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Title: Evaluation of Extracellular Matrices and Proteinase Interactions in Bovine and Porcine Endodermal Cell Migration In Vitro

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Extraembryonic endoderm is formed by the proliferation of endodermal cells from the inner cell mass (ICM) and the migration of these cells over an extracellular matrix (ECM) to form a continuous layer adjacent to the trophectoderm. To evaluate ECM interactions and proteinases involved in bovine and porcine endodermal cell migration, ICM were cultured for 96 h on ECM of collagen IV, fibronectin, or laminin to determine the optimal substrate for supporting attachment and cellular outgrowth. Fibronectin was the superior matrix ($P<0.05$) for supporting cellular outgrowth from bovine ICM compared to collagen IV or laminin. Bovine ICM were also cultured on fibronectin in medium containing 0 or 10 μg/ml recombinant human TIMP-2 (rhTIMP-2). Regression analysis revealed decreased ($P<0.05$) rates of bovine endodermal cell migration (0.2 vs 0.7 cells /h) and change in outgrowth areas (815.2 μ^2/h vs 2397.9 μ^2/h) in 0 vs 10 μg/ml rhTIMP-2.

To identify the ECM providing the greatest support of porcine endodermal cell migration, isolated porcine ICM were cultured on matrices

of collagen IV, fibronectin or laminin. Percentages of porcine ICM generating cellular outgrowth on fibronectin and laminin were similar ($P>0.10$), however, collagen IV failed to support cellular migration from the ICM ($P<0.05$).

Porcine ICM were also cultured on fibronectin or laminin in the presence or absence of the RGD-peptide. Cell numbers in the outgrowth and ICM and outgrowth areas for porcine ICM cultured on fibronectin in medium containing 500 μg/ml RGD-peptide were not different ($P>0.10$) compared to 0 μg/ml. Porcine ICM cultured on laminin in 500 μg/ml of RGD-peptide had fewer ($P<0.05$) cells in the outgrowths(15.4±2.0) and slower ($P<0.05$) rates of cell migration (-0.1 cells/h) compared to 0 μg/ml RGD-peptide (28.2±5.5 and 0.5 cells/h respectively). Porcine ICM were also cultured on laminin in medium containing 0 or 10 μg/ml rhTIMP-2 however no differences ($P>0.05$) were observed due to treatment.

The results suggest that fibronectin is the optimal ECM for bovine ICM outgrowth and bovine endodermal cells respond to rhTIMP-2 with increased rates of migration and plasminogen activator production. Porcine ICM generate cell outgrowth on fibronectin and laminin and addition of rhTIMP-2 did not affect cell outgrowths on laminin. Addition of the RGD-peptide inhibited cell outgrowth on laminin but not fibronectin. The lack of gelatinase activity during cell migration further suggests that interactions between endodermal cells and the ECM do not depend on extracellular proteinase activity to facilitate migration.

EVALUATION OF EXTRACELLULAR MATRICES AND PROTEINASE INTERACTIONS IN BOVINE AND PORCINE ENDODERMAL CELL MIGRATION IN VITRO

by

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INTRODUCTION

The preimplantation mammalian blastocyst is organized as a single celled layer of trophectoderm surrounding a core of inner cell mass (ICM) and a fluid-filled cavity called the blastocoel. After the emergence of ICM and trophectoderm, endodermal cells appear on the blastocoelic side of the ICM. As the embryo continues to develop, endodermal cells migrate away from the ICM over an extracellular matrix (ECM) and eventually surround the blastocoelic side of the trophectoderm to form extraembryonic endoderm (Richoux et al., 1989; Betteridge and Fléchon, 1988). Mesodermal cells from the embryonic disc will also migrate between the newly formed endodermal layer and the trophectoderm (Richoux et al., 1989). The ECM is known to have a key role in cell-matrix interactions (Duband et al., 1987). In particular, the ECM glycoproteins, fibronectin and laminin, have been identified in the early stages of embryonic development and are involved in cell migration. In porcine embryos, fibronectin has been detected prior to endodermal cell migration and has been suggested as the substrate over which these cells migrate (Richoux et al., 1989). After the extraembryonic endoderm is established, laminin appears in the blastocyst and is proposed as the signal for completion of migration and to stabilize cell-matrix interactions (Richoux et al., 1989).

Cell-ECM interactions are mediated primarily by a family of cell surface receptors called integrins. Integrins are a large family of α/β heterodimeric cell surface glycoproteins (Hynes, 1992). The ability of a cell

to bind to the ECM depends on the cell expressing a specific integrin for the cell binding site on the large proteins that comprise the ECM. By binding the cells weakly, but simultaneously to the ECM, integrins allow cells to move in their environment without losing attachment. However, recent studies indicate that integrins may serve other purposes other than cell adhesion to the ECM. Integrins are excellent candidates for the transduction of signals from the extracellular environment to the interior of the cell (Hynes, 1992). Interactions between the ECM and integrins may also regulate gene expression during differentiation.

During preimplantation embryonic development, several processes have been proposed to require the production of extracellular proteinases, including endodermal and mesodermal cell migration and expansion and elongation of the blastocyst. These processes have in common remodeling of the ECM. The matrix metalloproteinases (MMP) and the plasminogen activator (PA)-plasmin systems are known for their involvement in ECM remodeling and cellular migration. Tissue inhibitors of matrix metalloproteinases (TIMP) are endogenous proteinase inhibitors that control the degree of ECM degradation by inhibition of MMP activity. PA and MMP are involved in implantation as both murine trophoblast and invasive human cytotrophoblast cells synthesize both MMP and PA during endometrial invasion (Librach et al., 1991). Based on the evidence supporting the involvement of extracellular proteases in the migration of endodermal cells over or through an extracellular matrix, the objective of the research in this thesis was to evaluate these factors in the migration of bovine and porcine endodermal cells in vitro.

REVIEW OF THE LITERATURE

Preimplantation Embryonic Development in Cattle and Swine

Preimplantation embryonic development in mammals involves a detailed and complex set of processes that begin with fertilization and end with the attachment or implantation of the developing embryo into the endometrium. Prior to fertilization, the female gamete, the oocyte, is surrounded by an acellular glycoprotein matrix known as the zona pellucida. The zona pellucida provides a site for sperm recognition in addition to a protective coating during early development. The fertilized egg undergoes a series of cleavage divisions that result in the embryo being composed of a cluster of cells, or blastomeres, which to a point, will retain totipotency. These cells sequentially develop cytoplasmic and plasma membrane polarity with distinct apical and basolateral regions. Polarization of these cells is the initial step in embryonic differentiation. Further cell division results in the formation of the morula and reallocation of the polarized cells to produce two distinct cell populations: polar cells on the periphery of the embryo and apolar cells on the inside of the embryo. During blastocyst formation, polar cells develop into trophectoderm and the apolar cells develop into inner cell mass. Trophectoderm and inner cell mass will eventually give rise to the fetal membranes and the embryo proper, respectively. Also characterizing the blastocyst stage, is the development of the fluid-filled cavity called the blastocoel. As development progresses, the blastocoel continues to expand and the embryo sheds or "hatches" from the zona pellucida. After hatching, the embryo continues to expand, and in most livestock species, the blastocyst begins elongating into a filamentous-type morphology. After a period of elongation, the embryo

adheres and attaches to the endometrium to establish fetal-maternal contact.

Several embryonic and extraembryonic tissues are formed during the period extending from blastocyst formation to endometrial attachment. In bovine embryos, blastocyst formation occurs on Day 6 (Day 0= onset of estrus) and on Day 8 endodermal cells migrate away from the ICM, and completely surround the blastocoelic side of the trophectoderm by Day 10 (Betteridge and Fléchon, 1988). In ungulate embryos, the extraembryonic endoderm seems to form a homologous cellular monolayer. Hatching occurs on Day 10 and bovine embryos develop from a 3 mm sphere on Day 11 to a 25 cm filament by Day 17 (Betteridge and Fléchon, 1988). On Day 14, 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 Fléchon, 1988). The spherical to filamentous transformation of bovine blastocysts occurs over several days, but the rate of elongation and expansion of the pig blastocyst is unsurpassed.

In the pig blastocyst, primitive endodermal cells appear in the ICM on Day 7, and migrate over the blastocoelic surface of the trophectoderm, eventually covering it with a continuous monolayer of endoderm (Stroband et al., 1984). On Day 12, mesodermal cells leave the embryonic disc and migrate between the newly formed endoderm and the trophectoderm (Richoux et al., 1989) to form a continuous layer by Day 18.

Livestock embryos, unlike primate and rodent embryos, undergo a period of rapid expansion and elongation after hatching from the zona pellucida and prior to implantation. In porcine embryos, this morphological change is typified by the increase in size from a 150 μm sphere on Day 6 to a 9 mm sphere on Day 11. Cellular hyperplasia is apparent during this time of development, and the growth rate is 0.25

mm/h (Geisert et al., 1982). On Day 12, the growth rate dramatically increases to a rate of 35 to 45 mm/h, resulting in a 100 cm filament by Day 14 (Geisert et al., 1982). Geisert et al. (1982) proposed that elongation of the blastocyst occurs through alterations in microfilaments and junctional complexes of the trophectodermal cells and the formation of filopodia by endodermal cells. Proteinase involvement in the cellular restructuring required for these alterations is probable as specific extracellular proteinases participate in ECM remodeling during cellular migration and differentiation.

Cell-Extracellular Matrix Interactions in Differentiation and Development

Differentiation is a continuously regulated process and interactions between the cell and its environment play a major role in maintaining stable expression of differentiation-specific genes. An important component of the cellular environment is the ECM, which is composed of glycoproteins, proteoglycans, and glycosaminoglycans that are secreted and assembled locally into an organized network to which cells adhere (Adams and Watt, 1993). An ECM is present in mammalian embryos from the two-cell stage and is a component of all cellular micro-environments. However, the composition of the ECM and the spatial relationships between cells and the ECM differ between tissues. It has been clear for some time that the ECM plays a key role in regulating the differentiation of cells, but the mechanisms involved remained largely a mystery until the recent discovery of cell binding sites within the ECM glycoproteins and specific ECM receptors on cells. There are at least three mechanisms by which the ECM can regulate cell behavior. One is through the composition of the ECM. The second is through interactions between growth factors and matrix

molecules. The third is through the cell surface receptors that mediate adhesion to ECM components.

The Extracellular Matrix

The fact that laminin and fibronectin are the first ECM proteins to appear in embryo development, has lead to detailed investigations as to their importance in the process of cellular development and tissue organization. Laminin is a large (~850 kDa), flexible complex of three long polypeptide chains arranged in the shape of an asymmetric cross and held together by disulfide bonds. Like many other proteins in the ECM, it consists of a number of functional domains: including collagen type IV, heparin sulfate, and entactin, a constituent of the basement membrane, and two more domains that bind to laminin receptor proteins on the cell surface. Like type IV collagen, laminin molecules can self-assemble *in vitro* into a sheet, largely through interactions between the ends of the laminin arms (Alberts et al., 1994). Laminin is known to influence cell attachment, movement, differentiation, and growth, and several cell-surface receptors have been shown to mediate these processes (Mecham, 1991). The interaction between laminin and cell surface receptors is determined by functional domains in the molecule. As a multi-domain protein with multiple biolological functions, it is reasonable to believe that more than one class of receptor would be available to mediate laminin's various biological activities. In *Drosophila*, mutations that result in embryonic developmental defects have been identified in both ECM proteins and receptors. A null mutation in the laminin A chain gene in *Drosophila* embryos is a recessive lethal in late embryogenesis (Hortsch and Goodman, 1991).

Fibronectins are a group of closely related glycoproteins encoded by a single gene. Fibronectin is a large dimer (460 kDa) composed of two large subunits joined by a pair of disulfide bonds near their carboxyl termini. Each subunit is folded into a series of functionally distinct, rodlike domains separated by regions of flexible polypeptide chains. Fibronectins are a major component of the ECM and promote cell adhesion, cell migration, and cytoskeletal organization (Hynes,1990). Because expression of fibronectin is widespread in mammalian embryos, it is believed that fibronectin plays an important role in development. In particular, fibronectin has been shown to promote the migration of many cell types in vitro and is present in areas of active cell migration in embryos. Fibronectin appears before or at gastrulation in all vertebrates and is abundant during gastrulation, neural crest cell migration, primordial germ cell migration, and of great relevance to this study, endodermal cell migration from the ICM. Direct evidence that fibronectin is required for cell movements is demonstrated by inhibition of gastrulation by injecting antibodies to fibronectin into the blastocoel (Boucaut et al., 1984). George et al. (1993) demonstrated that mouse embryos null for fibronectin resulted in embryonic lethality due to defects in mesoderm, neural tube and vascular development. Gastrulation in the salamander and neural crest migration in avian embryos has also shown to be inhibited by the injection of RGD (Arg-Gly-Asp)-containing peptides (Boucaut et al.,1984) that competitively bind to the cell surface receptors for fibronectin, providing more evidence that cell-matrix interactions are essential for cellular differentiation and migration. Whole-embryo experiments have demonstrated a role for fibronectin in morphogenic cell movements and cell culture provides a method of determining the effects of fibronectin and other matrix proteins on the migration of individual cell types.

In order to describe the interactions between endodermal and mesodermal cell migration and the ECM, Richoux et al. (1989), investigated the distribution of fibronectins and laminin in the early porcine embryo. In porcine embryos fibronectin appears on the blastocoelic surface of the ICM at Day 6 of development. Fibronectin can be found lining the complete blastocoelic surface of the trophectoderm by Day 8. It is along this matrix that endodermal cells leave the ICM and form a continuous layer of endoderm by Day 10. Laminin appears in the blastocyst when the endoderm layer is established. On Days 12-13, the layer of fibronectin that lies between the endoderm and trophectoderm, gradually thickens to facilitate mesodermal cell migration away from the embryonic disc. At this point in development, laminin is found at the junction of endoderm and mesoderm and trophectoderm and mesoderm (Richoux et al.,1989). It has been proposed that endodermal and mesodermal cells use fibronectin to migrate, and that laminin is secreted when migration is complete to stabilize cell-matrix interactions. It is likely that this specific tissue remodeling involves extracellular proteinase activity because that endodermal and mesodermal cell migration occurs over or through an ECM.

Evidence for the importance of cell-matrix interactions during development is provided in studies of mutations affecting ECM proteins and their receptors in a variety of species. In *Ceanorhabditis elegans* mutations in over 50 genes that affect the morphology of the organism have been identified and several of these genes are now known to encode ECM proteins (Adams and Watt, 1993). The majority of ECM gene mutations mapped in vertebrates have been identified in humans, where several disorders have been studied. Mutations in type I collagen result in bone fragility, while mutations in collagen type II result in cartilage malformations during limb development. Mutations in the α1 chain of type II collagen result in skeletal defects ranging from abnormal limb to craniofacial development (Metsäranta

et al., 1992). ECM proteins are important for supporting embryonic cell proliferation during morphogenesis. Cells of mouse blastocysts attach and outgrow on fibronectin, laminin, vitronectin, collagen and thrombospondin (Armant et al., 1986). Ovine ICM have also been shown to attach and migrate on matrices of fibronectin and collagen is coincident with elevated levels of PA production (Bartlett and Menino, 1995). The observation of embryonic cell outgrowth supports the idea that cells migrate either over or through an ECM protein, as noted in the work performed by Richoux et al. (1989). The involvement of proteolysis in cell migration, also incorporates the involvement of the accompanying ECM. In each case where the ECM is degraded, the process involves extracellular proteolytic enzymes that are secreted locally by cells. The majority of these enzymes belong to one of two classes: matrix metalloproteinases (MMP), or the plasminogen activator (PA)- plasmin system, and are reviewed later in this section. The process by which these enzyme families degrade the ECM involves cleaving particular proteins at a small number of sites, which are often structured in such a fashion that the structural integrity of the ECM is destroyed in a relatively limited process. In this way, cell migration can take place with a relatively small amount of proteolysis.

Growth Factors and the Extracellular Matrix

Growth factors and ECM molecules interact in a number of ways to regulate cell behavior. One type of interaction is the binding of growth factors to the ECM, which affects the concentration and biological activity of the growth factors. Secondly, growth factors have profound effects on the production of ECM proteins and their receptors. Finally, ECM molecules themselves can be mitogenic and influence the responsiveness of cells to

growth factors. ECM binding of growth factors can induce several biological influences. Growth factors can bind to the ECM via the glycosaminoglycan side chains or the protein cores of specific matrix molecules. By limiting diffusion, the ECM provides a store of growth factor that continues after the production of the growth factor has ceased. Matrix-bound growth factors can be released by proteolysis of proteoglycans, although it is not clear how this mechanism operates physiologically (Klagsbrun and Baird, 1991). Growth factor-ECM interactions also have implications in the regulation of specific gene expression. Induction of fibronectin expression is a primary response to epidermal growth factor (EGF) stimulation of mouse embryo fibroblasts (Blatti et al.,1988). Adhesion of neutrophils to fibronectin leads to increased tumor necrosis factor (TNF) production (Nathan and Scorn, 1991). Transforming growth factor-β (TGFß) regulates the expression of collagenase and the tissue inhibitor of matrix metalloproteinases, (TIMP) which have been implicated in ECM remodeling (Edwards et al., 1987). Some ECM proteins possess intrinsic growth factor activity as well. Several ECM proteins contain repeated EGF-like sequences (Hardingham and Fosang, 1992), and one of these proteins, laminin, has been reported to possess mitogenic activity (Panayotou et al., 1989). In the case of laminin, the activity is localized to the domain containing the EGF-like repeats; however, since laminin and EGF do not compete for the same cell binding site, it is not clear whether laminin acts via the EGF-receptor (Panayotou et al., 1989). The ECM can regulate the mitogenic response of cells to growth factors by regulating cell shape. Over expression of certain cell adhesion receptors can cause changes in cell growth properties (Giancotti and Ruoslahti, 1990). These observations support the idea that growth factors and ECM proteins collaborate in creating cellular environments that regulate proliferation and differentiation.

Integrins and the Extracellular Matrix

Cells in tissues exist in a structural formation that involves other cells and an ECM, in addition to growth factors, hormones and ECM-remodeling enzymes. Recent biological and genetic research have reinforced the idea that cells respond to the ECM through cell surface adhesion receptors that maintain a role of regulating adhesive strength, cell shape, and of great importance to this study, cell migration and motility. Cellular migration through an ECM is a fundamental part of the morphogenesis of many tissues including the cornea (Toole and Trelstad, 1975), and all the derivatives of the neural crest (Weston, 1983; Erickson, 1986). In embryonic development, the earliest detectable adhesive interactions between cells occurs at the eight-cell stage when blastomeres of the mouse embryo undergo compaction (Ducibella and Anderson, 1975).

Cell-ECM interactions are mediated primarily by a family of cell surface receptors called integrins. The ability of a cell to bind to the extracellular matrix depends on its expressing a cell membrane receptor for the cell binding site on the large proteins that comprise the ECM. The ligands for integrins include a number of ECM proteins such as fibronectin, laminin, and collagens. Integrins differ from cell surface receptors for hormones and for other signaling molecules in that they bind their ligand with a relatively low affinity and are usually present at about 10- to 100-times higher concentration on the cell surface. Binding cells weakly, but simultaneously, to large numbers of matrix molecules allows cells to move in their environment without losing attachment.

However, recent in vitro studies have concluded that integrins have several functions other than cell adhesion to the ECM. Integrins comprise a large family of α/ß heterodimeric cell surface glycoproteins (Hynes, 1992). This family has been classified into subgroups according to the identity of

the ß subunit, based on the finding that different α subunits in combination with the same ß subunit form different receptor specificity. Multiple integrins recognize each of the major ECM glycoproteins, but the functional significance of this redundancy is still unclear. A simple model suggests that each site is recognized by a different receptor, which has specific functions. In the case of fibronectin for example, the EILDV adhesive sequence is recognized by the $\alpha_4ß_1$ integrin (Hynes, 1992) and the RGD site is recognized by the $\alpha_5ß_1$ integrin (Rouslahati and Peirschbacher, 1987). The RGD site is also recognized by five other integrins, and individual cells can simultaneously express more than one RGD-binding integrin (Humphries, 1990). A more likely explanation, therefore, for the expression of multiple integrins is that occupancy of different receptors by the same cell-binding site may convey different signals to the cell. Considerable progress is being made in defining the integrin recognition sites in the ligands and counter receptors. The first binding site to be defined was the Arg-Gly-Asp (RGD) sequence present in fibronectin, vitronectin, and a variety of other adhesive proteins. This tripeptide sequence is recognized by several integrins. Table 1-1 summarizes a portion of the integrin receptor family, their binding sites, and respective ligands.

Table 1-1. Integrin receptors, binding sites and respective ligands.

Subunits		Ligands	Binding Sites[a]
$ß_1$	α_1	Collagens, Laminin	ND[a]
	α_2	Collagens, Laminin	DGEA
	α_3	Fibronectin, Laminin, Collagens	RGD
	α_4	Fibronectin	EILDV
	α_5	Fibronectin	RGD
	α_6	Laminin	ND[a]
	α_7	Laminin	ND[a]
	α_v	Vitronectin, Fibronectin	RGD
$ß_5$	α_v	Vitronectin	RGD
$ß_6$	α_v	Fibronectin	RGD
$ß_7$	α_4	Fibronectin, VCAM-1	EILDV

[a]ND indicates that the binding site has not yet been determined

Integrins are excellent candidates for the transduction of signals from the extracellular environment to the interior of the cell. They are all transmembrane heterodimeric glycoproteins that bind extracellular ligands and the cytoskeleton. In order for cell-ECM interactions to cause changes in differentiated gene expression, ECM receptors must be able to transduce signals to the cell nucleus. With the exception of the $ß_4$ subunit, integrin α and ß subunits have short cytoplasmic domains of up to 53 amino acids. The high interspecies conservation of the $ß_1$ subunit (82% to 90% among vertebrates) suggests that it may have some conserved role in signaling. The low sequence homologies (20 to 30%) of the different α subunits that form heterodimers with $ß_1$ suggest that they may confer specificity of signal

transduction (Adams and Watt, 1993). The large number of integrin genes, possible combinations of α and ß dimers, redundancies in ligand-recognition preferences, amplifies their potential for diversity. There is also evidence that the occupancy of different heterodimers by the same ligand may result in different functions.

Receptor partnerships involving integrin-ECM interactions also regulate gene expression in differentiating systems. In pregnant mouse mammary gland terminal differentiation of alveolar structures and production of milk proteins require both lactogenic hormones and contact with the basement membrane. The signal from the basement membrane is integrin-mediated, as antibodies against the $ß_1$ integrin family block ß-casein production (Strueli et al., 1991). Similarly, at the time of involution, expression of milk proteins are lost when basement membranes are degraded by MMP. Milk protein expression can be restored when these proteinases are inhibited by tissue inhibitors of MMP (Talhouk et al,. 1992). These data suggest that the loss of the ECM initiates apoptosis that accompanies involution. Several other developing systems have revealed a similar ECM requirement to regulate differentiation such as muscle, skin, kidney, placenta, spermiogenesis, and fertilization.

ECM-initiated signal transduction is a proposed mechanism in the regulatory role of ECM remodeling. This was first documented by Werb et al. (1989) in studies showing that rabbit fibroblasts cultured on fibronectin produce low levels of MMP, and the addition of anti-α_5 antibody resulted in upregulation of MMP expression. This suggests that the $\alpha_5ß_1$ integrin as a fibronectin receptor transduces a signal that induces MMP expression. It has also been proposed that $\alpha_5ß_1$ integrin may play an important role during embryogenesis and differentiation. $\alpha_5ß_1$ is widely expressed at high levels in early *Xenopus* and chicken embryos (Whittaker and DeSimone, 1993) and the expression in adult tissues is depressed in comparison. Yang et

al. (1993) reported that a null mutation of the murine α_5 integrin gene is a recessive embryonic lethal. The α_5 -null embryos had defects in developing mesodermal structures, indicating a role for this integrin in mesodermal formation or movement. However, these embryos developed further than embryos null for fibronectin, for which $\alpha_5ß_1$ is a receptor, suggesting the role of other fibronectin receptors. Mutant cells from the integrin-null embryos were able to assemble fibronectin matrix, form contacts, and migrate on fibronectin despite the complete absence of the $\alpha_5ß_1$ fibronectin receptor integrin, providing evidence that cellular functions involving fibronectin can take place using other receptors. In another study by Yang et al. (1995) embryos were generated with a null mutation in the gene for the α_4 subunit that also lead to embryonic lethality. α_4 can associate with two ß subunits, $ß_1$ and $ß_7$. Both $\alpha_4 ß_1$ and $\alpha_4 ß_7$ are fibronectin receptors that bind to a non-RGD peptide on fibronectin. It has been suggested that α_4 integrins may also play a role in development as studies have shown the α_4 integrin is expressed in a number of embryonic tissues including somites, neural crest derivatives and heart, suggesting multiple roles in embryogenesis. The $\alpha_3ß_1$ integrin has been studied and it has been reported to play an important role in modulating the assembly of ECM proteins, similar to $\alpha_5ß_1$ integrin. However, $\alpha_3ß_1$ integrin binding was not inhibited by antibodies binding to the RGD domain of fibronectin, demonstrating that the two are mechanically distinct processes.

Extracellular Proteinase Involvement in Developmental Processes

The two major extracellular proteinase systems believed to be involved in early embryonic development are the plasminogen activator(PA)-plasmin system and the MMP family of enzymes.

Plasminogen Activators and Plasmin

PA are serine proteases that activate the serum proenzyme, plasminogen into plasmin. Two types of PA have been elucidated based on molecular weight, immunological reactivity, and fibrin affinity: urokinase - type PA (uPA) and tissue-type PA (tPA). The two types of PA are independent gene products and have been mapped to different chromosomes. uPA has a molecular mass between 31 and 55 kDa while tPA has a molecular mass of 70 kDa. Tissue-type PA is most commonly involved in fibrinolysis and is enhanced by the presence of fibrin whereas uPA is not. uPA was originally found in high concentration in human urine and is believed to be the principal PA involved in cell migration (Danõ et al., 1985). Proteolytic cleavage resulting in a disulfide-linked molecule determines conversion to active uPA or tPA. PA are also known to activate some latent MMP.

Plasminogen Activator in Early Embryonic Development

Embryonic production of PA has been identified in a variety of species. In embryonic developmental processes where PA has been

observed, uPA predominates. In early embryos, PA is expressed in correlation with hatching, endodermal and mesodermal cell migration, and implantation. Unlike mice and rats, bovine and porcine embryos do not undergo an invasive-type implantation. Instead, these embryos exhibit placentation that involves interdigitation of microvilli between epithelial surfaces of the trophoblast and endometrium. Mullins et al. (1980) began to investigate the relationship between PA and implantation in the porcine embryo. By isolating and culturing embryos in the early stages of implantation (Day 12), Mullins et al. (1980) observed a time-dependent increase in PA activity in conditioned medium. In the same study, uterine flushings were examined at days 8-18 of pregnancy. Mullins et al. (1980) found low PA activity, particularly after day 12. Because embryos collected at this time and subsequently cultured *in vitro* secreted PA, Mullins et al. (1980) hypothesized that embryonic PA was suppressed in utero. In uterine flushings of gilts, Mullins et al. (1980) found high PA activity on Days 3 and 18 of the cycle, but lower levels at Days 12 and 15. To determine whether a PA/plasmin inhibitor was present during mid-cycle, samples of Day 15 uterine flushings with negligible levels of PA were mixed with samples from Day 3 and 18. The results revealed a significant decrease in PA activity of Day 3 and 18 uterine flushings in the presence of the Day 15 flushings, suggesting a mid-cycle PA/plasmin inhibitor. To determine whether the inhibitor was hormone induced, uterine flushings were obtained from ovariectomized gilts maintained on progesterone or estrogen. Low levels of PA activity were found in the uterine flushings from the progesterone treated gilts, suggesting that the inhibitor was progesterone induced. Because porcine embryos are not invasive in uterine tissues, but are invasive if transplanted into an ectopic site (Samuel et al., 1971), Mullins et al. (1980) concluded that the progesterone-induced inhibitor may possibly play a role in preventing invasive implantation.

The porcine blastocyst elongates from a sphere to a long, thread-like morphology between Days 10-16. Fazleabas et al. (1983) observed a biphasic pattern of PA secretion during this period. The initial phase occurred between Days 10-12, synchronous with the elongation phase, while the secondary phase occurred on Days 14-16 and coincided with the time of increased DNA synthesis. Fazleabas et al. (1983) found that between the two times of secretion, Days 12 and 13, the endometrium secreted high levels of plasmin inhibitor into the uterine lumen. This inhibitor may serve to protect the uterus against proteolysis by PA produced by the porcine blastocyst. PA produced by the porcine blastocyst is not associated with implantation as was once implicated in the rodent embryo, however it is believed to be associated with the period of intense tissue remodeling and proliferation occurring during the elongation phase of development.

Menino and Williams (1987) were the first to investigate PA production in the bovine embryo and its role in development. Sixteen-cell to early morula stage embryos were cultured in five concentrations of plasminogen ranging from 0-120 μg/ml. PA production was low from 24-48 h of culture, increased between 48-120 h, and remained constant thereafter. Percentages of embryos developing to the initiating hatching blastocyst, hatched blastocyst, attached blastocyst, and attached blastocyst with trophoblastic outgrowth stage did not differ between treatments. However, as plasminogen levels increased in the culture medium, initiation and completion of hatching accelerated. These results suggest that PA may facilitate hatching, perhaps by weakening the zona pellucida proteolytically.

Kaaekuahiwi and Menino (1990) evaluated the relationship of PA production to cell stage, cell number, and changes in overall diameter and zona pellucida thickness. Total PA production was positively correlated with embryonic size, developmental stage and cell number, and a negative

correlation was found between PA production and zona pellucida thickness. More total PA production was seen in embryos that completed hatching, supporting the hypothesis of Menino and Williams (1987) that PA is involved in the hatching process. Dyk and Menino (1991) partially characterized the tissue source and type of PA produced by Day 12-14 bovine embryos. Using SDS-PAGE and zymography, light (41.5-47.0 kD) and heavy (86.1-92.2) forms of PA were detected. Based on molecular mass classification, the light form was classified as uPA and the heavy, being too large for tPA, was suggested to be a complex between uPA and PAI. Berg and Menino (1992) used antibodies against uPA and tPA and amiloride, a competitive inhibitor of uPA, to demonstrate that the light form of PA elucidated by Dyk and Menino (1991) was uPA and not tPA. Embryos treated with anti-uPA had abolished PA activity, whereas embryos treated with anti-tPA antibodies had no effect on PA activity, suggesting that Day 12-14 bovine embryos produce only uPA. Further confirmation as to the identity of the PA as uPA was demonstrated by the elimination of PA activity by amiloride.

Matrix Metalloproteinases

Matrix metalloproteinases 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). Most MMP have a hempoexin domain, with similar properties to vitronectin and the heme-binding protein, and a fibronectin and/or collagen binding domain, which are believed to have a possible role in substrate binding (Matrisian, 1990, 1992). Matrix metalloproteinases are secreted

into the extracellular environment and are thought to be involved in normal ECM degradation. Enzymes belonging to the MMP family must degrade at least one component of the ECM, contain a zinc ion, be inhibited by chelating agents, be secreted in zymogen form and require activation for proteolytic activity. They must also be inhibited by physiological specific tissue inhibitors of MMP (TIMP). Matrix metalloproteinases are classified into three groups of proteinases based on substrate specificities: interstitial collagenases, gelatinases, and stromelysins (Matrisian, 1990, 1992). Interstitial collagenases are comprised of MMP-1 and MMP-5. MMP-1 is a 52 kDa enzyme and degrades collagens type I, II, III, VII, and X (Woessner, 1991; Matrisian 1992). MMP-5 is a 53 kDa enzyme and is specific for collagens type I, II, and III. MMP-2 and MMP-9 are included in the gelatinase subfamily of matrix metalloproteinases. Substrates for MMP-2 and MMP-9 include gelatins, collagen IV and V, and elastin. Stromelysins consist of MMP-3 (stromelysin-1; 54 kDa), MMP-10 (stromelysin 2; 54.1 kDa) and MMP-7 (matrilysin; 26.7 kDa). Substrate specificity for the stromelysin subclass of enzymes is quite broad in comparison and consists of ECM proteoglycans, laminin, fibronectin, gelatin and the globular portion of the basement membrane collagens (Matrisian, 1992).

Regulation of MMP activity occurs at three levels: gene transcription, activation of the secreted latent enzyme, and inhibition of the active enzyme. Translation and secretion are closely coupled, as most MMP are not stored in those cells that secrete the enzyme. Typically, MMP-secreting cells receive a regulatory signal that initiates transcription, or conversely, inhibits transcription. The initiation signals range from cell fusion, heat shock, UV radiation, to interferons, interleukin, autocrine substances, and cAMP. Retinoic acid, glucocorticoids, estrogen and progesterone are included in the list of repressing signals.

Various proteinases and chemical substances allow the activation of

MMP, including plasmin, trypsin, organomercurials, and sodium dodecyl sulfate (Woessner, 1991). A "cysteine switch" mechanism has been proposed by Springman et al. (1990) as the form of MMP activation. A highly conserved cysteine at residue 73 in human fibroblast collagenase is associated with a zinc atom held in the catalytic domain. This mechanism is consistent with the proposed idea that all MMP are activated by a disruption of this bond, allowing a functional active site to form (Springman et al., 1990). Alternatively, dissociation of the zinc atom from the Cys^{73} may be the role of chemical agents in activation, causing the active site to be exposed. Once the active site is exposed, by either mechanism, the enzyme can autolytically cleave the peptide on the carboxyl terminal side of the cysteine residue, permanently activating the enzyme (Springman et al., 1990).

TIMP gene family is a major component of MMP regulation. These inhibitors can regulate the amount of ECM degradation by the respective MMP. The TIMP family is comprised of four proteins, TIMP-1, -2, -3 and -4 (Birkedhal-Hansen et al., 1993, Leco et al, 1994; Greene et al.,1996). TIMP-1 and -2 share a sequence homology of 40% and complete conservation of six disulfide bonds (Matrisian, 1992). TIMP-3 also has six disulfide bonds. TIMP-1 is a 28 kDa protein found in many tissues and is the key inhibitor involved in the regulation of MMP. It reversibly binds to MMP-1 and -3 in a 1:1 molar ratio, and has also been found to complex with the pro-form of MMP-9 (Matrisian, 1990). TIMP-2 is a smaller, unglycosylated protein. It has been associated with MMP-2 from cultured tumor cells and secreted in complexes with MMP-9 (Woessner, 1991). TIMP-2 also binds in a 1:1 molar ratio to active MMP, however a second molecule of TIMP-1 or -2 is required for complete inhibition of ECM degradation. TIMP-3 is a 24 kDa protein and unlike TIMP-1 and -2 is localized to the ECM. In contrast to TIMP-1, TIMP-3 is expressed at high levels in kidney, lung and brain, and lower levels in

bone; suggesting a tissue specific function (Leco et al., 1994). TIMP-4 has been recently isolated and exerts the greatest tissue specificity of the TIMP, having been found only in cardiac muscle (Greene et al., 1996). It has been proposed that TIMP may bind to a region close to the scissile bond or interact with the zinc atom of the MMP, rendering the enzyme inactive, however the exact mechanism of MMP inhibition is not known.

Matrix Metalloproteinases in Early Embryo Development

Embryo development at several stages shows implications for MMP involvement. Hatching, endodermal and mesodermal cell migration and implantation are all speculated to utilize the MMP family in these developmental processes. In terms of hatching, MMP are prime candidates for embryo escape from the zona. Sea urchin embryos express a collagenase-like hatching enzyme gene (Lepage and Gache, 1990). The gene for this enzyme has been sequenced and encodes a pre- and pro- sequence similar to that of mammalian MMP and has homologies to the activation center and active site of mammalian collagenase, gelatinase and stromelysin (Lepage and Gache, 1990). In addition, Zucotti et al. (1991) reported that hamster zona pellucida can be completely dissolved by collagenase.

The process by which endodermal and mesodermal cells migrate suggest the likely involvement of MMP. Mouse embryos secrete active collagenase and stromelysin during peri-implantation development and endoderm differentiation (Brenner et al., 1989). Maternal transcripts for collagenase, stromelysin and TIMP mRNA were found in the unfertilized ova. mRNA for these proteins was also found at the zygote and cleavage stages, and levels of mRNA increased at blastulation and endoderm differentiation (Brenner et al., 1989). Adler et al. (1990) showed that the differentiation of

embryonal carcinoma cells to embryoid bodies was accompanied by increased levels of MMP production. Also, increased levels of TIMP secretion was detected as differentiation increased (Adler et al., 1990). More important to the scope of this thesis project is the likely substrates for MMP, fibronectin and laminin. These ECM glycoproteins are found lining the blastocoelic cavity and are believed to be the matrix across which endodermal and mesodermal cells migrate (Richoux et al.,1989).

Plasminogen activator and Matrix Metalloproteinases in Implantation

As mentioned previously, the ability of PA and MMP to degrade the ECM make them a likely candidates for proteinase involvement during implantation. For invasive-type embryos, invasion of the endometrium during implantation requires ECM degradation. During this process, components of the uterine epithelium basement membrane must be degraded to allow invasion of uterine stroma by embryonic trophoblast cells. For implantation to be successful, the uterine stroma must be invaded by the embryonic trophoblast cells. Thus, enzymes capable of degrading these components, such as the MMP and PA are necessary for invasion. Plasmin, which is activated from plasminogen via PA, may degrade ECM proteins directly, or may be the initiator of a proteolytic cascade by activating latent forms of MMP. Several studies that have investigated the mechanisms by which trophoblast cells invade basement membranes *in vitro* implicate MMP and PA in the process. Of these, the most significant proteinases produced by the implanting embryo are uPA, stromelysin, MMP-2 and MMP-9 (Sappino et al.,1989; Brenner et al., 1989). After the implanting embryo establishes close contact with the endometrium, the invasion process begins, at least in part, through interactions of the embryo

with the ECM in the endometrium. Zhang et al. (1995) determined whether embryo interaction with the ECM components would effect the secretion of PA *in vitro.* Mouse embryos were collected at Day 3.5, just prior to implantation, and were cultured on dishes precoated with fibronectin. It was found that the embryos cultured on fibronectin secreted significantly more PA than those without fibronectin. The effect of fibronectin was inhibited by the addition of hexapeptides containing the integrin recognizing ARG-GLY-ASP (RGD)sequence. This suggests that the action of fibronectin enhancing PA secretion is mediated through its integrin receptors on the embryo. In this study laminin was as effective as fibronectin in promoting PA secretion. Interestingly, the onset of expression of laminin receptor-type integrin in mouse embryos also coincides with the onset of embryo implantation (Glasser et al., 1987). These findings indicate that the interactions of the implanting embryos with their ECM may regulate trophoblast invasion by a signal-transduction pathway responsive to integrin activation, and that one of the down-stream events in this pathway is uPA gene expression and secretion. In vitro studies using inhibitors of uPA have shown a decrease in the extent of trophoblast attachment and outgrowth (Kubo et al., 1981), and high levels of human chorionic gonadotropin lead to reduced uPA and collagenase activity that correlate with decreased trophoblast invasion *in vitro* (Yagel et al., 1993). Although there is much evidence that implicate PA in normal embryo implantation events, gene targeting studies have suggested that in the absence of PA, other proteinases are available to compensate or substitute for PA deficiencies. Urokinase PA is not the only proteinase required for tissue invasion. Other proteinases, particularly the MMP, are important for breaching the basement membrane and facilitating embryo invasion (Fisher and Damsky, 1993).

Human cytotrophoblasts secrete both uPA and MMP (Leibrach et al., 1991). In addition, neutralizing antibodies directed to MMP-9 and TIMP completely inhibited ECM degradation by trophoblasts in vitro (Behrendtsen et al., 1992). First trimester human trophoblasts, during the time of establishment of maternal-fetal contact, both secreted MMP-2 and -9 in relatively large amounts compared to third trimester trophoblast cells (Shimonovitz et al., 1994). Other studies have shown that antibodies against MMP-9 and TIMP-1 block degradation of the basement membrane and invasion of human cytotrophoblasts and mouse blastocyst outgrowths (Librach et al., 1991; Behrendtsen et al., 1992). During the most invasive period of mouse implantation, days 6 to 9, studies investigating TIMP gene expression *in vivo* showed that concentrations of TIMP-1 mRNA are at their highest in uterus, decidua and placenta (Waterhouse et al., 1993). TIMP-2 is expressed at a relatively constitutive rate, with concentrations increasing after day 10, when trophoblast invasion has stopped. By neutralizing TIMP-1 with antibodies, studies in decidual cell cultures led to increased trophoblast invasion (Lama and Graham 1990; Graham and Lama, 1992). It is evident that from such studies tissue invasion can be regulated at many levels. These include the synthesis of specific inhibitors, production of latent proteinases, and by activation of pro-MMP's by the uPA/plasmin/MMP cascade. Harvey et al. (1995) compared PA and MMP production in parthenogenic derived blastocyts to blastocysts derived from fertilized eggs. Parthenogenic embryos cleave, develop to the blastocyst stage, and are morphologically similar to *in vitro* produced embryos, however the majority of parthenogenic embryos exhibit perturbations of both the embryonic and extra-embryonic cell lines at the time of implantation (Peterson et al., 1993). Harvey et al. (1995) found that uPA and MMP-9 activity in conditioned medium from parthenogenic blastocyst outgrowths was less than in conditioned medium from blastocyst

outgrowths from normal zygotes. These reductions in activity may reflect the loss of functional trophoblast cells in the parthenogenic blastocyst outgrowths.

Trophoblast cells express both uPA and MMP-9 at the time of active invasion into the uterine tissue during implantation. Of these two activities, MMP-9 is likely to be the most important for the ECM degradation that accompanies implantation since antibodies and exogenous TIMP block this invasion and degradation of the basement membrane. It has been suggested that leukemia inhibitory factor (LIF) and EGF induce uPA and MMP-9 production (Harvey et al., 1995). LIF is a secreted glycoprotein that has multiple effects on cells *in vitro* and inhibits of embryonic stem cell differentiation (Rathjen et al., 1990). LIF is produced by the endometrial glands and levels are at peak production in the mouse at day 4, the time just prior to implantation (Bhatt et al., 1991). Trophectodermal cells express receptors that bind LIF (Robertson et al., 1990). LIF has been suggested to be a "trigger" for the blastocyst to initiate implantation. Harvey et al. (1995) found that when LIF was added to the cultures of embryonic outgrowths, LIF increased the activity of both uPA and MMP-9. After 9 or 10 days in culture, LIF selectively reduce expression of uPA and MMP-9 by mouse blastocysts compared to control or EGF treatments (Harvey et al., 1994). These data suggest that LIF could be the regulating cytokine which induces the ECM degrading machinery needed for implantation. EGF has also been detected in the mouse uterus and is believed to be regulated hormonally. The exact role of EGF in the implantation process is unknown, however, EGF stimulated (Harvey et al., 1995) secretion of uPA and MMP-9 in embryonic outgrowth similar to that seen with LIF. Again, Harvey et al. (1995) found that the addition of EGF to culture medium of embryonic outgrowths resulted in the stimulation of uPA and MMP-9 similarly to that seen with LIF.

Based on the proposed interactions between the ECM, integrins, and extracellular proteinases during cell migration, a comparative study was undertaken to elucidate the involvement of these components in bovine and porcine endoderm formation.

EVALUATION OF EXTRACELLULAR MATRICES AND PROTEINASE INTERACTIONS IN BOVINE ENDODERMAL CELL MIGRATION IN VITRO

Abstract

Effects of extracellular matrix-type and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) on bovine endodermal cell outgrowth in vitro were determined. Bovine inner cell masses (ICM) were isolated using immunosurgery from embryos harvested at gestational Day 7 (Day 0= onset of estrus). In Experiment 1, ICM were cultured on matrices of collagen IV, fibronectin or laminin. In Experiment 2, ICM were cultured on fibronectin in medium containing 0 or 10 μg/ml human recombinant TIMP-2 (rhTIMP-2). At 24-h intervals, ICM were observed for attachment and outgrowth, photographed and conditioned medium was recovered. Numbers of cells migrating away from the ICM were counted and ICM and outgrowth areas were measured. In Experiment 1, fibronectin (10/14; 71%) was the superior matrix ($P<0.05$) for supporting cellular outgrowth from the ICM compared to collagen IV (0/16; 0%) or laminin (2/13; 15%). ICM and outgrowth areas and cell numbers in the outgrowths increased ($P<0.05$) with time in culture. Mean cell numbers in outgrowths supported on laminin (29.0±11.3) were similar ($P>0.10$) to fibronectin (15.9±3.0). ICM and outgrowth areas were greater ($P<0.05$) on fibronectin compared to laminin and collagen IV. An interaction was observed between matrix type and time where ICM and outgrowth areas at 72 h were greater ($P<0.05$) on fibronectin compared to collagen IV and at 96 h of culture, were greater ($P<0.05$) on fibronectin compared to laminin and collagen IV. Gelatinase activity was not detected in conditioned medium recovered from cultures on any of the three matrices. In Experiment 2, no differences ($P>0.10$) in the number of ICM attaching or generating cellular outgrowth were observed in 0 and 10 μg/ml rhTIMP-2. ICM and outgrowth areas and numbers of cells in the outgrowth increased ($P<0.05$) with time in culture but no difference ($P>0.10$) was observed to rhTIMP-2. A significant interaction was observed at 96 h of

culture where numbers of cells in outgrowths in 10 μg/ml were greater ($P<0.05$) than in 0 μg/ml rhTIMP-2. Regression analysis revealed increased ($P<0.05$) rates of endodermal cell migration and change in ICM and outgrowth areas in 10 compared to 0 μg/ml rhTIMP-2. PA activity in conditioned medium from embryos cultured in 10 μg/ml rhTIMP-2 was greater ($P<0.05$) compared to 0 μg/ml rhTIMP-2. Gelatinase activity was not detected in conditioned medium from ICM cultured in the presence or absence of TIMP-2. These results demonstrate that fibronectin, and to a lesser extent, laminin, are supportive of cellular outgrowth from bovine ICM. Cell migration on fibronectin and PA production were enhanced by rhTIMP-2 suggesting a possible stimulatory effect on endodermal cell proliferation in this species.

Introduction

The preimplantation mammalian blastocyst is organized as a single celled layer of trophectoderm surrounding a fluid-filled cavity, the blastocoel, and a core of inner cell mass (ICM). In bovine embryos, primitive endodermal cells appear in the inner face of the ICM, migrate away from the ICM on Day 8, and completely surround the blastocoelic side of the trophectoderm on Day 10 (Betteridge and Fléchon, 1988). The extracellular matrix (ECM) glycoprotein fibronectin has been detected on the blastocoelic surface of the trophectoderm in swine and rats before endodermal cell migration and has been suggested as the substrate responsible for supporting movement of these cells (Richoux et al., 1989; Carnegie, 1991). After the endodermal layer is established in swine, mesodermal cell migration takes place on Day 11 and another extracellular matrix glycoprotein, laminin, appears at the junction of endoderm and mesoderm,

and trophectoderm and mesoderm (Richoux et al., 1989). Richoux et al. (1989) proposed that endodermal and mesodermal cells use fibronectin to migrate and that laminin is secreted when migration is complete to stabilize cell-matrix interactions. Cell-ECM interactions are mediated primarily by a family of cell surface receptors called integrins. The ability of a cell to bind to the ECM depends on its expressing a cell membrane receptor for the cell binding site on the large proteins that comprise the ECM. In addition to cell-ECM interactions, various matrix-degrading proteinases have been implicated in cell migration, including urokinase-type plasminogen activator (uPA) and the matrix metalloproteinases (MMP) (Danõ et al., 1985; Quax et al., 1992; Behrendtsen, 1992). Sherman et al. (1976) and Strickland et al. (1976) have suggested PA involvement in cellular migration events occurring during early mouse embryogenesis. uPA activity has been detected in cattle embryos during hatching and endodermal cell migration (Menino and Williams, 1987; Berg and Menino, 1992). MMP may also be involved in endodermal cell migration as mouse embryos secrete active MMP during peri-implantation development and endodermal differentiation (Brenner et al., 1989). In addition, Chamberlin (1995) and Chamberlin and Menino (1995) observed MMP activity, specifically, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in conditioned medium from swine, cattle and sheep embryos during the time of endodermal and mesodermal cell migration. Tissue inhibitors of MMP (TIMP) may participate in cell migration, as these inhibitors regulate the amount of ECM degradation by MMP in addition to exhibiting growth factor activity. Because PA and MMP have been implicated in the limited proteolysis of the ECM associated with cellular migration (Quax et al., 1992), the production of these proteinases, in concert with ECM-integrin interaction, may facilitate endodermal cell migration over the ECM. Therefore, the objectives of this research were to identify the ECM most supportive of cellular outgrowth from the bovine ICM

and evaluate MMP and PA production and the effects of TIMP during bovine endodermal cell migration in vitro.

Materials and Methods

Embryo Collection and Culture

Crossbred beef cows were estrous synchronized and superovulated with $PGF_2\alpha$ (Lutalyse; Upjohn Co., Kalamazoo, MI) and pFSH (Schering-Plough, Kenilworth, NJ) or Super-Ov (Wyeth-Ayerst Montreal Inc., Canada). Two 25 mg injections of $PGF_2\alpha$ were administered i.m. twelve days apart (Day 0 = first Lutalyse injection). For cows receiving pFSH injections 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. For cows receiving Super-Ov, injections were administered twice daily on days 11, 12, and 13 at doses of 1.6 ml per injection. Cows were observed for estrus at 12-h intervals beginning 24 h after the second Lutalyse injection. Cows were either handmated to one of two bulls at estrus and at 12-h intervals thereafter for as long as the cow would accept the bull, or artificially inseminated with two straws of semen at 0, 12, and 24 h after the onset of estrus. Embryos were recovered nonsurgically 7 d after mating by flushing the uterus with Dulbecco's phosphate buffered saline (DPBS) containing 2 ml/l heat-treated cow serum (HTCS) and 10 ml/l antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, MO). Embryos were recovered from the flushes by aspiration, washed three times in microdrops of Ham's F-12 with 1.5% BSA (Ham's F-12+BSA; Sigma) under paraffin oil (Fisher Scientific Co., Tustin, CA) and evaluated for normal morphology at 200X magnification with an

inverted stage phase contrast microscope. Embryos were cultured overnight until ICM isolation.

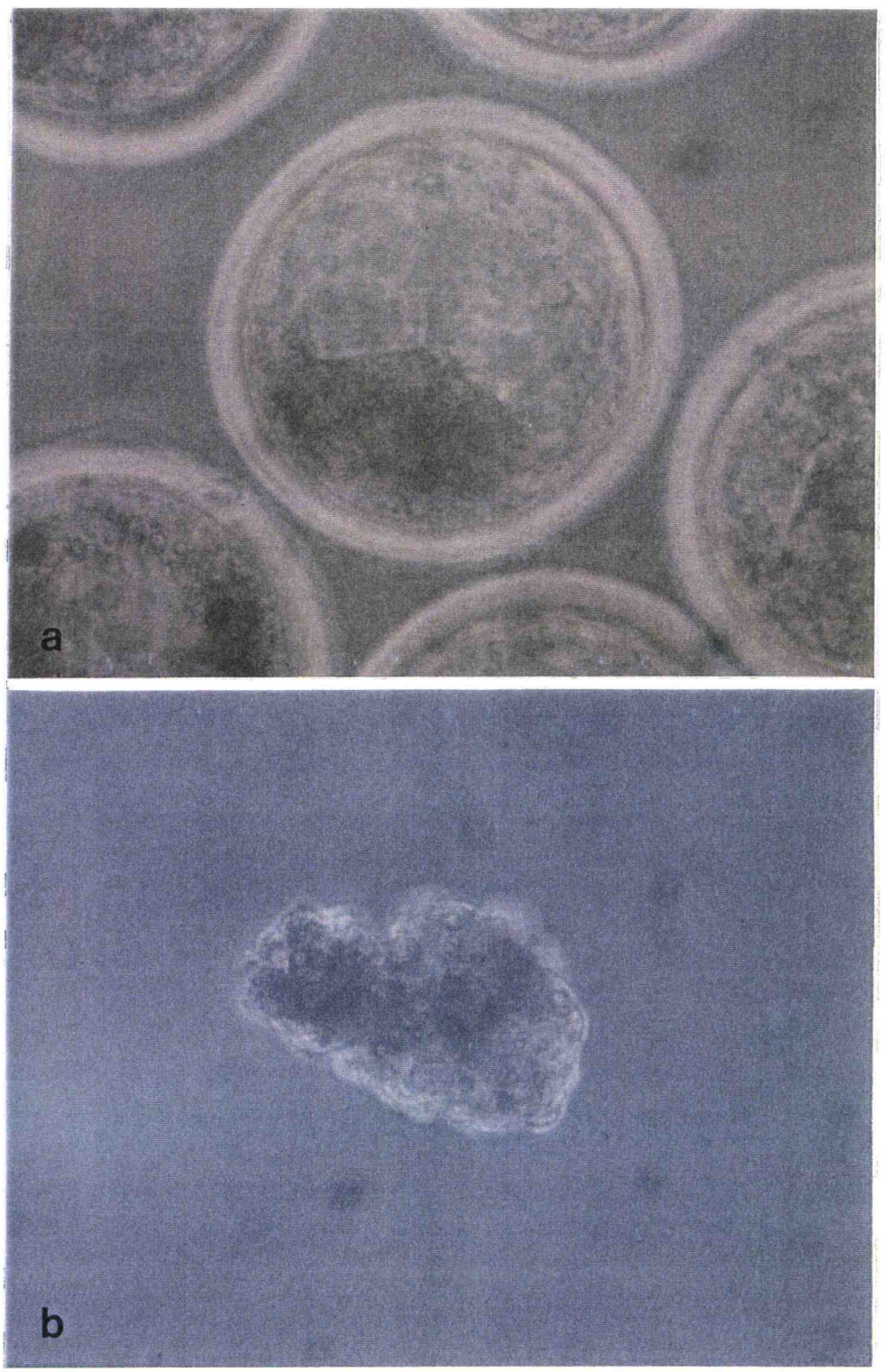
Inner Cell Mass Isolation

For embryos which failed to complete the hatching process during the overnight culture, zonae pellucidae were removed by brief exposure to acidified PBS (pH 2.0) (Figure 2-1). Embryos were subjected to immunosurgery using techniques described by Solter and Knowles (1975) and Bartlett and Menino (1995). Embryos were incubated in microdrops of rabbit anti-bovine serum (diluted 1 to 4 in Ham's F-12 + BSA) for 1 h, washed in three changes of Ham's F-12+ BSA, incubated for 1 h in guinea pig complement (diluted 1 to 4 in Ham's F-12 + BSA), washed three times and placed in microdrops of 25 mM HEPES-buffered Ham's F-12 + BSA for isolation of the ICM. All incubations were conducted at 39° C in a humidified atmosphere of 5% CO_2 in air and isolation of the ICM was performed at room temperature. ICM were isolated by repeatedly aspirating the embryo into a finely drawn siliconized capillary pipette until the lysed trophectodermal cells were clearly removed from the ICM.

Experimental Procedures

In Experiment 1, development of bovine ICM on ECM of collagen IV, fibronectin and laminin was evaluated. ICM were cultured on 60 x 15 mm plastic tissue culture dishes (Becton Dickinson and Company, Lincoln Park, NJ) in 25-µl microdrops coated with type IV collagen, fibronectin or laminin (Sigma). Culture dishes were prepared by aliquoting 25 µl of 10 µg/ml

Figure 2-1. Bovine blastocyst (a) and isolated inner cell mass (b).



solutions of type IV collagen, fibronectin or laminin in sterile PBS with 1% antibiotic-antimycotic solution (Sigma) onto the surface of the dish, covering the drops with paraffin oil and incubating at 39° C overnight. The solutions were removed the following morning and each drop was rinsed three times with Ham's F-12 + 1.5% BSA before aliquoting 25 µl of culture medium. Inner cell masses were cultured in a humidified atmosphere of 5% CO_2 in air at 39° C. Starting at the initiation of culture and at 24-h intervals, ICM were observed for attachment and cellular outgrowth with an inverted-stage phase-contrast microscope and photographed. Numbers of cells migrating away from the ICM core were also counted, and length and width measurements of the ICM and respective outgrowth were measured with an ocular micrometer. Areas of the ICM and outgrowth were determined by tracing the photomicrograph with a compensating polar planimeter (model L-20-M, LASICO, Los Angeles, CA) and computing the actual microscopic area. At 24-h intervals, 15 µl of conditioned medium was recovered from each microdrop and replaced with fresh medium. Conditioned medium was stored at -20° C until assayed for gelatinase activity.

In Experiment 2, development of bovine ICM on matrices of fibronectin in medium containing human recombinant TIMP-2 (rhTIMP-2) was evaluated. ICM were cultured in 15 x 60 mm plastic tissue culture dishes in 25-µl microdrops coated with fibronectin as described in Experiment 1. Culture media included the addition of 0 or 10 µg/ml rhTIMP-2, graciously provided by Dr. Keith E. Langley, of Amgen, CA. ICM and their respective outgrowths were evaluated at 24-h intervals as described in Experiment 1. Conditioned medium (15 µl) was recovered at 24-h intervals and replaced with fresh medium. Conditioned medium was stored at -20° C until assayed for gelatinase and PA activity.

SDS-PAGE and Zymography

All reagents for SDS-PAGE and zymography were obtained from Sigma Chemical Co., unless otherwise stated. Electrophoresis was performed at 4° C in 10% SDS-polyacrylamide gels copolymerized with 1% gelatin under non-reducing conditions as described by Brenner et al. (1989). 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 into one dimensional slab gels. Low range molecular mass markers (97.4 to 14.4 kDa; BioRad, Richmond, CA) were used as standards. After electrophoresis, gels were incubated in Triton X-100 for 1 h 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 at room temperature for 36 to 48 h. Gels were fixed and stained with 1 g/L amido black (BioRad) in 10:30:60 acetic acid:methanol:water for 1 hour and destained in 10:30:60 acetic acid:methanol:water. The appearance of clear lytic zones or bands against a dark staining background was used to indicate the presence of gelatinase activity.

Plasminogen Activator Assay

Determination of the PA activity in pooled conditioned medium was determined as described by Kaaekuahiwi and Menino (1990) using a caseinolytic agar gel assay with urokinase (E.C.3.4.21.73, American Diagnostica Inc, Greenwich, CT) as the standard. Ten microliters of urokinase standard or conditioned medium were combined with 15 µl of 120 µg/ml human plasminogen (Sigma) and the mixture was incubated for

30 min at 37°C. Twenty microliters of this solution was then pipetted into wells in the casein agar gel for determination of PA activity. Plates were incubated at room temperature for 24 h and fixed with 3% acetic acid. Lytic zones in the plate were measured by the use of a digital caliper, and each respective diameter recorded. Correlation-regression analysis was used to determine the PA activities in the conditioned medium from plots of lytic ring diameters versus log urokinase concentrations.

Statistical Analysis

Differences in the percentages of ICM undergoing attachment or outgrowth due to treatment were evaluated by Chi-square procedures. ICM and outgrowth areas and numbers of cells in the outgrowths were analyzed by repeated measures analysis of variance. Correlation-regression analysis was used to evaluate treatment effects on rates of cell proliferation and changes in ICM outgrowth and areas. All analyses were performed using the NCSS statistical software program (Number Cruncher Statistical System; version 4.1, 1984, Hintze, JL, Kaysville, UT).

Results

Experiment 1. Effects of ECM Type on ICM Outgrowth

Incidences of attachment by ICM to the ECM did not differ ($P>0.10$) among the ECM evaluated (Table 2-1). However, fibronectin was a superior matrix ($P<0.05$) for supporting cellular outgrowth compared to collagen IV or laminin. No cellular outgrowth was observed on collagen IV.

ICM areas supported by collagen IV and laminin were similar ($P>0.10$) and both ECM supported smaller ($P<0.05$) ICM outgrowth areas than fibronectin (Table 2-2). ICM and outgrowth areas increased ($P<0.05$) over time in culture, where ICM and outgrowth areas at 0, 24 and 48 h were less ($P<0.05$) than at 96 h of culture (Table 2-2). A significant interaction was also observed between ECM-type and time in culture. Over the first 48 h, all ECM supported similar ($P>0.10$) outgrowth areas however, at 72 h of culture, areas supported on fibronectin were similar ($P>0.10$) to laminin, but greater ($P<0.05$) than collagen IV (Figure 2-2). Fibronectin supported greater ($P<0.05$) areas of cellular outgrowth compared to collagen IV and laminin at 96 h of culture. Regression analysis revealed greater ($P<0.05$) rates of change in ICM and outgrowth areas on fibronectin (538.4 μ^2/h) compared to laminin (79.6 μ^2/h), and collagen IV (0.2 μ^2/h). .

Despite the reduced incidence of cellular outgrowth on laminin, mean cell numbers over the 96 h of culture in bovine outgrowths supported on laminin (29.0±11.3) were similar ($P>0.10$) to fibronectin (15.9±3.0) (Table 2-2). Numbers of cells in outgrowths increased ($P<0.05$) with time in culture where cell numbers at 24 and 48 h were less ($P<0.05$) than at 72 and 96 h. A significant interaction was observed between matrix-type and time ($P<0.05$) as ICM cultured on laminin had more ($P<0.05$) cells in outgrowths at 72 h than fibronectin (Figure 2-3). Regression analysis revealed no difference ($P>0.10$) in the rates of cell migration for ICM cultured on laminin (0.82 cells/h) and fibronectin (0.34 cells/h). A representative sequence of cellular outgrowth for bovine ICM is depicted in Figure 2-4. Gelatinase activity could not be detected in conditioned medium recovered from ICM cultured on collagen IV, fibronectin or laminin.

Table 2-1. Incidences of attachment and cellular outgrowths for bovine ICM cultured for 96 h on ECM of collagen IV, fibronectin or laminin.

ECM	Attachment [a]	Outgrowth [a]
Collagen IV	10/16 (62)[b]	0/16 (0)[b]
Fibronectin	12/14 (86)[b]	10/14 (71)[c]
Laminin	9/13 (69)[b]	2/13 (15)[b]

[a]Values presented are the number (percents) of ICM that attached or generated cellular outgrowth/total number of ICM cultured.
[b,c]Values in the same column without common superscripts are different ($P<0.05$).

TABLE 2-2. ICM and outgrowth areas ($\mu^2 x10^3$) and numbers of cells in the outgrowth for bovine ICM cultured on ECM of collagen IV, fibronectin or laminin.

	ICM and outgrowth areas		Number of cells	
Main effects	Mean	SE	Mean	SE
ECM				
Collagen IV	4.96[a]	0.53	---	---
Fibronectin	25.03[b]	6.27	15.9[a]	3.0
Laminin	7.93[a]	2.05	29.0[a]	11.3
TIME				
0	5.24[a]	0.42	---	---
24	8.00[a]	1.72	2.8[a]	1.6
48	9.68[a]	2.44	10.8[a]	3.2
72	19.23[a,b]	7.22	28.7[b]	8.0
96	30.36[b]	11.86	30.2[b]	6.4

[a,b]Means within a main effect without common superscripts are different ($P<0.05$).

Figure 2-2. Changes in ICM and outgrowth areas for bovine ICM cultured on collagen IV, fibronectin or laminin. Standard errors of the regression for ICM cultured on collagen IV, fibronectin and laminin are 0.50 x 10^3 microns^2, 8.32 x 10^3 microns^2, and 1.90 x 10^3 microns^2, respectively.

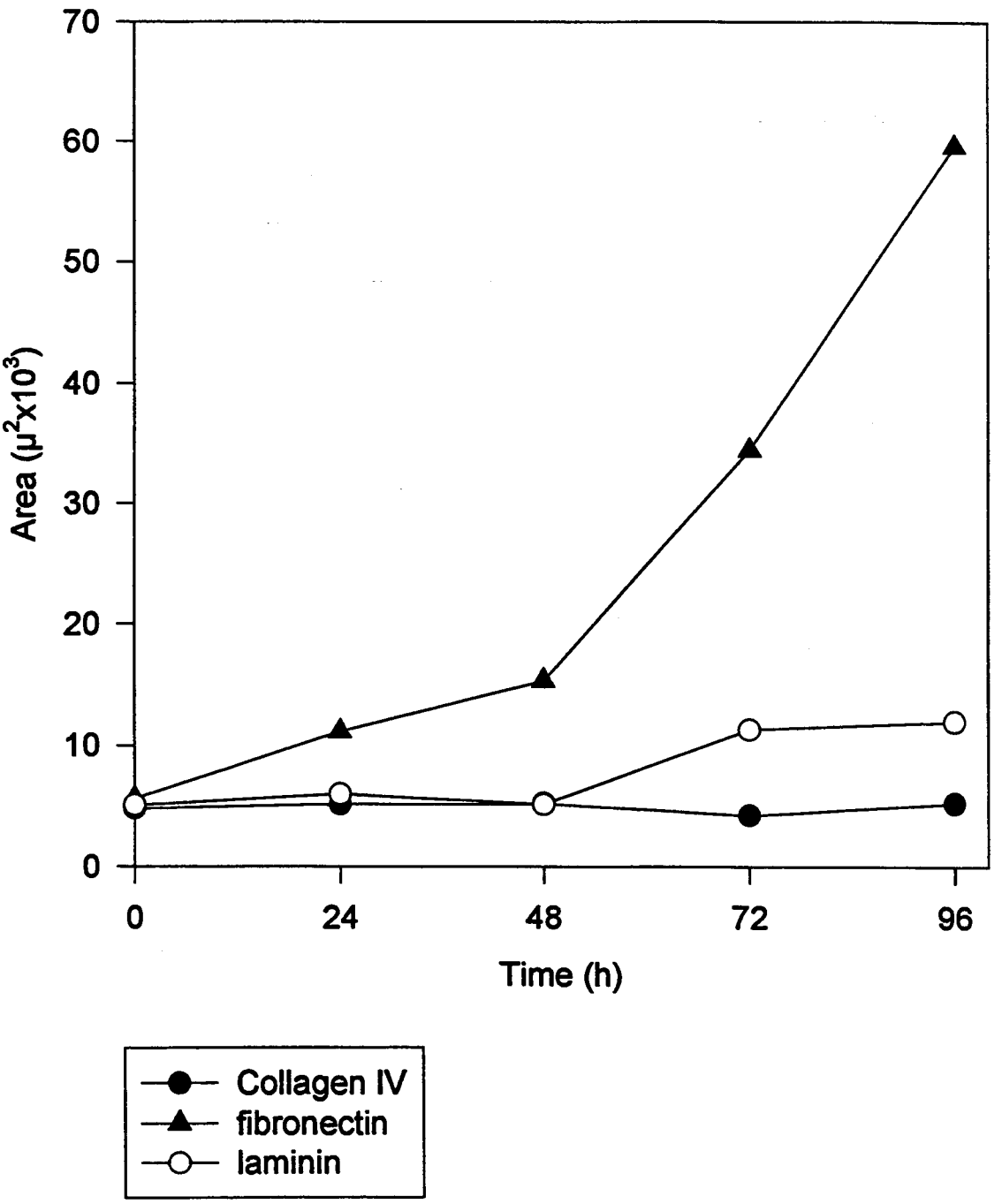


Figure 2-3. Numbers of cells in outgrowths from bovine ICM cultured on fibronectin or laminin. Standard errors of the regression for ICM cultured on fibronectin and laminin are 0.6 and 28.0 cells, respectively.

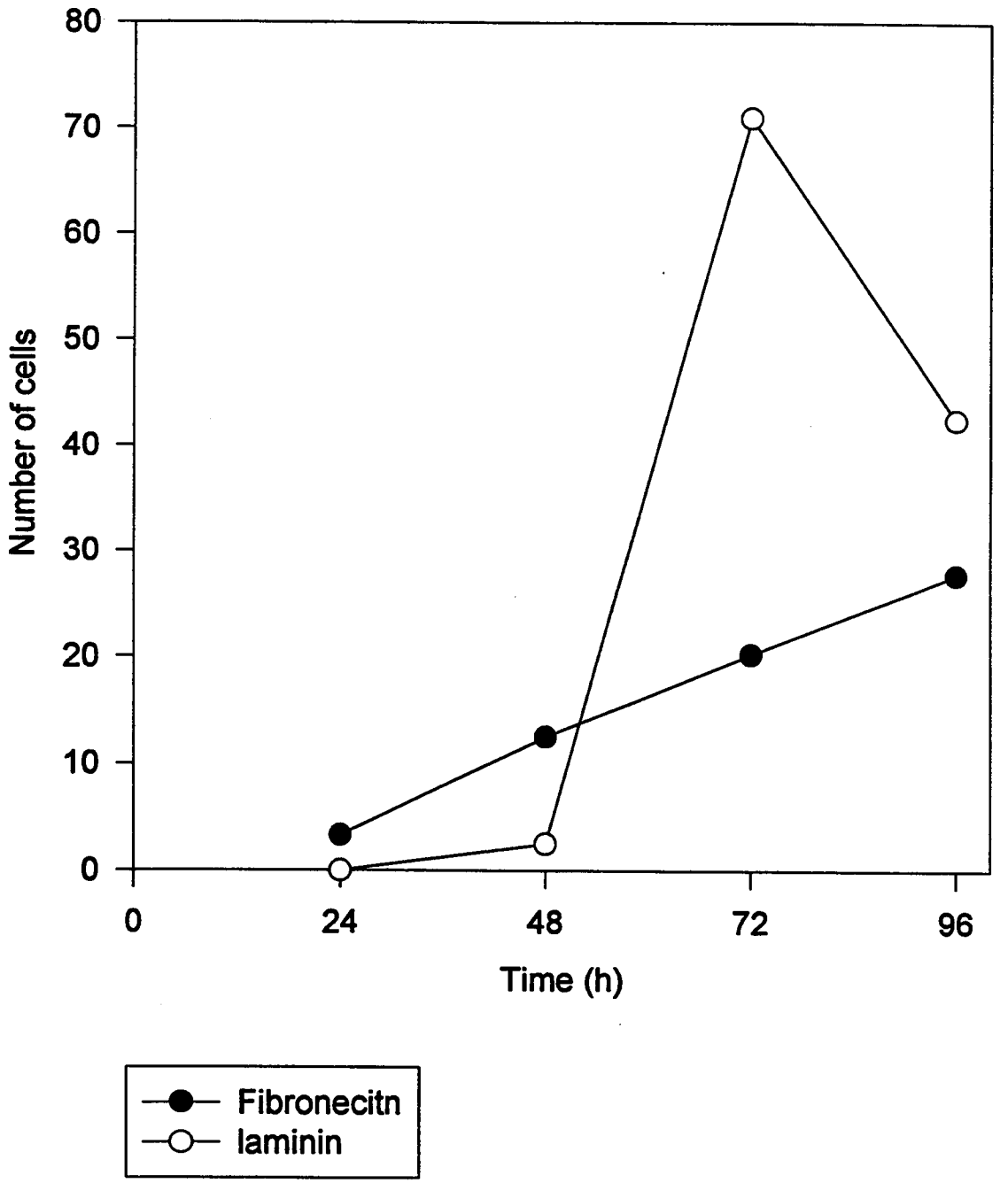
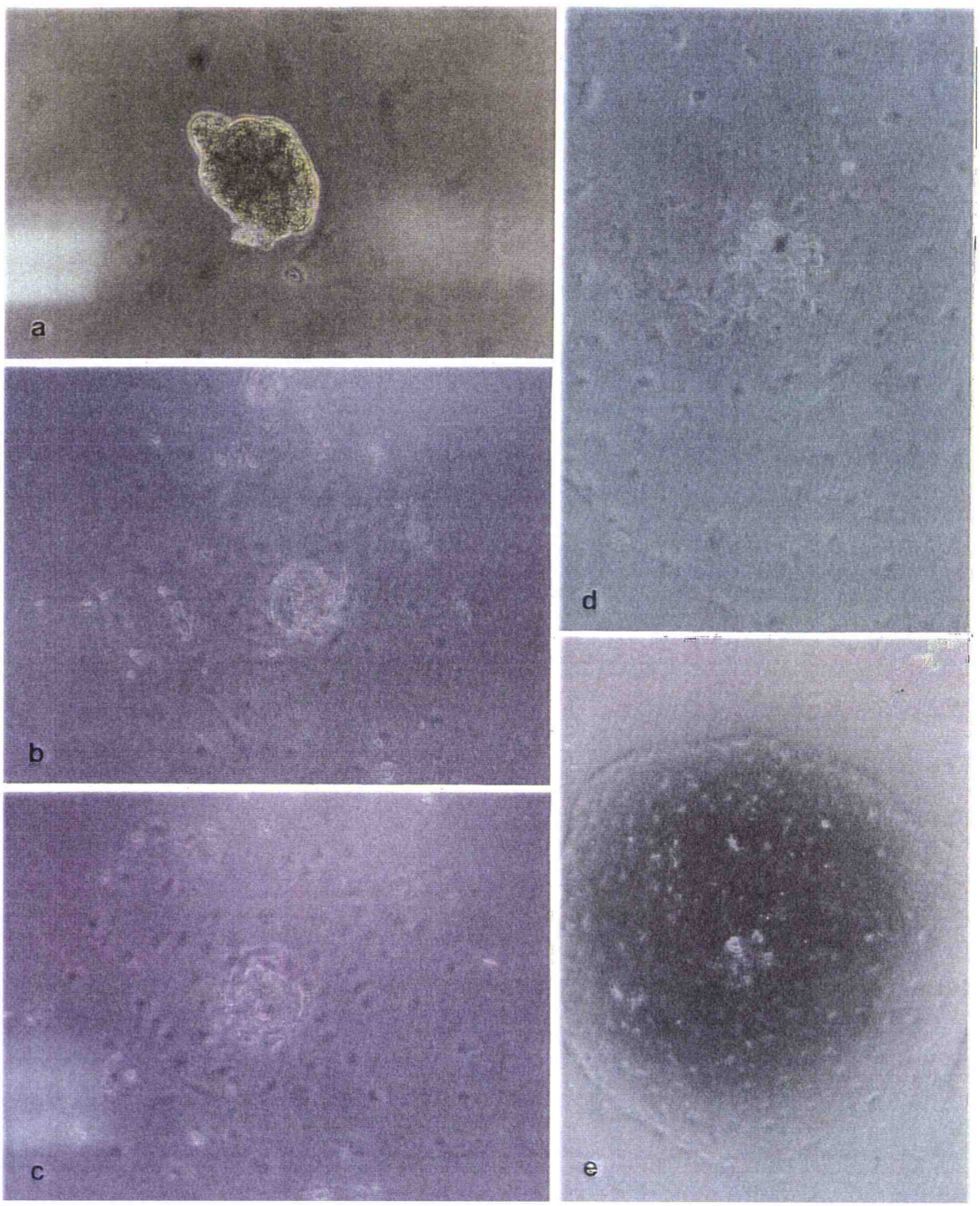


Figure 2-4. Bovine ICM at 0 h (a) and accompanying outgrowths on laminin at 24 (b), 48 (c), 72 (d) and 96 h (e) of culture.



Experiment 2. Effects of rhTIMP-2 on ICM Outgrowth on Fibronectin

Recombinant human TIMP-2 did not inhibit bovine ICM outgrowth on fibronectin. Incidences of attachment and outgrowth on fibronectin were similar (P>0.10) for ICM cultured in 0 or 10 μg/ml rhTIMP-2 (Table 2-3). No differences (P>0.10) were observed in ICM and outgrowth areas due to rhTIMP-2 and the treatment x time interaction was not significant (Table 2-4). ICM and outgrowth areas increased (P<0.05) over time in culture where areas were different (P<0.05) at 0 vs 72 and 96 h and 24 and 48 vs 96 h of culture (Figure 2-5). The rate of change in ICM and outgrowth areas in 10 μg/ml rhTIMP-2 (2397.9 μ^2/h) was accelerated (P<0.05) compared to 0 μg/ml of rhTIMP-2 (815.2 μ^2/h). No differences (P>0.10) were observed in numbers of cells in the outgrowth in 0 compared to 10 μg/ml rhTIMP-2 however, cell numbers increased with time (P<0.05) (Table 2-4). Numbers of cells in the outgrowth differed (P<0.05) between 24 vs 72 and 96 h, and 48 vs 96 h of culture. The time x treatment interaction was significant where numbers of cells in outgrowths at 96 h were greater (P<0.05) in 10 μg/ml rhTIMP-2 compared to 0 μg/ml rhTIMP-2 h (Figure 2-6). Regression analysis revealed greater (P<0.05) rates of endodermal cell migration in 10 (0.71 cells/h) compared to 0 μg/ml (0.18 cells/h) rhTIMP-2. MMP activity was not detected in conditioned medium from ICM cultured in 0 or 10 μg/ml rhTIMP-2.

PA production (Figure 2-7) was greater (P<0.05) in ICM cultured in 10 μg/ml (0.016±0.005 IU/ml) rhTIMP-2 compared to 0 μg/ml (0.007±0.001 IU/ml) rhTIMP-2. PA production increased with time and was greater (P<0.05) at 96 h compared to 24 h of culture. The treatment x time interaction was significant and PA production at 96 h in 10 μg/ml rhTIMP-2 was greater (P<0.05) than 0 μg/ml rhTIMP-2.

TABLE 2-3. Incidences of attachment and cellular outgrowth for bovine

Table 2-3. ICM cultured for 96 h on fibronectin in medium containing 0 or 10 μg/ml rhTIMP-2.

rhTIMP-2	Attachment[a]	Outgrowth[a]
0 μg/ml	13/19 (68)	6/19 (32)
10 μg/ml	9/18 (50)	9/18 (50)

[a]Values presented are the number (percents) of ICM that attached or generated cellular outgrowth/total number of ICM cultured.

TABLE 2-4. ICM and outgrowth areas ($\mu^2 x10^3$) and numbers of cells in the outgrowths for bovine ICM cultured on fibronectin in medium containing 0 or 10 μg/ml rhTIMP-2.

Main effect	ICM and outgrowth areas		Number of cells	
	Mean	SE	Mean	SE
rhTIMP-2				
0 μg/ml	42.96[a]	14.70	23.8[a]	4.9
10 μg/ml	87.89[a]	28.99	29.0[a]	6.6
TIME				
0	7.03[a]	0.75	---	---
24	20.16[a,b]	6.67	10.5[a]	3.0
48	41.70[a,b]	15.77	18.7[a,b]	4.4
72	100.56[b,c]	37.30	32.9[b,c]	8.4
96	170.17[c]	72.25	45.7[c]	13.3

[a,b,c]Means within a main effect without common superscripts are different ($P<0.05$).

Figure 2-5. Changes in ICM and outgrowth areas for bovine ICM cultured on fibronectin in 0 (-) or 10 (+) μg/ml rhTIMP-2. Standard errors of the regression for cultures with 0 or 10 μg/ml rhTIMP-2 are 3.71 x 10^3 microns^2 and 40.99 x 10^3 microns^2, respectively.

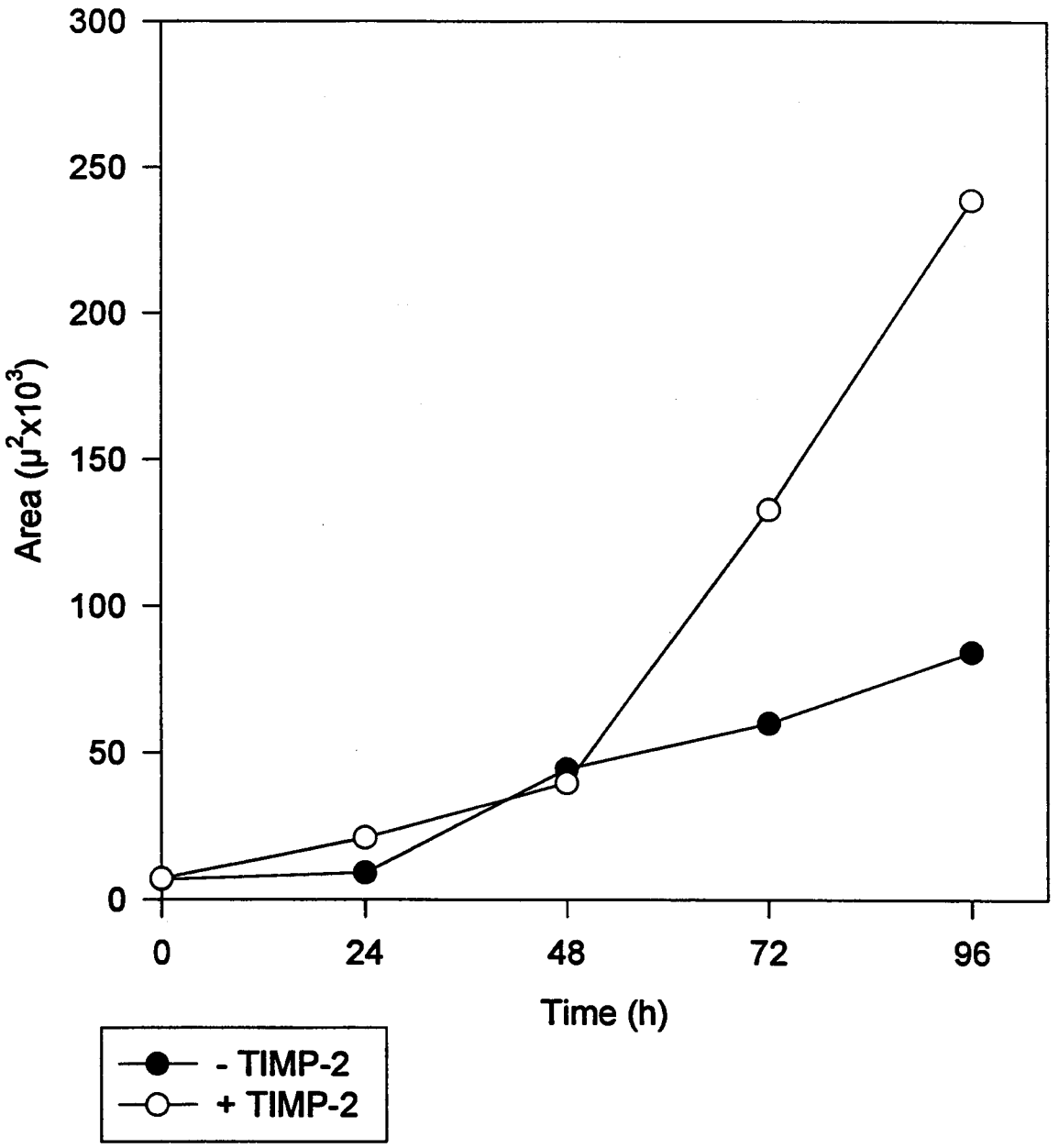


Figure 2-6. Numbers of cells in outgrowths from bovine ICM cultured on fibronectin in 0 (-) or 10 (+) µg/ml rhTIMP-2. Standard errors of the regression for cultures with 0 or 10 µg/ml rhTIMP-2 are 0.3 cells and 3.5 cells, respectively.

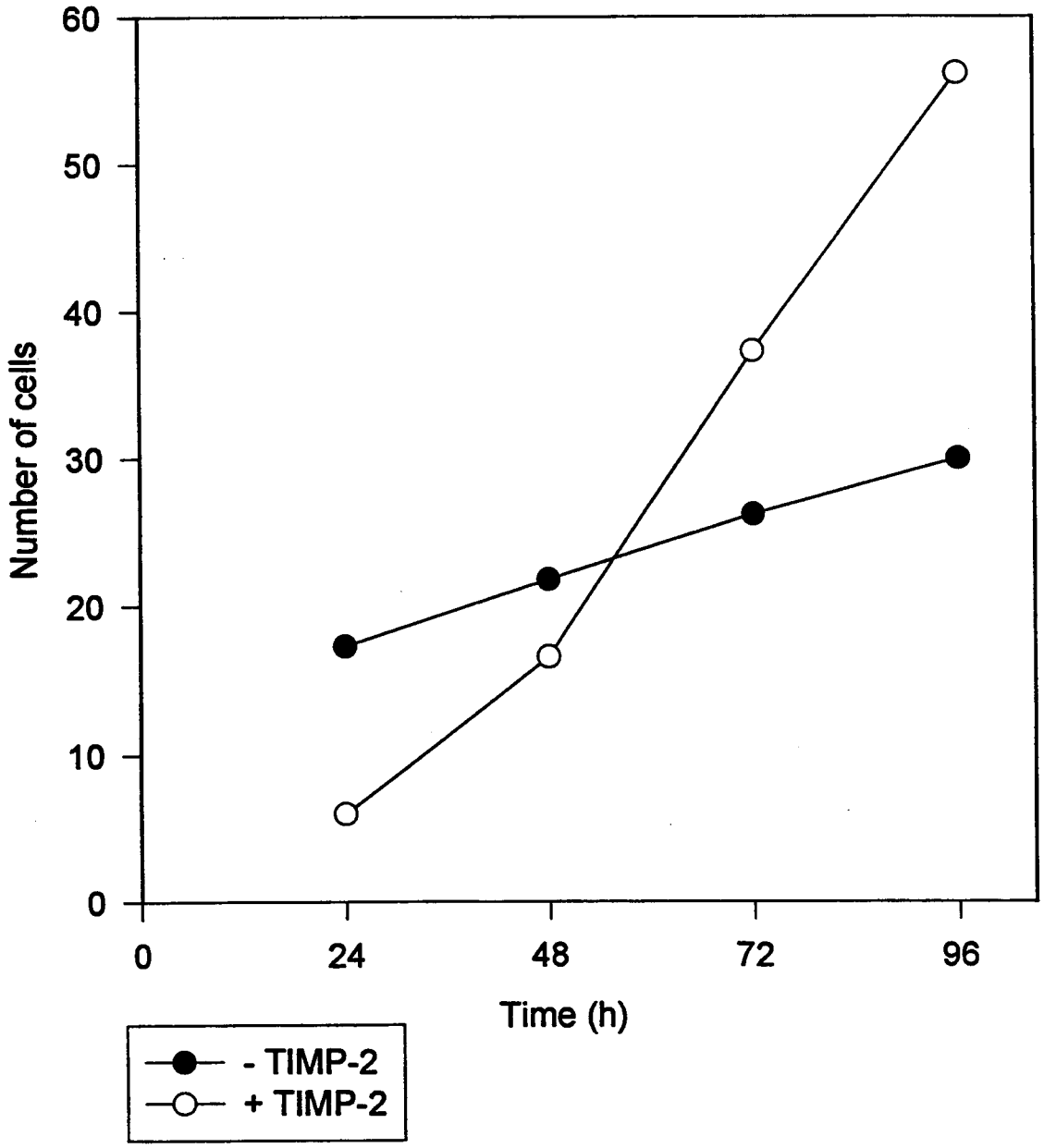
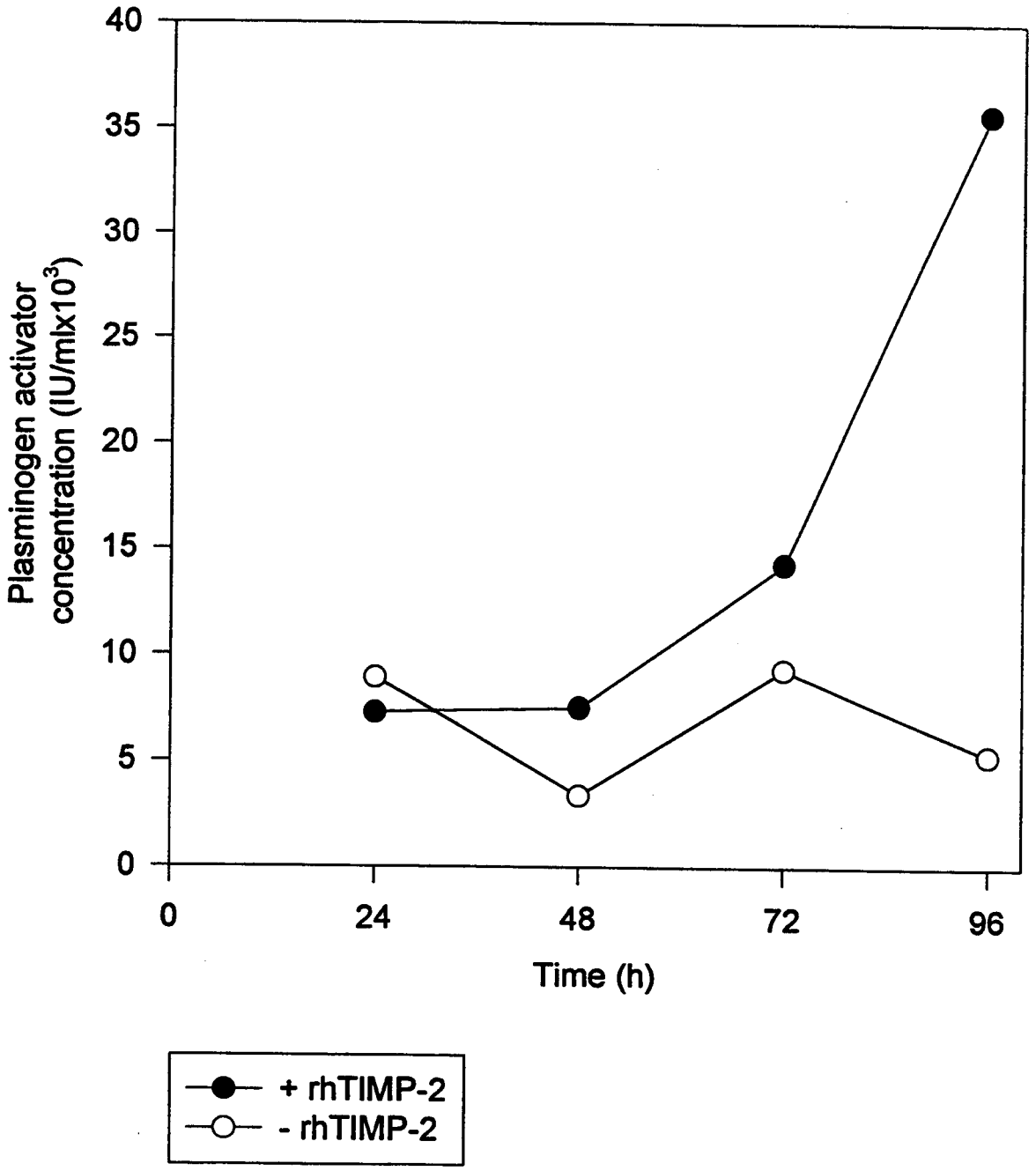


Figure 2-7. Plasminogen activator concentration (IU/ml x 10^3) in conditioned medium from bovine ICM cultured in 0 (-) or 10 (+) µg/ml rhTIMP-2. Standard errors of the means for 0 and 10 µg/ml rhTIMP-2 are 0.001 and 0.005 IU/ml, respectively.



Discussion

In the present study, fibronectin and laminin, but not collagen IV, supported outward cellular migration of bovine ICM. Fibronectin was the superior ECM as the incidence of cellular outgrowths generated on fibronectin was three-fold greater than on laminin. Fibronectin and laminin appear early in the embryo and have been implicated in key processes in cellular development and tissue organization (Wartiovaara et al., 1979). In swine and rat embryos (Carnegie, 1991), fibronectin is present on the blastocoelic surface of the trophectoderm prior to endoderm formation and is postulated to provide support for endodermal cell migration from the ICM (Richoux et al., 1989). Laminin is secreted by endodermal cells following formation of the endoderm in swine embryos (Richoux et al., 1989) and collagen IV and laminin are secreted by mouse and rat endodermal cells during formation of Reichert's membrane (Smith and Strickland, 1981; Fowler et al., 1990). Carnegie and Cabaca (1991) observed mouse endodermal cell migration on ECM of collagen IV, fibronectin and laminin. Similar results were reported by Armant et al. (1986) who observed that fibronectin and laminin support mouse trophoblast outgrowth in vitro. Apparent species differences exist because in a similar study, Bartlett and Menino (1995) observed sheep endodermal cell migration on collagen IV and fibronectin, but not laminin. However, in that study, sheep trophectodermal cells attached and generated cellular outgrowths on collagen IV, fibronectin and laminin. The observation that bovine endodermal cells proliferate over fibronectin and to a lesser extent, laminin, implies that bovine endoderm possess the necessary integrins for interaction with these two ECM. Integrins are a large interchangeable family of ECM receptors (Hynes, 1992; Simon et al.,1996) involved in cell-cell and cell-ECM adhesion. The $\alpha_5\beta_1$ is a commonly expressed fibronectin

receptor (Hynes, 1992) and bovine embryos express the $\alpha_5\beta_1$ integrin as early as the morula stage (MacLaren and Wildeman, 1995). MacLaren and Wildeman (1995) also demonstrated intense immunoreactivity for $\alpha_5\beta_1$ in Day 14 bovine endoderm but failed to detect fibronectin in Day 14 to 21 bovine embryos. Considering that the $\alpha_5\beta_1$ integrin is exclusive for fibronectin, it is somewhat odd that the receptor is present in the absence of the ligand.

Addition of rhTIMP-2 to the culture medium was stimulatory to ICM development and cellular outgrowth. Interestingly, TIMP have been reported to have growth factor effects separate from their roles as MMP inhibitors. Human TIMP-2 is 94.8% homologous to bovine TIMP-2 (Birkedahl-Hansen et al., 1993), hence, the effects observed appear physiologic. Satoh et al. (1994) reported that TIMP-1 produced by granulosa and oviductal cells enhanced in vitro development of bovine embryos and concluded that the stimulatory effect of bovine TIMP was due to enhanced cell proliferation and/or stabilization of the ECM via inhibition of MMP activity. Hayakawa et al. (1994) reported that TIMP-2 has potent growth-promoting activity for a wide variety of cells at an optimal concentration of 10 ng/ml. Hayakawa et al. (1994) report that the cell-proliferating activity of TIMP-2 seems to be a result of its direct interaction with the cell membrane and appears to be unrelated to its MMP inhibitory activity. Both TIMP lose their growth-promoting properties when they form a complex with their corresponding proMMP (Kodama et al., 1990; Fujimoto et al., 1993). Therefore, proMMP may act as regulators to control the growth factor activity of TIMP. These reports also suggest that TIMP may be one of the many unknown serum factors that play key roles in regulating in vivo cell maintenance and proliferation. Hayakawa et al. (1994) also demonstrated that TIMP-1 and TIMP-2 are constitutive components of human serum and fetal calf serum (FCS), and that cell

proliferation was remarkably suppressed in TIMP-free serum. This stimulative property was restored however, by the addition of TIMPs, suggesting that both TIMPs are potent growth factors in serum. The growth factor effects of TIMP in FCS differed from one cell line to another suggesting that proliferation may depend either on the amount of TIMP produced by the cell lines themselves, which would stimulate cell proliferation by an autocrine mechanism, or specific receptor characteristics of each cell type.

TIMP-2 is also speculated to be secreted into the extracellular environment by the migrating cell coincident with pro-forms of MMP. TIMP-2 then binds to MT-MMP, a transmembrane component of the migrating cell with greater affinity than MMP. In this position, TIMP-2 is bound to the membrane by MT-MMP and has a free carboxyl terminus binding site to which it can bind MMP-2. This complex may facilitate cell migration by forming a membrane bound MMP, with TIMP-2 being the intermediate between the membrane and the active enzyme (Imai et al., 1996). This interaction of MT-MMP, TIMP-2 and MMP-2 may be the mechanism by which bovine endodermal cell migration is enhanced on fibronectin in the presence of rhTIMP-2. However, MMP activity could not be detected in conditioned medium despite previous reports from our laboratory where MMP-2 and -9 were observed in D 12-14 bovine embryos (Chamberlin, 1995). ICM cultured on fibronectin in the presence of rhTIMP-2 produced more PA than ICM on fibronectin alone. PA production by mouse endoderm is correlated with cell migration (Strickland et al., 1976) and PA production is characteristic of endodermal cells. Sheep endodermal cells cultured on fibronectin produced more PA than cells cultured on collagen IV or laminin (Bartlett and Menino, 1995). Signal transduction through the fibronectin receptor has been reported to stimulate MMP expression (Werb et al., 1989). However, in the present

study, neither MMP-2 nor -9, were detected in conditioned medium from cultured bovine endodermal cells. The increased PA activity in conditioned medium with 10 μg/ml rhTIMP-2 may also be due in part to the greater number of cells at 96 h of culture compared to 0 μg/ml rhTIMP-2 as well as trophic effects of rhTIMP-2 on endodermal cells.

The results of the present study demonstrate that the ECM protein fibronectin, and to a lesser extent, laminin, support migration of endodermal cells. TIMP-2 is stimulatory to cell proliferation and migration and the enhanced cell proliferation results in increased PA production. MMP-2 and -9 were not detected by migrating endodermal cells, hence, endodermal cell-matrix interactions during migration may not involve this class of MMP.

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FACTORS AFFECTING PORCINE ENDODERMAL CELL MIGRATION IN VITRO

Abstract

During early embryonic development, endodermal cells leave the inner cell mass (ICM) and migrate over an extracellular matrix (ECM) located on the blastocoelic side of the trophectoderm to form a continuous layer of endoderm. In an effort to elucidate the mechanisms involved in porcine endodermal cell migration, the following experiments were conducted. In Experiment 1, isolated porcine ICM were cultured on matrices of collagen IV, fibronectin or laminin. Percentages of ICM generating cellular outgrowth on fibronectin (5/11; 45%) and laminin (4/10; 40%) were similar ($P>0.10$), however collagen IV (0/10; 0%) failed to support cellular migration from the ICM ($P<0.05$). ICM and outgrowth areas and numbers of cells in ICM with outgrowths increased ($P<0.05$) with time in culture, but no differences were observed due to matrix ($P>0.10$). In Experiment 2, porcine ICM were cultured on fibronectin or laminin in 0 or 500 μg/ml of the RGD-peptide, or on laminin in 0 or 10 μg/ml recombinant human tissue inhibitor of matrix metalloproteinase-2 (rhTIMP-2). ICM and outgrowth areas and cell numbers in the outgrowth for ICM cultured on fibronectin in medium containing 0 or 500 μg/ml RGD-peptide were not different ($P>0.10$). Porcine ICM cultured on laminin in 500 μg/ml RGD-peptide had fewer cells in the outgrowths and slower rates of cell migration compared to 0 μg/ml ($P<0.05$). No differences ($P>0.10$) in ICM and outgrowth areas and numbers of cells in the outgrowths were observed in porcine ICM cultured on laminin in medium containing 0 or 10 μg/ml rhTIMP-2. The results of these experiments suggest that optimal porcine endodermal cell migration is supported by fibronectin and laminin. Because cell migration over fibronectin was not inhibited by the RGD-peptide endodermal cells must use an integrin that recognizes an alternative sequence in fibronectin. Porcine endodermal cell

migration on laminin was inhibited by the RGD-peptide suggesting either endodermal cells recognize an RGD-sequence in laminin or stimulation by the RGD-peptide in the presence of laminin alters proliferation. rhTIMP-2 has no effect on cell outgrowth from the ICM suggesting that the MMP system is not directly involved in endodermal cell migration.

Introduction

Embryonic differentiation is a continuously regulated process and interactions between developing cells and the surrounding extracellular matrix (ECM) play a major role in the continued development of these cells. In the preimplantation mammalian embryo, the blastocyst is organized as a core of inner cell mass (ICM) surrounded by a single layer of trophectoderm. Soon after the emergence of these two cell types, endodermal cells appear on the inner face of the ICM and migrate on the blastocoelic side of the trophectoderm to form a monolayer. Studies in the mouse suggest that cell-cell and cell-ECM interactions may have a key role in differentiation of endodermal cells (Gardner, 1983; Rosenstrauss et al., 1983).

The ECM proteins, fibronectin and laminin, have been identified in the early embryo and have been reported to be involved in cell migration events. Fibronectin has been detected on the blastocoelic surface of the trophectoderm in swine and rodents before endodermal cell migration and it has been suggested as the substrate responsible for supporting movement of these cells (Smith and Strickland, 1981; Richoux et al., 1989; Carnegie, 1991). Laminin is known to influence cell attachment, movement, differentiation, and growth and several cell-surface receptors mediate these effects (Mecham, 1991). Cell-ECM interactions are mediated

primarily by a family of cell-surface receptors called integrins. A key structural element in the fibronectin molecule, the tripeptide sequence Arg-Gly-Asp (RGD), appears to function as a recognition element for integrin binding to this protein. Based on studies that utilize this peptide as an inhibitor of cell adhesion, it has been deduced that this receptor, and probably other receptors, recognize the RGD sequence in a variety of proteins that are involved in cell adhesion and migration (Pierschbacher and Ruoslahti, 1984; Hynes, 1992).

Extracellular proteinases are also believed to participate in cell migration through degradation of the ECM. The two candidate systems for extracellular proteolysis in cell migration are the plasminogen activator (PA)-plasmin system and the matrix metalloproteinases (MMP) (Vassali and Pepper, 1994). In each case where the ECM is degraded, the process involves extracellular proteolytic enzymes that are secreted locally by the migrating cells. The process by which the MMP degrade the ECM involves cleaving proteins at a small number of sites, so that the structural integrity of the ECM is destroyed in a relatively limited process. The tissue inhibitors of MMP (TIMP) gene family is a major regulator of MMP proteolysis and can limit the amount of ECM degradation by the specific MMP. PA and MMP-2 and -9 have been detected in porcine embryos during the period of endodermal and mesodermal cell proliferation but it is not known if these enzymes directly participate in cell migration events in the embryo (Fazleabas et al., 1983; Chamberlin and Menino, 1995).

In an effort to elucidate the mechanisms by which porcine endodermal cells migrate, two experiments were conducted in vitro. In Experiment 1, cellular attachment and proliferation of porcine ICM were investigated on matrices of collagen IV, fibronectin and laminin. In Experiment 2, the effects of the RGD-peptide and TIMP-2 on ICM outgrowth on matrices of fibronectin and laminin were investigated.

Materials and Methods

Embryo Collection and Culture

Crossbred gilts were checked daily for estrus and handmated to one of two boars. Embryos were recovered at slaughter or surgically 5-6 days after estrus (Day 0=onset of estrus). The reproductive tracts were recovered at slaughter and transported into the laboratory where embryos were collected by flushing the uteri with the alpha modification of Eagle's Minimum Essential Medium (αMEM; Sigma Chemical Co., St. Louis, MO). Surgical recovery of embryos was performed by induction of anesthesia in gilts by injection of 5 ml of a 1:1 mixture of xylazine (Rompum, Lloyd Laboratories, Shenandoah, IA) and ketamine HCl (Ketaset, Fort Dodge Laboratories, Fort Dodge IA) into the ear vein and anesthesia was maintained during surgery via inhalation of oxygen and halothane (Fluothane; Fort Dodge Laboratories, Inc., Fort Dodge, IA). The reproductive tract was exteriorized via midventral laparotomy and uteri were flushed with αMEM supplemented with 25 mM HEPES and 1.5% BSA (Sigma). Morulae to hatched blastocysts were recovered from the uterine flushings by aspiration and transferred to screw-cap tissue culture tubes containing 10 ml HEPES-buffered αMEM and 1.5% BSA at 39° C and transported to the laboratory. Embryos were evaluated for morphology using an inverted stage phase contrast microscope (100-200X). Embryos were washed three times and cultured overnight in microdrops containing αMEM with 1.5% BSA (αMEM+BSA) under paraffin oil (Fischer Scientific Co., Tustin, CA, U.S.A.) in a humidified atmosphere of 5% CO_2 in air at 39° C.

ICM Isolation

For embryos which failed to complete the hatching process during the overnight culture, zonae pellucidae were removed by rapid exposure to acidified PBS (pH 2.0). ICM were isolated from blastocysts using immunosurgical procedures as described by Solter and Knowles (1975) and Bartlett and Menino (1995). Embryos were incubated in microdrops of rabbit anti-porcine serum (diluted 1 to 4 in αMEM) for 1 h, washed in three changes of αMEM+BSA, and incubated in guinea pig complement (diluted 1 to 4 in αMEM+BSA) for 1 h, washed three times and placed in microdrops of 25 mM HEPES-buffered αMEM+BSA for isolation of ICM. All incubations were conducted at 39° C in a humidified atmosphere of 5% CO_2 in air. ICM were isolated by repeated aspiration through a finely drawn siliconized glass capillary pipette until the lysed trophectodermal cells were clearly removed from the ICM.

Experimental Procedures

In Experiment 1, ICM were cultured on 60 x 15 mm plastic tissue culture dishes (Becton Dickinson and Company, Lincoln Park, NJ) in 25-µl microdrops coated with type IV collagen, fibronectin or laminin. Culture dishes were prepared by aliquoting 25 µl of 10 µg/ml solutions of type IV collagen, fibronectin or laminin prepared in sterile PBS with 1% antibiotic (Sigma) onto the surface of the dish, covering the drops with paraffin oil and overnight incubation. The solutions were removed the following morning and each drop was rinsed three times with αMEM+BSA and then overlaid with αMEM+BSA. Inner cell masses were cultured for 96 h in a humidified atmosphere of 5% CO_2 in air at 39° C. At 24-h intervals, ICM

were observed for attachment and cellular outgrowth with an inverted stage phase contrast microscope and photographed. Numbers of cells migrating away from the ICM core were also counted, and length and width measurements of the ICM and respective outgrowth were determined with an ocular micrometer. Also at 24 h intervals, 15 µl of conditioned medium was recovered from each microdrop, replaced with fresh medium and stored at -20°C until assayed for gelatinase activity. ICM and outgrowth areas were determined by tracing the photomicrograph with a compensating polar planimeter (model L-20-M, LASICO, Los Angeles, CA) and computing the actual microscopic area.

In Experiment 2, development of porcine ICM on matrices of fibronectin or laminin in medium containing the RGD-peptide or rhTIMP-2 were evaluated. Inner cell masses were harvested and processed as described above, however, preparation of the culture medium included the addition of 500 µg/ml RGD-peptide (Sigma) or 10 µg/ml recombinant human TIMP-2 (rhTIMP-2) graciously provided by Dr. Keith E. Langley of Amgen Inc., CA. At 24-h intervals, ICM and the respective outgrowths were measured and photographed and numbers of cells in the outgrowths counted and conditioned medium was recovered as described.

SDS-PAGE and Zymography

All reagents for SDS-PAGE and zymography were obtained from Sigma, unless otherwise stated. Electrophoresis was performed at 4° C in 10% SDS-polyacrylamide gels copolymerized with 1% gelatin under non-reducing conditions as described by and Brenner et al. (1989). Electrophoresis through the stacking gel and separating gel was conducted for 1 h at 20 mA for and 3 h at 30 mA respectively. 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 into one dimensional slab gels. Low range molecular mass markers (97.4 to 14.4 kDa; BioRad, Richmond, CA) were used as standards. After electrophoresis, gels were incubated in Triton X-100 for 1 h 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 at room temperature for 36 to 48 h. Gels were fixed and stained with 1 g/L amido black (BioRad) in 10:30:60 acetic acid:methanol:water for 1 hour and destained in 10:30:60 acetic acid:methanol:water. The appearance of clear lytic zones or bands against a dark staining background was used to indicate the presence of gelatinase activity.

Statistical Analysis

Differences in the percentages of ICM undergoing attachment or outgrowth due to treatment were evaluated by Chi-square procedures. Morphometric data and numbers of cells in the outgrowth were analyzed by analysis of variance with repeated measures. Relationships between cell proliferation and changes in outgrowth areas and time were evaluated by correlation-regression analysis. All analyses were performed by using the NCSS statistical software program (Number Cruncher Statistical System; version 4.1, 1984, Hintze, JL, Kaysville, UT).

Results

Experiment 1. Effects of ECM-Type on ICM Outgrowth

ICM recovered from morulae cultured to the blastocyst stage or expanded blastocysts attached to fibronectin or laminin at a low frequency (1/13; 8%; and 2/14; 14%, respectively) and failed to generate cellular outgrowth (0/13; and 0/14, respectively). Incidences of attachment by ICM isolated from hatched blastocysts did not differ ($P>0.10$) with respect to the ECM-type (Table 3-1). Incidences of cellular outgrowth were similar for fibronectin (5/11; 45%) and laminin (4/10; 40%) however, collagen IV (0/10; 0%) failed to support cellular outgrowth (Table 3-1). ICM and outgrowth areas increased over time where areas at 0 and 24 h were less ($P<0.05$) than at 96 h of culture (Table 3-2). No difference ($P>0.10$) was observed in ICM and outgrowth areas due to matrix-type, and the treatment x time interaction was not significant (Figure 3-1). Regression analysis of ICM and outgrowth areas over time revealed that the rate of increase in area on fibronectin (556.6 μ^2/h) and laminin (410.5 μ^2/h) were similar ($P>0.10$) and both were greater ($P<0.05$) than collagen IV (-9.9 μ^2/h).

Numbers of cells in the outgrowths increased ($P<0.05$) over time in culture, where cell numbers at 24 h were less ($P<0.05$) than at 72 and 96 h (Table 3-2). Numbers of cells in outgrowths on fibronectin and laminin were not different ($P>0.10$) (Table 3-2). A significant interaction was observed between matrix-type and time where cell numbers in outgrowths generated on laminin were greater ($P<0.05$) than fibronectin at 72 and 96 h of culture (Figure 3-2). The rate of cellular migration on laminin (0.75 cells/h) was accelerated ($P<0.05$) compared to fibronectin (0.19 cells/h). A representative sequence of cellular outgrowth for porcine ICM is depicted in Figure 3-3. Gelatinase activity was not detected in conditioned medium

TABLE 3-1. Incidences of attachment and cellular outgrowth for porcine ICM cultured for 96 h on ECM of collagen IV, fibronectin or laminin.

ECM	Attachment [a]	Outgrowth [a]
Collagen IV	3/10 (30)[b]	0/10 (0)[b]
Fibronectin	8/11 (73)[b]	5/11 (45)[c]
Laminin	6/10 (60)[b]	4/10 (40)[c]

[a]Values presented are the number (percents) of ICM that attached or generated cellular outgrowth/total number of ICM cultured.
[b,c]Values in the same column without common superscripts are different ($P<0.05$).

TABLE 3-2. ICM and outgrowth areas ($\mu^2 x10^3$) and numbers of cells in the outgrowths for porcine ICM cultured on ECM of collagen IV, fibronectin or laminin.

Main effects	ICM and outgrowth areas Mean	SE	Number of cells Mean	SE
ECM				
Collagen IV	4.88[a]	0.79	---	---
Fibronectin	29.90[a]	9.89	14.4[a]	2.9
Laminin	24.53[a]	7.18	38.0[a]	9.4
TIME				
0	5.55[a]	0.75	---	---
24	10.20[a]	2.11	7.0[a]	2.0
48	27.23[a,b]	10.11	21.7[a,b]	5.1
72	28.23[a,b]	12.67	32.2[b]	7.9
96	43.48[b]	20.10	38.8[b]	15.6

[a,b]Means within a main effect without common superscripts are different ($P<0.05$).

Figure 3-1. Changes in ICM and outgrowth areas for porcine ICM cultured on collagen IV, fibronectin and laminin. Standard errors of the regression for ICM cultured on collagen IV, fibronectin and laminin are 0.65 x 10^3 microns^2, 7.29 x 10^3 microns^2, and 5.99 x 10^3 microns^2, respectively.

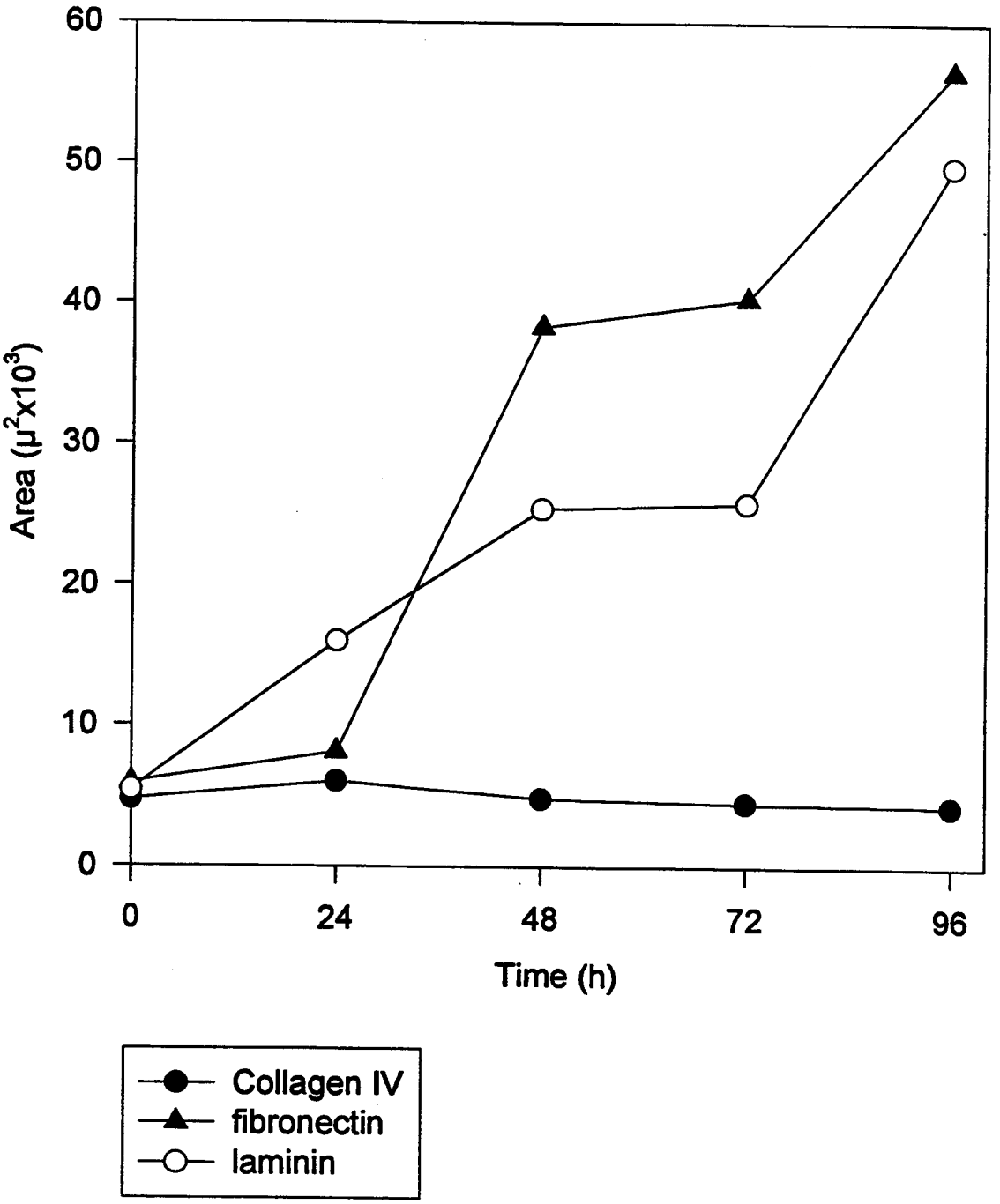


Figure 3-2. Numbers of cells in outgrowths from porcine ICM cultured on fibronectin and laminin. Standard errors of the regression for ICM cultured on fibronectin and laminin are 5.3 cells and 0.7 cells, respectively.

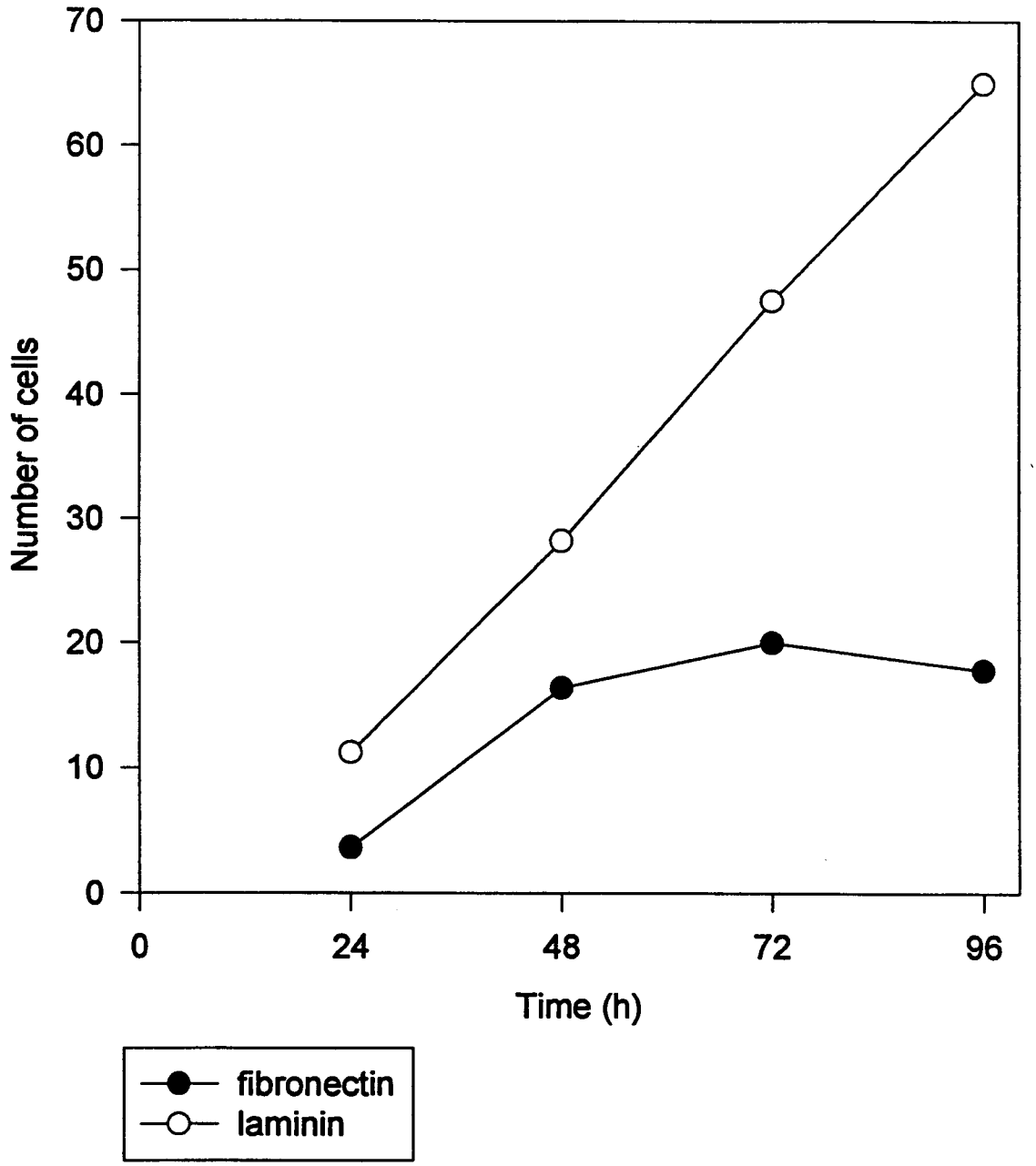


Figure 3-3. Porcine ICM at 0 h (a) and accompanying outgrowths on laminin at 24 (b), 48 (c), 72 (d) and 96 h (e) of culture.

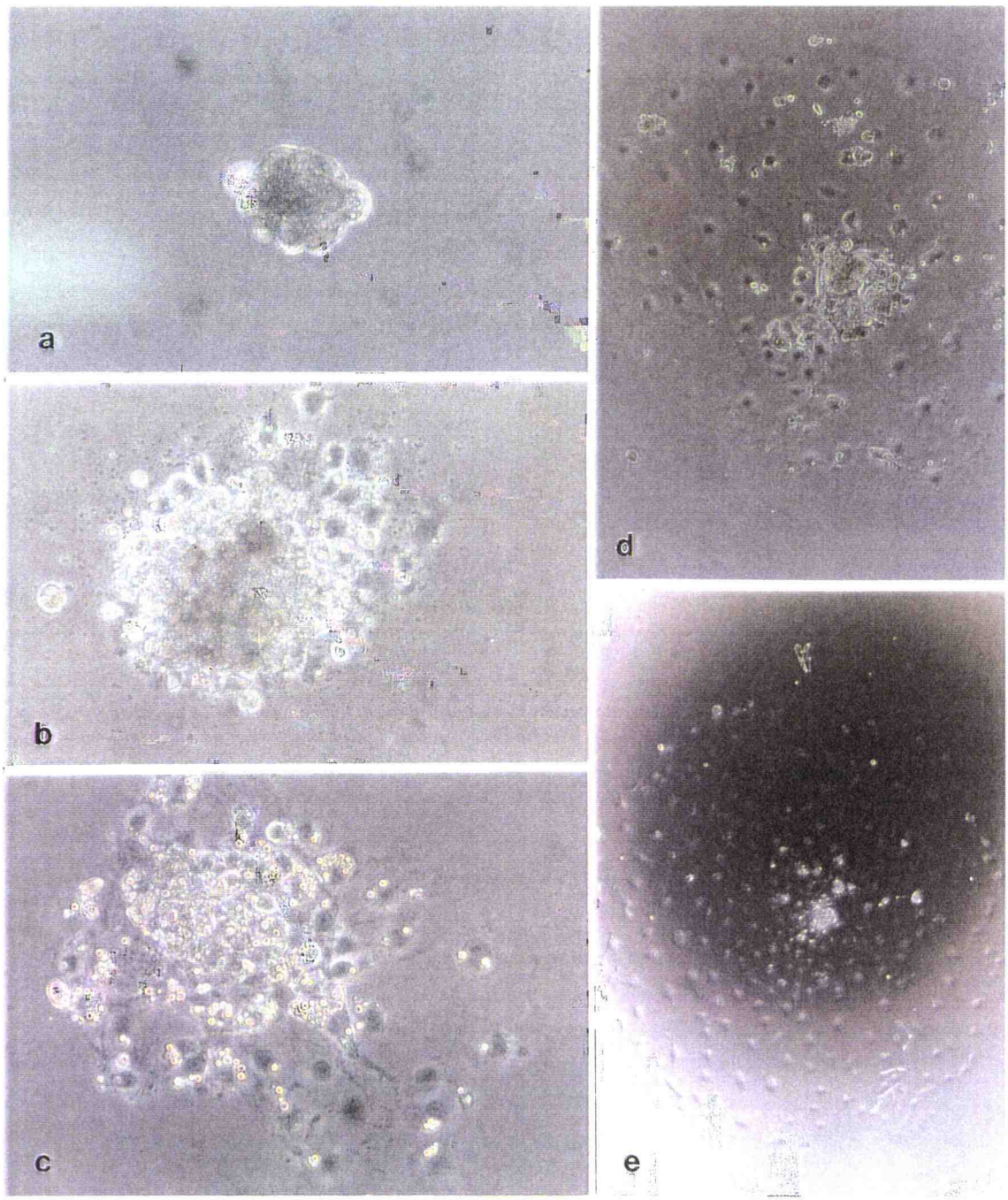
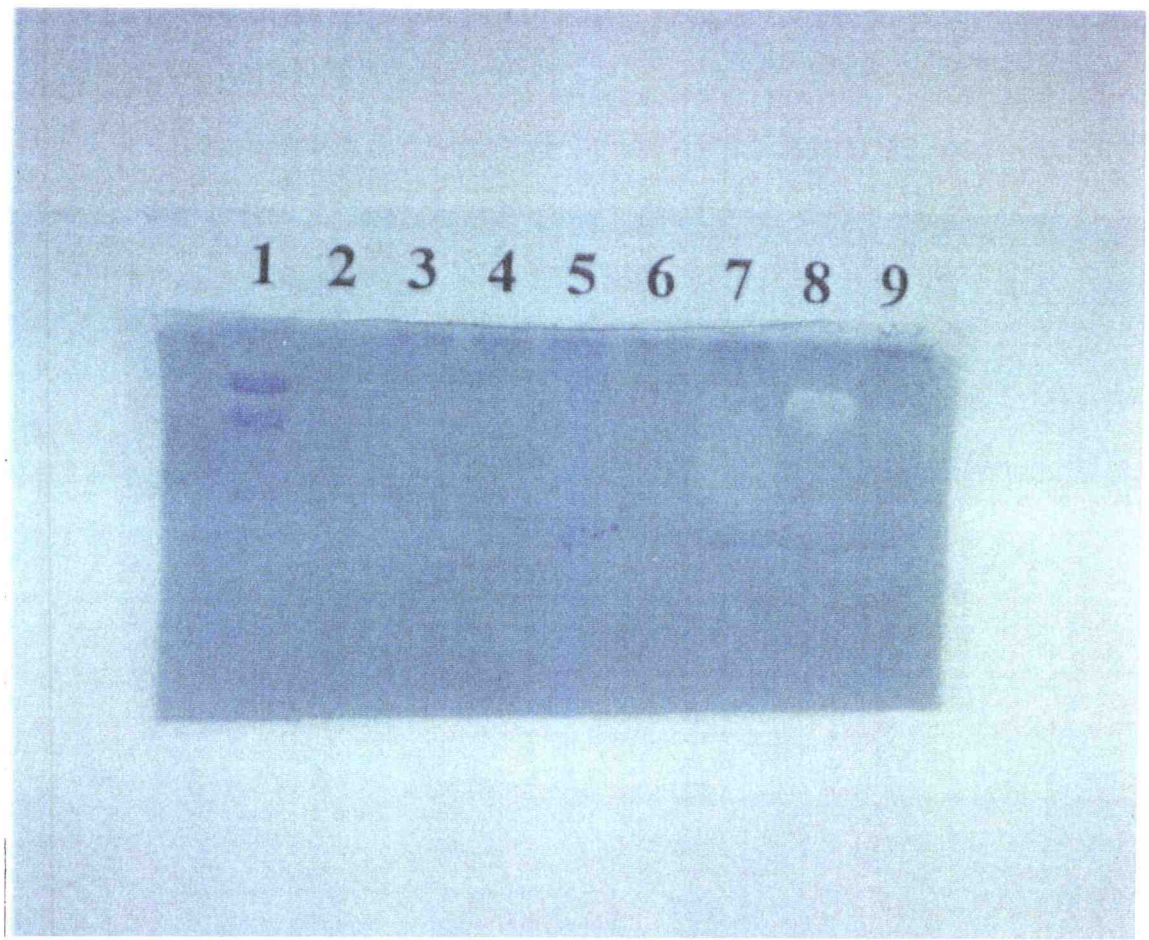


Figure 3-4. Zymographic analysis of conditioned medium recovered from porcine ICM at 24 (lane 3), 48 (lane 4), 72 (lane 5), 96 (lane 6), 120 (lane 7), and 144 h (lane 8) of culture. Molecular mass markers are in lane 1 and unconditioned medium in lane 9.



recovered over the 96 h culture period, however activity was observed in pooled conditioned medium recovered from 120 to 144 h of culture (Figure 3-4).

Experiment 2. Effects of the RGD-peptide on ICM Outgrowth on Fibronectin and Laminin and rhTIMP-2 on ICM Outgrowth on Laminin

Addition of the RGD-peptide to the culture medium did not inhibit endodermal cell migration on fibronectin. No differences ($P>0.10$) due to treatment were observed in the incidences of ICM attachment or outgrowth (Table 3-3). ICM and outgrowth areas increased ($P<0.05$) over time where areas at 96 h were greater ($P<0.05$) than at 0 through 72 h of culture (Table 3-4). No difference in ICM and outgrowth areas were observed due to the RGD-peptide ($P>0.10$) and the treatment x time interaction was not significant (Figure 3-5). The rate of change in ICM and outgrowth areas did not differ ($P>0.10$) in 500 μg/ml RGD-peptide (6857.0 μ^2/h) when compared to 0 μg/ml (9124.3 μ^2/h). Numbers of cells migrating away from the ICM increased ($P<0.05$) over time in culture where numbers of cells at 96 h were greater ($P<0.05$) than at 24 and 48 h of culture (Table 3-4). No difference ($P>0.10$) was observed due to the addition of the RGD-peptide and the treatment x time interaction was not significant (Figure 3-6). Rate of cellular migration was not different ($P>0.10$) in medium containing 500 μg/ml (.33 cells/h) compared to 0 μg/ml RGD (.55 cells/h).

In contrast to the results from the fibronectin experiment, addition of RGD inhibited endodermal cell migration on laminin. No differences ($P>0.05$) due to treatment were observed in the incidences of cellular attachment and outgrowth (Table 3-3). ICM and outgrowth areas increased over time where areas at 0 h were less ($P<0.05$) than at 48 through 72 h and areas at 24 and 48 h were less ($P<0.05$) than at 72 and 96 of culture

(Table 3-6). No difference (P>0.10) in ICM and outgrowth areas were observed due to the addition of the RGD-peptide and the treatment x time interaction was not significant (Figure 3-7). However, the rate at which the cellular outgrowth area enlarged was more moderate (P<0.10) with 500 μg/ml RGD-peptide (668.3 μ^2/h) when compared to 0 μg/ml RGD (1008.9 μ^2/h). Numbers of cells in the outgrowths were less (P<0.05) in cultures with 500 μg/ml RGD-peptide compared to 0 μg/ml (Table 3-4). Numbers of cells in outgrowths did not increase (P>0.10) over time, however a significant treatment x time interaction was observed at 72 and 96 h of culture where numbers of cells in outgrowths in 500 μg/ml RGD were less (P<0.05) than 0 μg/ml (Figure 3-8). Regression analysis revealed a decreased (P<0.05) rate of cellular migration by cells cultured with 500 μg/ml RGD (-0.07 cells/h) when compared to 0 μg/ml (0.52 cells/h). A representative culture of porcine ICM on laminin in the presence or absence of RGD is depicted in Figure 3-9.

No differences (P>0.05) in the incidences of attachment or cellular outgrowth were observed due to rhTIMP-2 (Table 3-5). ICM and outgrowth areas increased (P<0.05) over time in culture where areas at 0 through 48 h were less (P<0.05) than at 96 h of culture (Table 3-6). Rates of increase in ICM and outgrowth areas were similar (P>0.10) for ICM cultured in 0 (5750.5 μ^2/h) and 10 μg/ml rhTIMP-2 (9720.7 μ^2/h). Results observed for cell numbers in the outgrowths were consistent with morphometric observations. Numbers of cells in the outgrowths increased (P<0.05) over time where cell numbers were less (P<0.05) at 24 h vs 72 and 96 h and 48 h vs 96 h of culture. No differences in numbers of cells in the outgrowths were observed due to treatment and the treatment x time interaction was not significant. Cellular migration rates were similar (P>0.05) for endodermal cells in 10 μg/ml rhTIMP-2 (0.78 cells/h) compared to 0 μg/ml (0.62 cells/h).

TABLE 3-3. Incidences of attachment and cellular outgrowth for porcine ICM cultured for 96 h on fibronectin or laminin in medium containing 0 or 500 μg/ml RGD-peptide.

RGD	Attachment[a]	Outgrowth[a]
Fibronectin		
0 μg/ml	9/10 (90)	8/10 (80)
500 μg/ml	9/11 (82)	7/11 (64)
Laminin		
0 μg/ml	9/12 (75)	5/12 (42)
500 μg/ml	9/12 (75)	9/12 (75)

[a]Values presented are the number (percents) of ICM that attached or generated cellular outgrowth/total number of ICM cultured.

TABLE 3-4. ICM and outgrowth areas ($\mu^2 x10^3$) and numbers of cells in the outgrowths for porcine ICM cultured on fibronectin or laminin in medium containing 0 or 500 μg/ml RGD.

	ICM and outgrowth areas		Number of cells	
Main effect	Mean	SE	Mean	SE
Fibronectin/RGD				
0 μg/ml	295.78[a]	105.56	36.3[a]	8.6
500 μg/ml	248.46[a]	78.14	28.9[a]	6.9
TIME				
0	7.06[a]	0.70	---	---
24	71.14[a]	26.39	18.0[a]	4.0
48	122.53[a]	47.76	27.8[a]	5.5
72	302.54[a]	59.66	33.5[a,b]	9.4
96	904.65[b]	309.85	52.1[b]	18.6
Laminin/RGD				
0 μg/ml	48.42[a]	9.93	28.2[b]	5.5
500 μg/ml	41.26[a]	7.02	15.4[a]	2.0
TIME				
0	5.70[a]	0.28	---	---
24	22.40[a,b]	2.82	13.4[a]	2.4
48	42.28[b]	4.83	21.1[a]	3.1
72	73.09[c]	15.73	20.6[a]	3.8
96	79.13[c]	15.94	24.9[a]	8.1

[a,b,c]Means within a main effect without common superscripts are different.

Figure 3-5. Changes in ICM and outgrowth areas for porcine ICM cultured on fibronectin in 0 (-) or 500 (+) μg/ml RGD-peptide. Standard errors of the regression for cultures with 0 μg/ml and 500 μg/ml RGD-peptide are 243.34 x 10^3 microns^2 and 109.78 x 10^3 microns^2, respectively.

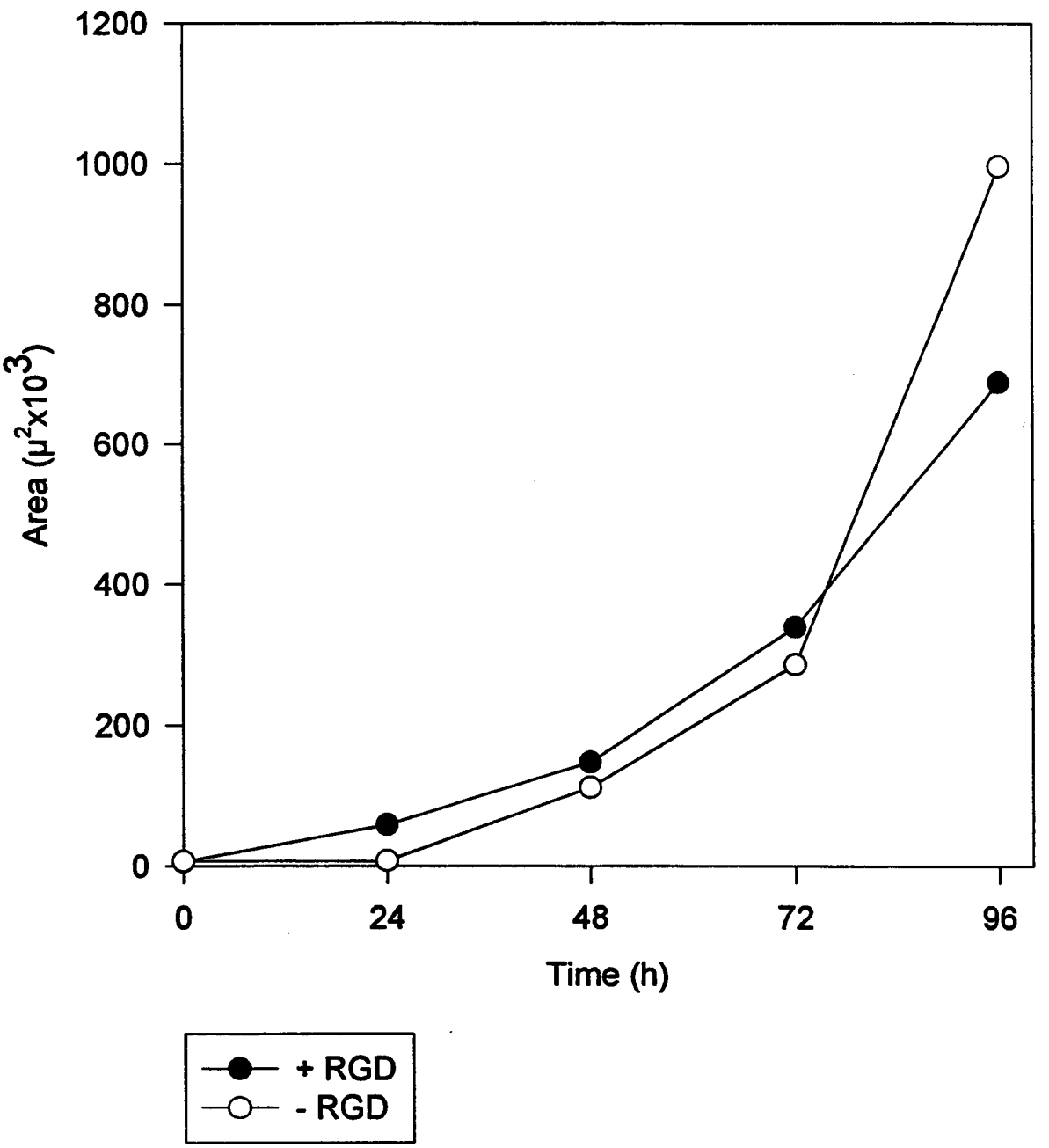


Figure 3-6. Numbers of cells in porcine outgrowths cultured on fibronectin in 0 (-) or 500 (+) µg/ml of the RGD-peptide. Standard errors of the regression for cultures with 0 µg/ml or 500 µg/ml of RGD are 3.8 and 4.6 cells, respectively.

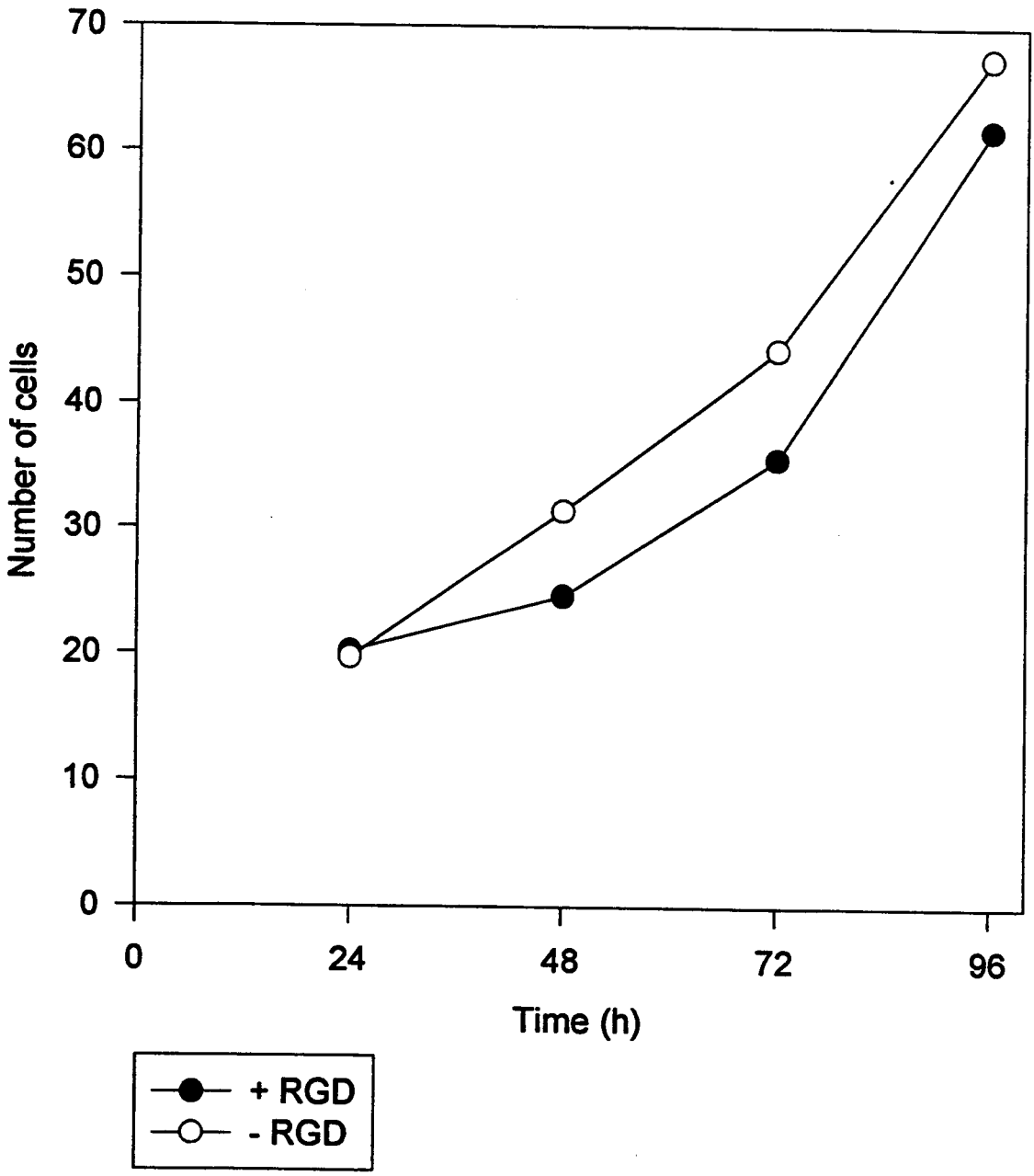


Figure 3-7. Changes in ICM and outgrowth areas for porcine ICM cultured on laminin in 0 (-) or 500 (+) μg/ml RGD-peptide. Standard errors of the regression for cultures with 0 μg/ml and 500 μg/ml RGD-peptide are 10.75 x 10^3 microns^2 and 4.34 x 10^3 microns^2, respectively.

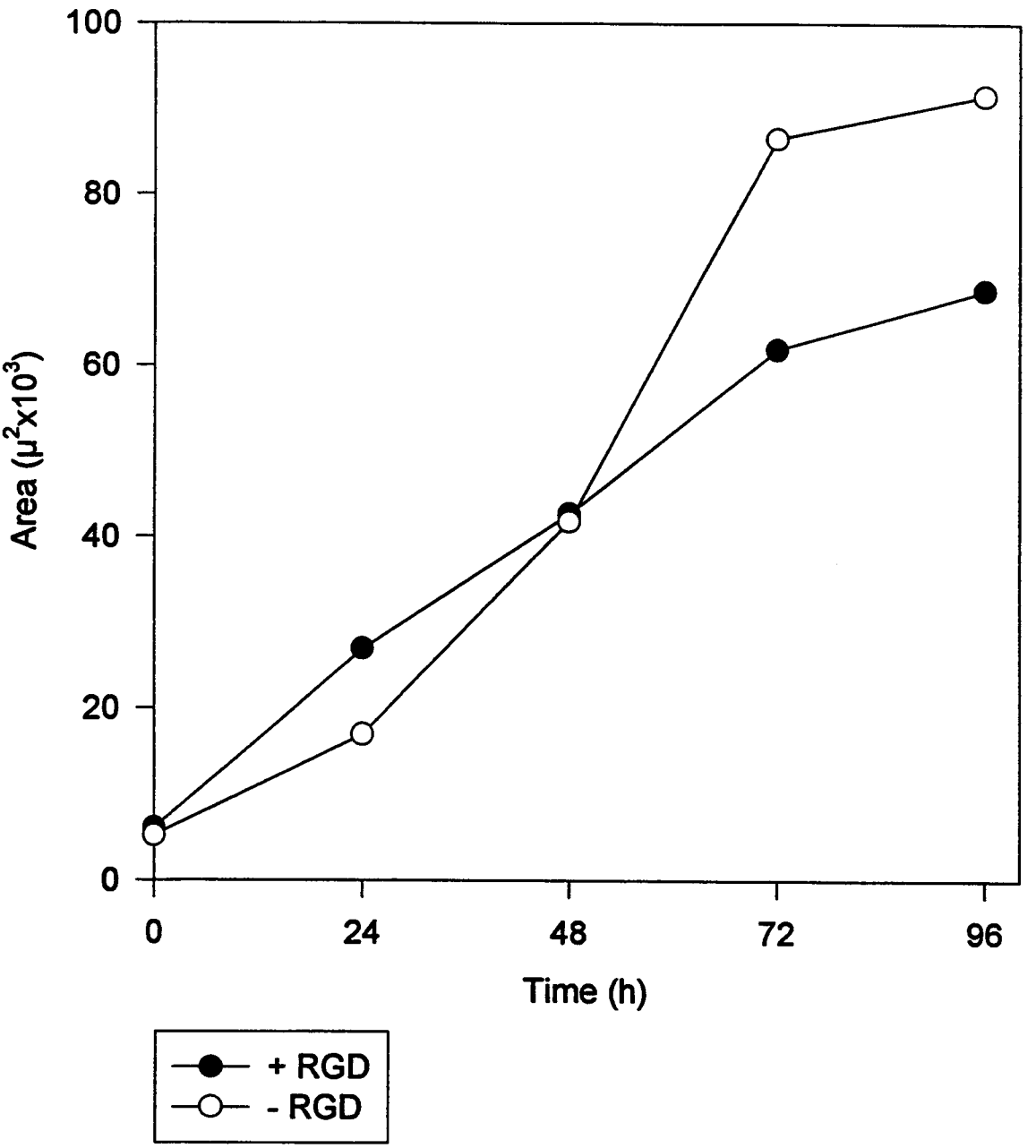


Figure 3-8. Numbers of cells in porcine outgrowths cultured on laminin in 0 (-) or 500 (+) µg/ml RGD-peptide. Standard errors of the regression for cultures with 0 µg/ml and 500 µg/ml RGD-peptide are 2.8 and 4.6 cells, respectively.

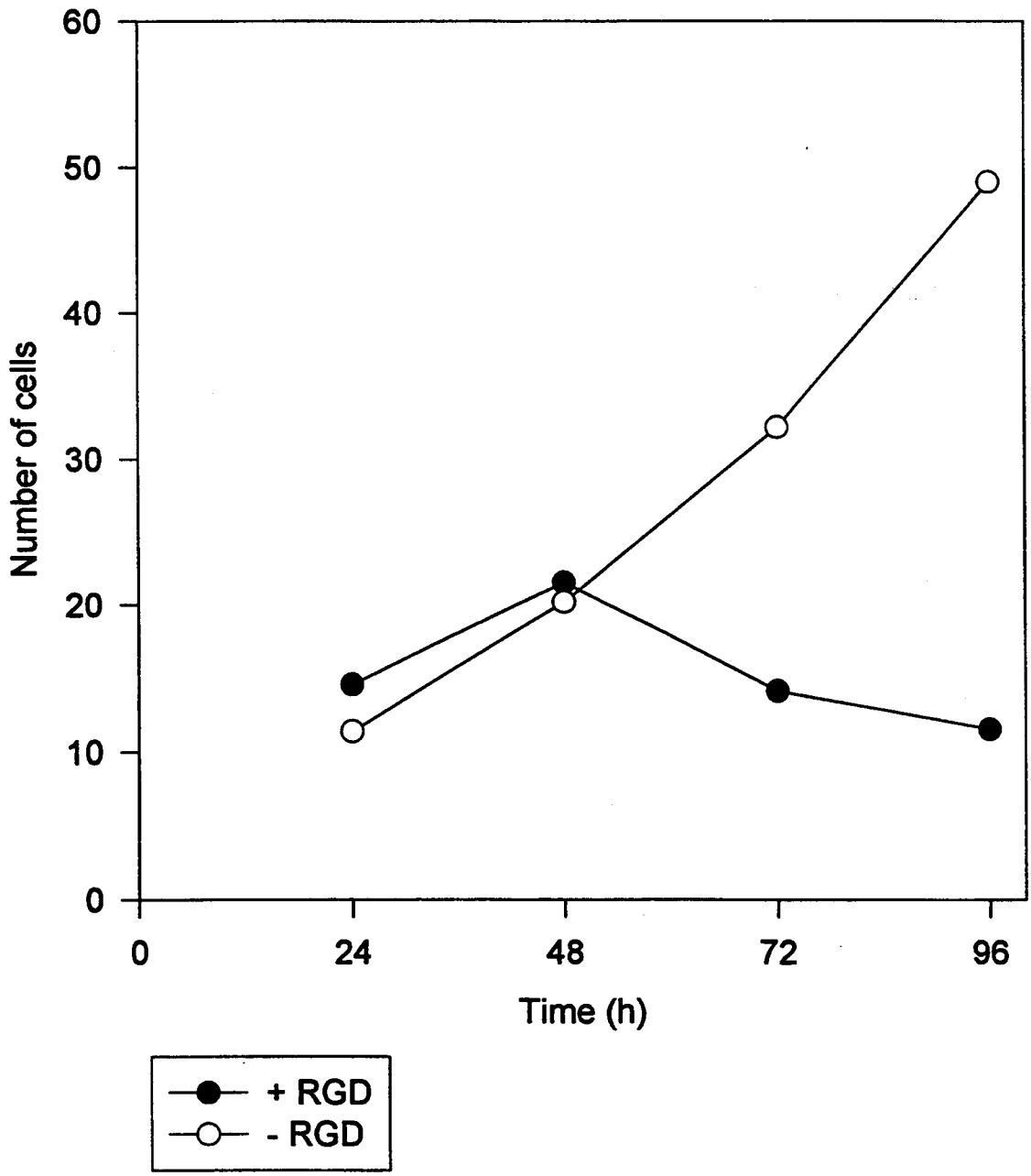


Figure 3-9. Porcine ICM cultured on laminin in 0 (a) and 500 µg/ml (b) RGD-peptide at 72 h of culture. ICM in (a) photographed at 100 X whereas the ICM in (b) was photographed at 200 X.

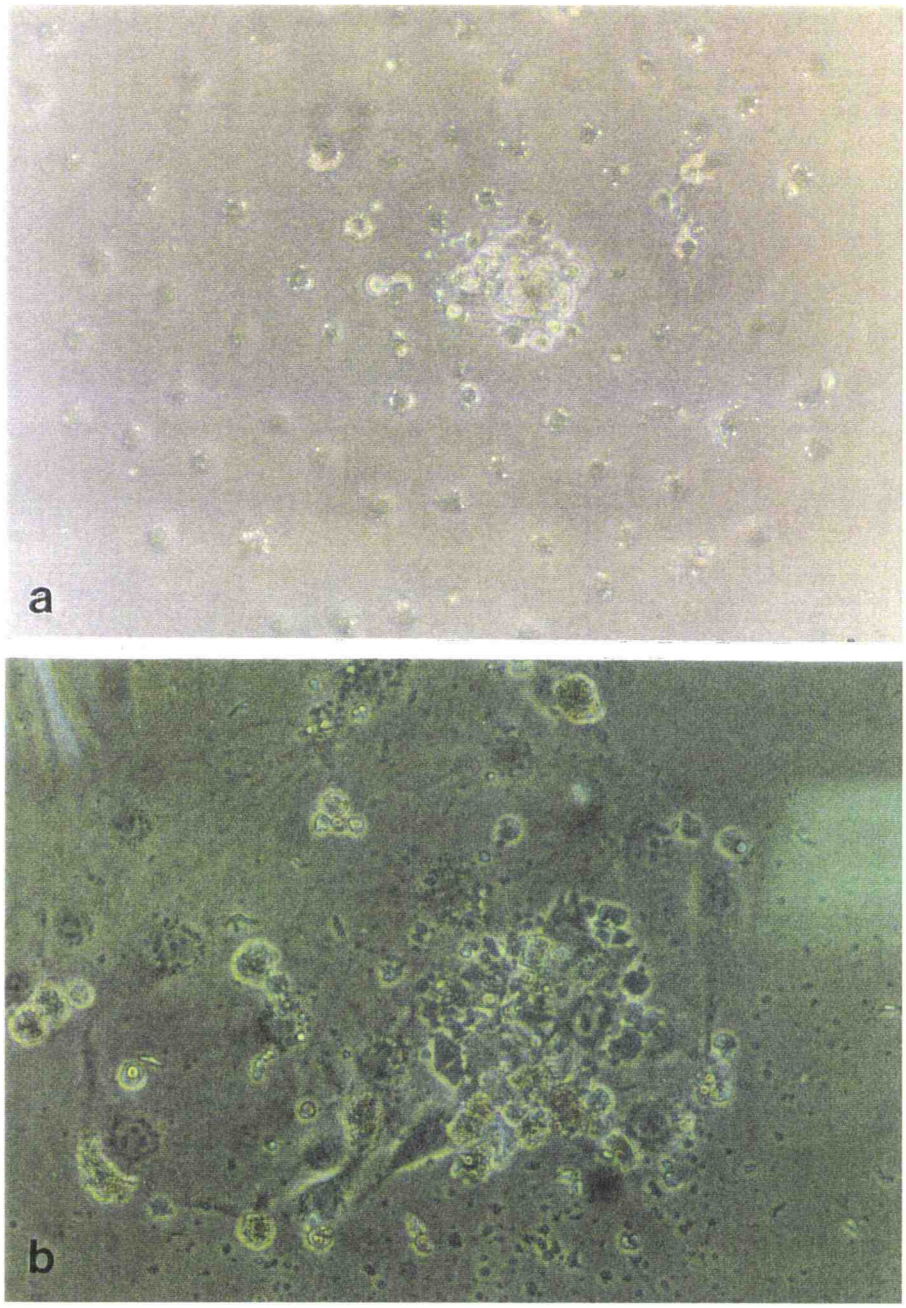


TABLE 3-5. Incidences of attachment and cellular outgrowth for porcine ICM cultured for 96 h on fibronectin in medium containing 0 or 10 μg/ml rhTIMP-2.

rhTIMP-2	Attachment[a]	Outgrowth[a]
0 μg/ml	9/11 (82)	8/11 (73)
10 μg/ml	5/11 (45)	4/11 (36)

[a]Values presented are the number (percents) of ICM that attached or generated cellular outgrowth/total number of ICM cultured.

TABLE 3-6. ICM and outgrowth areas ($\mu^2 x 10^3$) and numbers of cells in the outgrowths for porcine ICM cultured on laminin in medium containing 0 or 10 μg/ml rhTIMP-2.

Main effect	ICM and outgrowth areas Mean	SE	Number of cells Mean	SE
rhTIMP-2				
0 μg/ml	204.60[a]	86.08	30.1[a]	8.9
10 μg/ml	283.42[a]	134.29	29.7[a]	10.5
TIME				
0	5.98[a]	0.56	---	---
24	28.77[a]	10.31	4.4[a]	1.6
48	84.52[a]	48.42	23.2[a,b]	8.4
72	339.59[a,b]	142.74	39.6[b,c]	14.3
96	676.75[b]	262.33	52.7[c]	19.9

[a,b,c]Means within a main effect without common superscripts are different ($P<0.05$).

Figure 3-10. Changes in ICM and outgrowth areas for porcine ICM cultured on laminin in 0 (-) or 10 (+) µg/ml rhTIMP-2. Standard errors of the regression for cultures with 0 µg/ml rhTIMP-2 or 10 µg/ml rhTIMP-2 are 86.78 x 10^3 microns^2 and 259.86 x 10^3 microns^2, respectively.

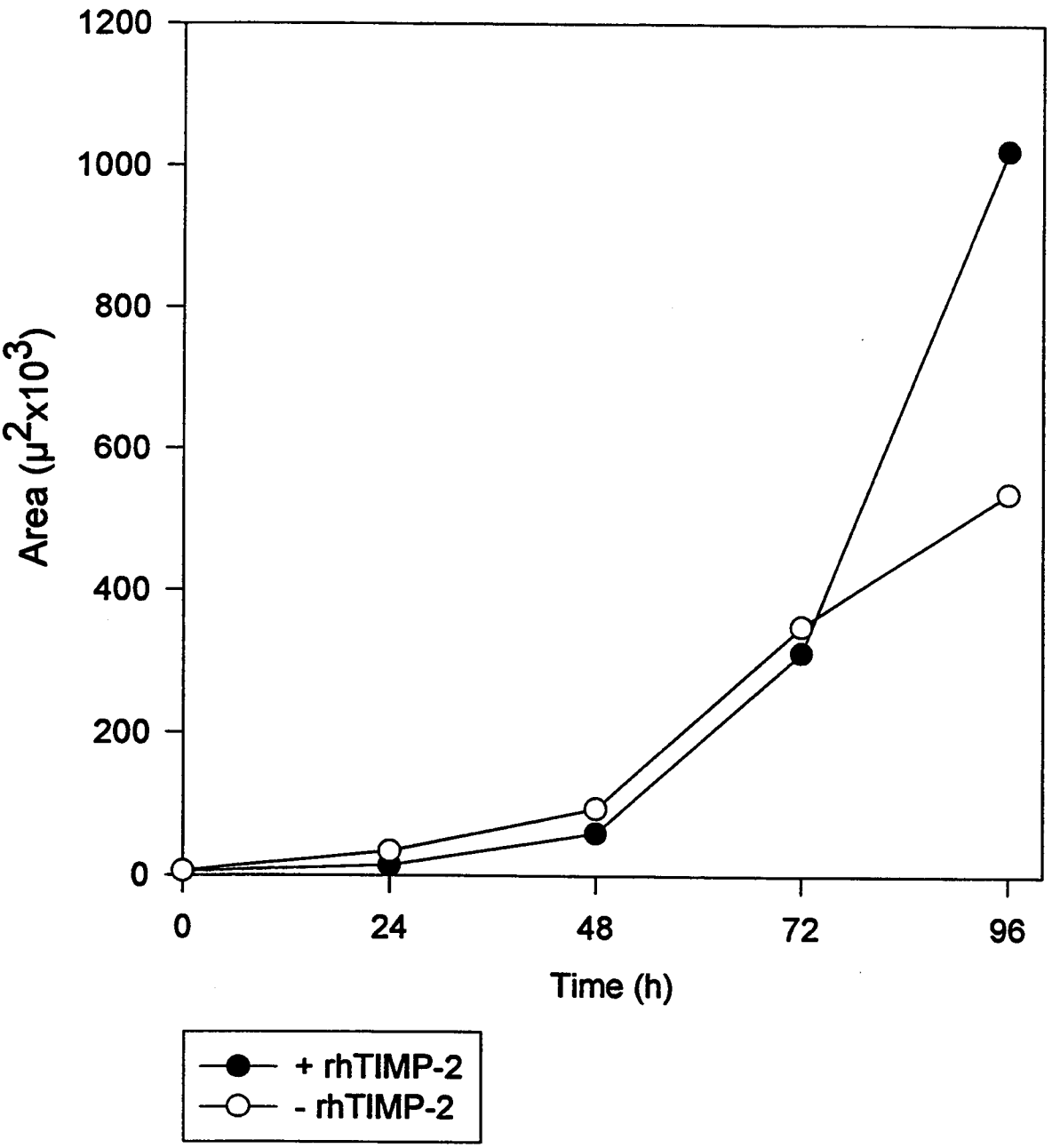
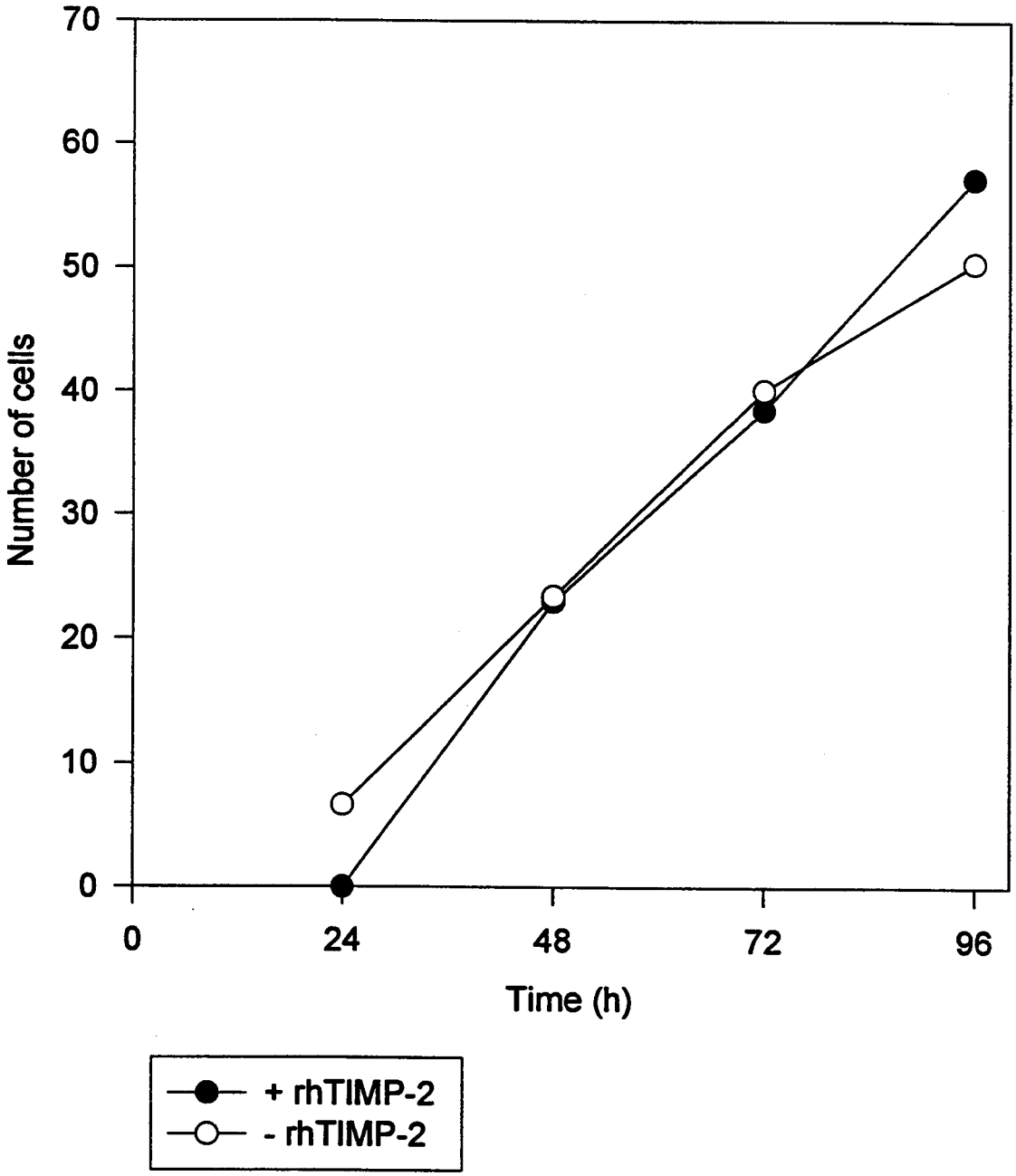


Figure 3-11. Numbers of cells in porcine outgrowths cultured on laminin in 0 (-) or 10 (+) μg/ml rhTIMP-2. Standard errors of the regression for cultures with 0 and 10 μg/ml rhTIMP-2 are 2.5 and 2.3 cells, respectively.



Discussion

In the present study, fibronectin and laminin supported outward cellular migration of porcine endodermal cells from the ICM. Fibronectin is present in mouse, rodent and swine embryos (Carnegie, 1991; Richoux et al., 1989) on the blastocoelic surface of the trophectoderm prior to endodermal formation. Richoux et al. (1989) has suggested that endodermal cells migrate over the fibronectin matrix to form the extraembryonic endoderm in the pig. Laminin is secreted by porcine endodermal cells following migration and is believed to stabilize cell-ECM interactions. Rodent parietal endoderm cells also secrete laminin during formation of Reichert's membrane, the thin basement membrane located between the trophectoderm and parietal endoderm (Smith and Strickland, 1981). Mesodermal cells follow a similar pattern of migration from the embryonic disc during formation of the extraembryonic mesoderm in the pig (Richoux et al., 1989). Mesodermal cells migrate through a network of fibronectin and also lay down laminin when migration is complete. Besides stabilizing cell-ECM interactions, laminin may also serve as a stop signal for cell migration (Coopman et al., 1991).

Cellular outgrowth in the presence of the RGD-peptide should be inhibited if endodermal cells utilize an integrin that recognizes the RGD-peptide (Hynes, 1992). The present findings suggest that porcine endodermal cells migrate on fibronectin without the use of an RGD-recognizing integrin. The $\alpha_5\beta_1$ integrin is the principal cellular receptor for fibronectin and recognizes the RGD-sequence. As no decrease in attachment and outgrowth is observed with RGD, it is possible that endodermal cells bind fibronectin via another integrin which recognizes fibronectin at an alternative site. The $\alpha_4\beta_1$ integrin binds fibronectin at the EILDV site and the α_4, β_1 subunits have been detected in D 11-15 pig

embryos (Bowen et al., 1996). Armant (1986) demonstrated inhibition of trophoblast attachment and outgrowth in a system containing fibronectin and hexapeptides containing the RGD sequence, but no blockage in culture systems containing laminin. These results deviate from our observations where porcine ICM cultured on laminin in the presence of RGD had fewer cells in the outgrowths and slower rates of cell migration. On laminin alone, porcine ICM attach and generate cell outgrowths, but in the presence of the RGD-peptide, attachment and outgrowth occur, but after 24 h, the cultures become static, and migration rates decline. With respect to previous studies, porcine endodermal cells may require exposure to the ECM in a specific sequence to replicate the in vivo process of migration and stabilization. A signal transduction pathway induced by RGD binding to the cell may mimic initial exposure to fibronectin and an alternative response to laminin. After the initial proliferative period, cell behavior on laminin is modified and cell migration ceases. In a crude manner, addition of the RGD peptide sequence may simulate the sequence of events that occurs in vivo, where cells initially migrate on fibronectin, and laminin later stabilizes cell interactions with the ECM.

Porcine preimplantation embryos produce MMP (Chamberlin and Menino, 1995) during the period of endodermal cell migration and it is known that TIMPs are secreted in concert with many proforms of MMP (Birkedahl-Hansen et al., 1992; Matrisian, 1992). MMP are involved in cellular migration and function to promote a limited and controlled amount of ECM degradation. Gelatinase activity was not detected in the present study until later in culture and may be due, in part, to the sensitivity of the zymograph techniques. TIMP are secreted in order to provide a balance for MMP degradation, and are also believed to participate in the regulated ECM degradation during cellular migration. Addition of rhTIMP-2 did not inhibit cellular migration as expected. Growth factor effects have also been

reported for TIMP (Satoh et al., 1994; Hayakawa et al., 1994) in bovine embryos as well as many other cell types. Satoh et al. (1994) reported that TIMP-1 produced by granulosa and oviductal cells enhanced in vitro development of bovine embryos and concluded that the stimulatory effect of bovine TIMP is directly attributed to enhanced cell proliferation and/or stabilization of the ECM via the inhibition of MMP activity. In this situation, MMP may be secreted in order to facilitate migration, and increased concentrations of TIMP produced later in development for stabilization of endodermal cells in their new environment. However, addition of rhTIMP-2 to the culture medium did not result in any significant changes in cell proliferation or migration. It appears that the principal mechanism involved in endodermal cell migration is integrin recognition and binding to the ECM.

The results of this study suggest that the ECM proteins, fibronectin and laminin, support migration of endodermal cells from isolated porcine ICM. An RGD recognizing integrin is not necessary for endodermal cell migration on fibronectin, and it is likely that porcine endodermal cells are expressing an $\alpha_4\beta_1$ integrin. Cell migration is impaired on laminin when ICM are cultured with RGD and may be due to either competition with an RGD site on laminin or to the perturbation of the in vivo sequence of ECM exposure. To elucidate the mechanisms supporting porcine endodermal cell migration and proliferation, subsequent studies should be directed at evaluating interactions between the ECM and their specific cellular receptors.

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CONCLUSION

This thesis investigated endodermal cell migration and the involvement of the ECM, integrins and extracellular proteinases in bovine and porcine embryos. Both fibronectin and laminin, but not collagen IV, supported proliferation of endodermal cells from the ICM. Fibronectin and laminin appear in early embryo development and have been implicated in key processes in cellular development and tissue organization (Wartiovaary et al., 1979). In swine and rat embryos (Carnegie, 1991) fibronectin is present on the blastocoelic surface of the trophectoderm prior to endoderm formation and is postulated to provide support for endodermal cell migration from the ICM (Richoux et al., 1989). Laminin is secreted by endodermal cells following formation of the endoderm in swine embryos (Richoux et al., 1989). The observation that bovine endodermal cells proliferate over fibronectin and to a lesser extent, laminin, implies that bovine endodermal cells possess the necessary integrins for interaction with these two ECM. Addition of rhTIMP-2 to the culture medium was stimulatory to ICM development and cellular outgrowth. Interestingly, TIMP have been reported to have growth factor effects separate from their roles as MMP inhibitors.

The present findings suggest that porcine endodermal cells can migrate on fibronectin without the use of an RGD-recognizing integrin. Cellular outgrowth in the presence of the RGD-peptide should be inhibited if endodermal cells utilize an integrin that recognizes the RGD-peptide. As no decrease in the incidence of cellular attachment and outgrowth is observed with the addition of RGD to the culture medium, it is possible that endodermal cells bind to fibronectin via another integrin such as the $\alpha_4\beta_1$ integrin which recognizes fibronectin at the EILDV site rather than the RGD site. On laminin alone, porcine ICM attach and migrate, but with the

addition of the RGD-peptide, at 24 h, the cultures become static, and migration rates decline. In light of previous studies, porcine endodermal cells may require exposure to the ECM in a specific sequence to replicate the in vivo process of migration and stabilization. A signal transduction pathway induced by RGD binding to the cell may initiate a brief period of cell migration on laminin by the use of an integrin that recognizes laminin. In a crude manner, this may simulate the series of events that takes place in vivo, as cells are introduced to fibronectin, migrate, and then introduced to laminin to stabilize their interactions with the basement membrane. Porcine ICM did not respond to rhTIMP-2 as did bovine ICM. Although the homology of porcine TIMP-2 with rhTIMP-2 is not known, it is possible that the molecules were sufficiently dissimilar as not to have an effect.

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