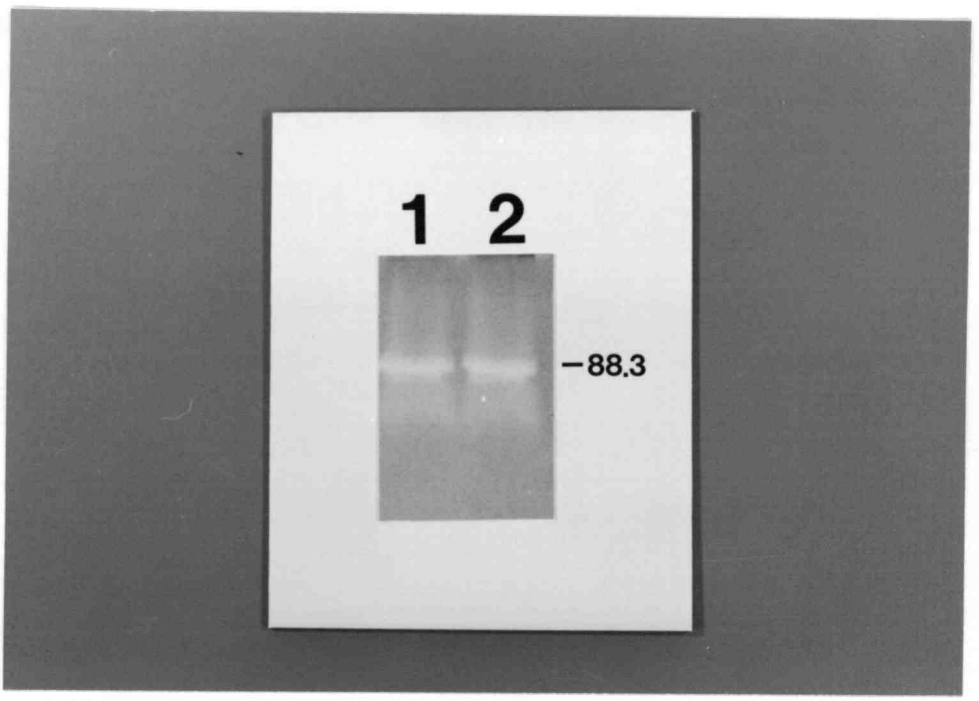

Fig. 2.8 Zymograph of Day 13-14 bovine embryonic tissues incubated with 0 (lane 1) or 1 mM aminophenyl-mercuric acetate (lane 2).

the protease inhibitors, NEM, benzamidine, soybean trypsin inhibitor and pepstatin A, did not have an inhibitory effect on gelatinase activity of bovine embryonic tissues or conditioned medium.

Members of the MMP family are secreted in zymogen form (Matrisian, 1990; Woessner, 1991). Addition of organomercurials or certain proteinases can activate latent enzymes by cleaving a portion of the pro-domain which allows a conformational change which destabilizes the association of a cysteine residue with the zinc atom activating the enzyme (Springman et al., 1990). In some members of the MMP family this leads to an autocatalytic reaction that cleaves the remainder of the pro-domain and results in a permanently active enzyme with a molecular mass approximately 10 kDa lower than the pro-enzyme form (Stetler-Stevenson et al., 1989). When bovine embryonic tissues were incubated with APMA, no difference in the banding pattern was observed. No gelatinase activity could be detected in the ovine embryonic tissues. This observation suggests that the bovine gelatinases are produced predominantly in active form. The optimal pH for the ovine and bovine gelatinases was between 7.9 and 8.5 and 8.1 and 8.5, respectively. MMP are active at a pH close to neutrality (Matrisian, 1992).

There are three classes of MMP based loosely on substrate specificity (Woessner, 1991; Matrisian, 1992), the collagenases, gelatinases and stromelysins. There are two

gelatinases, a 72 kDa enzyme known as MMP-2 and a 92 kDa enzyme known as MMP-9. Substrates for the gelatinases include collagen types IV and V and gelatin. The two bands observed in ovine conditioned medium were 68.7 and 86.1 kDa. It is possible that the 68.7 kDa gelatinase is MMP-2 and the 86.1 is MMP-9. Bovine embryonic tissue and conditioned medium expressed a high molecular mass band at 88.3 kDa possibly corresponding to MMP-9, and a doublet at 80.9 and 72.5 kDa, likely the pro-enzyme and active forms of MMP-2. The low molecular mass band at 43.0 kDa could be a collagenase or a stromelysin.

Gelatinolytic activity could not be detected in Days 7 or 9 ovine embryos, although, it was expressed in all day 11 conditioned medium pools. This expression corresponds with the onset of expansion and elongation of the ovine embryo. Interestingly, this is the same period of time that the ovine endometrium expresses TIMP-1 and -2 and does not express MMP-1 and -3 (Hampton et al., 1995). Also, ovine trophoblast interferon, which is expressed by the embryo during this period of development, inhibits the production of MMP-1 and -3 by cultured ovine endometrial cells (Salamonsen et al., 1994). The suppression of MMP and the induction of TIMP by the uterus occurs at a time when the embryo begins to produce MMP and initiates endometrial attachment. These observations suggest that the uterine environment may be focused to control proteolysis so as to finely regulate establishment of

maternal-embryonic contact. Although a similar scenario occurs for the bovine embryo with respect to uterine attachment, it is not known if endometrial MMP and TIMP activities change with approaching attachment.

Several embryonic cell migration events are also occurring during this period of development. Endodermal and mesodermal cell migration occur between Days 8-18 for both ovine and bovine embryos and migration is believed to occur through an extracellular matrix located at the basal surface of the trophectoderm (Wintenberger-Torres and Flechon, 1974; Betteridge and Flechon, 1988). In sheep embryos, collagen has also been localized to the trophectodermal-endodermal interface and may serve as the matrix supporting cell migration (Wintenberger-Torres and Flechon, 1974). It is indeed possible the gelatinases observed in the present study are involved in the cell migration events that accompany expansion and elongation in ovine and bovine embryos.

LITERATURE CITED

Behrendtsen, O., C. Alexander and Z. Werb. 1992. Metalloproteinases mediate extracellular matrix degradation by cells from mouse blastocyst outgrowths. Devel. 114:447-456

Betteridge, K. and J. Flechon. 1988. The anatomy and physiology of pre-attachment bovine embryos. Theriogenology. 29:155-187

Brenner, C., R. Adler, D. Rappolee, R. Pederson and Z. Werb. 1989. Genes for extracellular matrix-degrading metalloproteinases and their inhibitor, TIMP, are expressed during early mammalian development. Genes Devel. 3:848-859

Domon, M., M.C. Pinsker and B. Mintz. 1973. Thiocyanate assay for sublytic change in the zona pellucida of the mouse egg. Biol. Reprod. 9:246-253

Hampton, A., A. Butt, S. Riley and L. Salamonsen. 1995. Tissue inhibitors of metalloproteinases in endometrium of ovariectomized steroid-treated ewes and during the estrous cycle and early pregnancy. Biol. Reprod. 53:302-311

Heussen, C. and E. Dowdle. 1979. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Analy. Biochem. 162:196-202

Hoversland, R. and H. Weitlauf. 1982. In-vitro zona-lytic activity in uterine fluid from ovariectomized mice treated with oestradiol-17ß and progesterone. J. Reprod. Fert. 64:223-226

Joshi, M. and I. Murray. 1974. Immunological studies of the rat uterine fluid peptidase. J. Reprod. Fert. 37:361-365

Lepage, T. and C. Gache. 1989. Purification and characterization of the sea urchin embryo hatching enzyme. J. Biol. Chem. 264:4787-4793

Lepage, T. and C. Gache. 1990. Early expression of a collagenase-like hatching enzyme gene in the sea urchin embryo. EMBO 9:9;3003-3012

Librach, C.L., Z. Werb, M.L. Fitzgerald, K. Chiu, N. Corwin, R. Esteves, D. Grobelny, R. Galardy and C. Damsky. 1991. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. J. Cell Biol. 113:2;437-449

Matrisian, L. 1990. Metalloproteinases and their inhibitors in matrix remodeling. Trends Genet. 6:121-125

Matrisian, L. 1992. The matrix-degrading metalloproteinases. Bioessays 14:455-462

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

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

Nomura, K., H. Tanaka, Y Kikkawa, M. Yamaguchi and N. Suzuki. 1991. The specificity of sea urchin hatching enzyme (Envelysin) places it in the mammalian matrix metalloproteinase family. Biochem. 30:6115-6123

Perona, R. and P. Wassarman. 1986. Mouse blastocysts hatch in vitro by using a trypsin-like proteinase associated with cells of mural trophectoderm. Devl. Biol. 114:42-52

Roe, J. and W. Lennarz. 1990. Biosynthesis and secretion of the hatching enzyme during sea urchin embryogenesis. J. Biol. Chem. 265:8704-8711

Rosenfeld, M. and M. Joshi. 1977. A possible role of a specific uterine fluid peptidase in implantation in the rat. J. Reprod. Fert. 51:137-139

Salamonsen, L., A. Hampton, R. Suzuki and H. Nagase. 1994. Modulation of production of matrix metalloproteinases from ovine endometrial cells by ovine trophoblast interferon. J. Reprod. Fert. 102:155-162

Salamonsen, L., H. Nagase, R. Suzuki and D. Wooley. 1993. Production of matrix metalloproteinase 1 (interstitial collagenase) and matrix metalloproteinase 2 (gelatinase A: 72 kDA gelatinase) by ovine endometrial cells in vitro: different regulation and preferential expression by stromal fibroblasts. J. Reprod. Fert. 98:583-589

Sawada, H., K. Yamazaki and M. Hoshi. 1990. Trypsin-like protease from mouse embryos: evidence for the presence in culture medium and its enzymatic properties. J. Exp. Zoo. 254:83-87

Springman, E.B., E.L. Angleton, H. Birkedal-Hansen and H.E. Van Wart. 1990. Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys^{73} active-site zinc complex in latency and a "cysteine switch" mechanism for activation. Proc. Natl. Acad. Sci. USA. 87:364-368

Stetler-Stevenson, W., H. Krutzsch and M. Wacher, I. Margulies and L. Liotta. 1989. The activation of human type IV collagenase proenzyme. J. Biol. Chem. 264:1353-1356

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

Woessner, Jr. J. 1991. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J.. 5:2145-2154

Yasumasu, S., S. Katow, T. Hamazaki, I. Iuchi, K. Yamagami. 1992. Two constituent proteases of a telostean hatching enzyme: concurrent syntheses and packaging in the same secretory granules in discrete arrangement. Devel. Biol. 149:349-356

Zuccotti, M., U.A. Urch, R. Yanagimachi. 1991. Collagenase as an agent for dissolving the zona pellucida of hamster and mouse oocytes. J. Reprod. Fert. 93:515-520

CONCLUSION

This thesis partially characterized several gelatinases or MMP produced by porcine, ovine and bovine preimplantation embryos. The expression of these gelatinases coincides with embryonic expansion and elongation and mesodermal cell migration. MMP may have activity in both these processes as MMP are known to be involved in both tissue remodeling and cell migration (Woessner, 1991) In porcine embryos, gelatinase expression was greatest during the periods of mesodermal cell migration and rapid morphologic transformation from a spherical to elongated shape (Geisert et al., 1982; Richoux et al., 1989). Ovine embryos do not express gelatinase activity until Day 11 which corresponds to the onset of embryonic expansion (Wintenberger-Torres and Flechon, 1974). Incidentally, this is the same period of time that the ovine endometrium expresses TIMP-1 and -2 (Hampton et al., 1995). Bovine embryos express gelatinase activity on Days 12 to 14. Bovine embryos undergo mesodermal cell migration between Days 14 and 18 and elongation and expansion begins on Day 13. Although the function of MMP in the early embryo is not completely known, their production during periods of cell migration and morphologic transformation, when interaction with the ECM is apparent, suggests a role for MMP in these processes.

BIBLIOGRAPHY

Adler, R., C. Brenner and Z. Werb. 1990. Expression of extra cellular matrix-degrading metalloproteinases and metalloproteinase inhibitors is developmentally regulated during endoderm differentiation of embryonal carcinoma cells. Devl. 110:211-220

Anderson L. 1978. Growth, protein content and distribution of early pig embryos. Anat. Rec. 190:143-154

Bainton, D. 1981. The discovery of lysosomes. J. Cell Biol. 91:66s-76s

Behrendtsen, O., C. Alexander and Z. Werb. 1992. Metalloproteinases mediate extracellular matrix degradation by cells from mouse blastocyst outgrowths. Devel. 114:447-456

Betteridge, K. and J. Flechon. 1988. The anatomy and physiology of pre-attachment bovine embryos. Theriogenology. 29:155-187

Blair, R., C. Coughlin, J. Minton and D. Davis. 1994. Peri-oestrous hormone profiles, embryonic survival and variation in embryonic developmebt in gilts and primiparous sows. J. Reprod. Fert. 101:167-173

Brenner, C., R. Adler, D. Rappolee, R. Pederson and Z. Werb. 1989. Genes for extracellular matrix-degrading metalloproteinases and their inhibitor, TIMP, are expressed during early mammalian development. Genes Devel. 3:848-859

Carmeliet, P., L. Schoonjans, L. Kieckens, B. Ream, J, Degen, R. Bronson, R. De Vos, J. van den Oord, D. Collen and R. Mulligan. 1994. Physiological consequences of loss of plasminogen activator gene function in mice. Nature. 368:419-424

Coates, A.A. and A.R. Menino, Jr.. 1994. Effects of blastocoelic expansion and plasminogen activator activity on hatching and zona pellucida solubility in bovine embryos in vitro. J. Animal Sci. 72:2936-2942

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

Domon, M., M.C. Pinsker and B. Mintz. 1973. Thiocyanate assay for sublytic change in the zona pellucida of the mouse egg. Biol. Reprod. 9:246-253

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

Geisert, R., J. Brookbank, R.M. Roberts and F. Bazer. 1982. Establishment of pregnancy in the pig: II. cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biol. Reprod. 27:941-955

Graham, C. and P. Lala. 1991. Mechanism of control of trophoblast invasion in situ. J. Cell Phys. 148:228-234

Hampton, A., A. Butt, S. Riley and L. Salamonsen. 1995. Tissue inhibitors of metalloproteinases in endometrium of ovariectomized steroid-treated ewes and during the estrous cycle and early pregnancy. Biol. Reprod. 53:302-311

Herz, J., D. Clouthier and R. Hammer. 1992. LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. Cell. 71:411-421

Heussen, C. and E. Dowdle. 1979. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Analy. Biochem. 162:196-202

Hoversland, R. and H. Weitlauf. 1982. In-vitro zona-lytic activity in uterine fluid from ovariectomized mice treated with oestradiol-17ß and progesterone. J. Reprod. Fert. 64:223-226

Joshi, M. and I. Murray. 1974. Immunological studies of the rat uterine fluid peptidase. J. Reprod. Fert. 37:361-365

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

Laiho, M. and J. Keski-Oja. 1989. Growth factors in the regulation of pericellular proteolysis: a review. Cancer Res. 49:2533-2553

Leco, K., R. Khokha, N. Pavloff, S. Hawkes and D. Edwards. 1994. Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. J. Biol. Chem. 269:9352-9360.

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

Lepage, T. and C. Gache. 1989. Purification and characterization of the sea urchin embryo hatching enzyme. J. Biol. Chem. 264:4787-4793

Lepage, T. and C. Gache. 1990. Early expression of a collagenase-like hatching enzyme gene in the sea urchin embryo. EMBO 9:9;3003-3012

Librach, C.L., Z. Werb, M.L. Fitzgerald, K. Chiu, N. Corwin, R. Esteves, D. Grobelny, R. Galardy and C. Damsky. 1991. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. J. Cell Biol. 113:2;437-449

Matrisian, L. 1990. Metalloproteinases and their inhibitors in matrix remodeling. Trends Genet. 6:121-125

Matrisian, L. 1992. The matrix-degrading metalloproteinases. Bioessays 14:455-462

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

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

Moll, U. and B. Lane. 1990. Proteolytic activity of first trimester human placenta: localization of interstitial collagenase in villous and extravillous trophoblast. Histochem. 94:555-560

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

Nomura, K., H. Tanaka, Y Kikkawa, M. Yamaguchi and N. Suzuki. 1991. The specificity of sea urchin hatching enzyme (Envelysin) places it in the mammalian matrix metalloproteinase family. Biochem. 30:6115-6123

Paulij, W., H. Verhoof and J. DenucÉ. 1992. Partial purification and characterization of *Loligo Vulgaris* hatching enzyme obtained from hatching medium. Comp. Biochem. Physiol. 101B:617-622

Perona, R. and P. Wassarman. 1986. Mouse blastocysts hatch in vitro by using a trypsin-like proteinase associated with cells of mural trophectoderm. Devl. Biol. 114:42-52

Reith, A. and G. Rucklidge. 1992. Invasion of brain tissue by primary glioma: evidence for the involvement of urokinase-type plasminogen activator as an activator of type IV collagenase. Biochem. Biophys. Res. Comm. 186:348-354

Richoux, V., T. Derribere, J. Boucaut, J. Flechon and J. Thiery. 1989. Distribution of fibronectins and laminin in the early pig embryo. Anat. Record. 223: 72-81

Roe, J. and W. Lennarz. 1990. Biosynthesis and secretion of the hatching enzyme during sea urchin embryogenesis. J. Biol. Chem. 265:8704-8711

Rosenfeld, M. and M. Joshi. 1977. A possible role of a specific uterine fluid peptidase in implantation in the rat. J. Reprod. Fert. 51:137-139

Salamonsen, L., A. Hampton, R. Suzuki and H. Nagase. 1994. Modulation of production of matrix metalloproteinases from ovine endometrial cells by ovine trophoblast interferon. J. Reprod. Fert. 102:155-162

Salamonsen, L., H. Nagase, R. Suzuki and D. Wooley. 1993. Production of matrix metalloproteinase 1 (interstitial collagenase) and matrix metalloproteinase 2 (gelatinase A: 72 kDA gelatinase) by ovine endometrial cells in vitro: different regulation and preferential expression by stromal fibroblasts. J. Reprod. Fert. 98:583-589

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

Sawada, H., K. Yamazaki and M. Hoshi. 1990. Trypsin-like protease from mouse embryos: evidence for the presence in culture medium and its enzymatic properties. J. Exp. Zoo. 254:83-87

Sherman, M.I., S. Strickland and E. Reich. 1976. Differentiation of early mouse embryonic and

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

Shimonovitz, S., A. Hurwitz, M. Dushnik, E. Anteby, T. Geva-Eldar and S. Yagel. 1994. Developmental regulation of the expression of 72 and 92 type IV collagenases in human trophoblasts: a possible mechanism for control of trophoblast invasion. Am. J. Obstet. Gynecol. 171:832-838

Springman, E.B., E.L. Angleton, H. Birkedal-Hansen and H.E. Van Wart. 1990. Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys^{73} active-site zinc complex in latency and a "cysteine switch" mechanism for activation. Proc. Natl. Acad. Sci. USA. 87:364-368

Stetler-Stevenson, W., H. Krutzsch and M. Wacher, I. Margulies and L. Liotta. 1989. The activation of human type IV collagenase proenzyme. J. Biol. Chem. 264:1353-1356

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

Strickland, S. and W. Richards. 1992. Invasion of the trophoblasts. Cell. 71:355-357

Vassalli, J. and M. Pepper. 1994. Membrane proteases in focus. Nature. 370:14-15

Wassarman, P.M., J.M. Greve, R.M. Perona, R.J. Roller and G.S. Salzmann. 1984. How mouse eggs put on and take off their extracellular coat. Mol. Biol. Devel. 213-225

Werb, Z., P. Trimble, O. Behrendtsen, E. Crowley and C. Damsky. 1989. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell Biol. 109:877-889

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

Woessner, Jr. J. 1991. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J.. 5:2145-2154

Yasumasu, S., S. Katow, T. Hamazaki, I. Iuchi, K. Yamagami. 1992. Two constituent proteases of a telostean hatching enzyme: concurrent syntheses and packaging in the same secretory granules in discrete arrangement. Devel. Biol. 149:349-356

Zhang, X., G. Kidder, C. Zhang, F. Khamsi and D. Armstrong. 1994. Expression of plasminogen activator genes and enzymatic activities in rat preimplantation embryos. J. Reprod. Fert. 101:235-240

Zuccotti, M., U.A. Urch, R. Yanagimachi. 1991. Collagenase as an agent for dissolving the zona pellucida of hamster and mouse oocytes. J. Reprod. Fert. 93:515-520