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Regulation of Matrix-Degrading Proteinases in Rat Endometrium

by

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ABSTRACT

In mammals, embryo implantation is a critical process for successful pregnancy, and is associated with the decidual cell reaction (DCR) in humans and rodents. This process requires degradation of extracellular matrix (ECM) in the endometrium by a group of ECM-degrading enzymes. The present study was designed to determine the expression and regulation of some of these enzymes in the rat uterus. The results show that urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor 1 (PAI-1), matrix-metalloproteinase 2 (MMP-2), membrane-type metalloproteinase 1 (MT-MMP-1), and tissue inhibitor of metalloproteinase 3 (TIMP-3) are expressed during pregnancy. The expression of these enzymes and inhibitors are controlled by different mechanisms. uPA expression is up-regulated by the endocrine environment whereas TIMP-3 is associated with the process of decidualization. MMP-2 expression is controlled at the post-translational level while TIMP-3 is probably controlled at the transcriptional level. The presence of the embryo influences the expression of TIMP-3 and MT-MMP-1, but have little effect on that of other proteinases. The findings suggest that these matrix-degrading enzymes may participate in different aspects of embryo implantation and decidualization.

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DEDICATION

To my parent

and

my family

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LIST OF ABBREVIATIONS

APS	ammonium persulfate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cm	centimetre
cpm	counts per minute
DCR	decidual cell reaction
dCTP	deoxyadenosine triphosphate
DEPC	diethylpyrocarbonate
dH_2O	distilled water
dNTP	deoxynucleoside triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECM	extracellular matrix
E. coli	Escherichia coli
EDTA	ethylenediamine-tetraacetate
et al.	and collaborators
hr	hour(s)
i.p.	peritoneal injection

IU	international unit
kb	kilobase pairs
kDa	kilodalton
LB	Luria broth
M	molar
mA	milliampere
mg	milligram
min	minute(s)
ml	millilitre
mM	millimolar
mm	millimetre
MMP	matrix metalloproteinase
MOPS	3-(N-morpholino)propanesulfonic acid
Mr	relative molecular mass
mRNA	messenger ribonucleic acid
MW	molecular weight
ng	nanogram
NP-40	nonidet P-40
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAI	plasminogen activator inhibitor

pBS	pBluescript
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PMSF	phenylmethylsufony fluoride
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
s.c.	subcutaneous injection
SDS	sodium dodecyl sulfate
SE	standard error
sec	second(s)
SSC	saline sodium citrate
TEMED	*N. N. N. N*-tetramethyl-ethylenediamine
TIMP	tissue inhibitor of metalloproteinases
tPA	tissue-type plasminogen activator
Tris	tris(hydroxymethyl)aminomethane
uPA	urokinase-type plasminogen activator
V	volt
vol	volume
vs.	versus

v/v	volume/volume
w/v	weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
µg	microgram
µl	microlitre

CHAPTER ONE. INTRODUCTION

In mammals embryo implantation is a critical process for establishing a successful pregnancy. Between 30 and 70% of pregnancies in the human are lost prior to or during implantation (Hafez, 1967; Cooke, 1988). Genetic disorders are responsible for approximately half of the lost pregnancies (Hassold, 1986). The causes for the other half of lost pregnancies are not well understood. Embryo implantation involves a sequence of events which involves maternal-embryonic interactions, and is governed by the endocrine media in the mother. Understanding of the regulatory mechanisms of this maternal-embryonic interaction will help elucidate the causes of genetically unrelated pregnancy losses, thereby permitting the development of diagnosis and treatment strategies for this reproductive disorder. Because many aspects of embryo implantation are similar between the human and rodents, mice and rats are commonly used as experimental models for studying the control of embryo implantation.

In rodents the endometrial stromal cells proliferate and differentiate into decidual cells during embryo implantation. This process is controlled by both the endocrine milieu during early pregnancy and signals from the implanting embryos (Psychoyos, 1973). As the endometrium undergoes decidualization, the implanting embryos invade the endometrium in order to establish an intimate contact with the maternal blood supply (Schlafke and Enders, 1975; Welsh and Enders, 1991). Although the implanting embryos produce matrix-degrading enzymes for invasion, some evidence indicates that

degradation and turnover of extracellular matrix (ECM) in the endometrium are, at least partially, independent of the implanting embryos (Brown and Papaioannou, 1992; Mulholland *et al.*, 1992; Abrahamsohn and Zorn, 1993). Various types of ECM-degrading enzymes are produced in the human endometrium under the control of steroid hormones (Osteen *et al.*, 1994; Marbaix *et al.*, 1995). These endometrial enzymes may be important in many aspects of embryo implantation, such as the modulation of trophoblast invasion, angiogenesis within the decidua, and migration of blood-borne cells into the decidua.

The studies presented in this thesis were designed to determine factors that may control the expression of matrix-degrading enzymes and their inhibitors in the rat endometrium.

CHAPTER TWO. LITERATURE REVIEW

2.1. Sequence of events during early pregnancy in rats.

The female laboratory rat reaches puberty at around 4-6 weeks of age, and regular estrous cycles commence thereafter. Each cycle lasts around 4-5 days and typically consists of four small phases: proestrus, estrus, metestrus and diestrus. Follicle-stimulating hormone and luteinizing hormone from the pituitary are the principal regulators of ovarian development, and bring ovarian follicles to their final maturation during the proestrous phase. A preovulatory surge of luteinizing hormone triggers ovulation, which usually takes place around midnight. This is followed by estrus when the female becomes receptive for mating with a male. If mating does not occur, the female will go through the phases of metoestrus and dioestrus, to complete one cycle, and another cycle begins. If fertile mating occurs following ovulation and the ovulated eggs are fertilized, embryo development will ensue and the regular cycle will give rise to gestation, which lasts about 20-22 days. The physical stimulation of the cervix by copulation triggers a neuro-chemical reflex that, via hormonal products of the hypothalamus and pituitary gland, converts the ovulated follicles into gestational corpora lutea. The gestational corpora lutea secrete progesterone for almost the entire period of gestation, which is essential for the establishment and maintenance of pregnancy (Finn and Martin, 1970; Glasser and Clark, 1975).

Fertilization takes place in the ampulla region of the oviduct. The embryo spends the first 3-4 days of development in the oviduct. It arrives in the uterus on Day 4-5 of pregnancy (Day 1 = the day of mating), when the embryo has reached the blastocyst stage. The blastocyst consists of two distinctive cell types: the inner cell mass (ICM) and trophoblast cells. The process of implantation is initiated soon after the embryo arrives in the uterus. Implantation begins with the apposition of the embryo to the luminal surface of the endometrium. This apposition is followed by self-degeneration (apoptosis) and sloughing of the luminal endometrial epithelium adjacent to the implanting embryo, exposing the underlying stromal cells to the embryo (Parr *et al.*, 1987). Coincidentally, the stromal cells near the embryo undergo a transformation process to give rise to the decidua (Figure 2-1). The transformation of endometrial stroma to decidua is also termed the decidual cell reaction (DCR). The trophoblast cells of the embryo "invade" the endometrium by breaching the basement membrane and other extracellular matrix components in the stroma/decidua. This trophoblast invasion leads to the establishment of an intimate contact between the embryonic tissues and the maternal blood supply, allowing the exchange of the nutrients and wastes between the developing embryo and the nurturing mother. The invasion process is most active between Days 7 and 10 of pregnancy. Once the intimate maternal-foetal relationship is established, the invasion of the maternal tissue gradually ceases as the trophoblast cells undergo terminal differentiation at the maternal-foetal interface (Enders, 1972; Welsh and Enders; 1987; Weitlauf, 1994).

2.2. The decidual cell reaction (DCR)

2.2.1. The process of DCR

The decidual cell reaction (DCR) involves rapid cell proliferation and differentiation in the endometrial stroma adjacent to the implanting embryo (Weitlauf, 1994). DCR is always preceded by the degeneration and "sloughing" of the overlying epithelial cells. DCR starts on Day 5 of pregnancy from the antimesometrial region to form the primary decidua. DCR then spreads towards the mesometrial triangle. As the decidualized cells become "colonized" in the mesometrial region to form the so called "metrial gland", the decidualized cells in the anti-mesometrial region undergoes apoptosis (Gu *et al.*, 1994). By Day 12 of pregnancy, the metrial gland is transformed into the maternal component of the placental disc. The placental disc is the exchange site for nutrients and metabolic wastes between the maternal and foetal blood circulation. It is discharged along with the new-borns at birth.

The DCR resembles an inflammatory reaction in many aspects (Finn, 1986). For example, inflammation can be induced by traumatization or exposure to a foreign body. In rats and mice, the induction of DCR requires an implantation stimulus. The natural stimulus is the implanting embryo. DCR can also be induced artificially by a physical stimulus, including traumatization of the uterine lumen or injecting oil into the uterine lumen (Psychoyos, 1973; Finn, 1977). The artificially induced decidual tissue is called

"deciduoma", to distinguish it from the embryo-induced "decidua". Inflammation always begins with increased blood flow to the affected area and a local increase in vascular permeability that results in edema. Increased vascular permeability is probably the first discernible sign of DCR in the rat and mouse (Psychoyos, 1973). Inflammation is always associated with infiltration of white blood cells, which also occurs during DCR (Finn, 1986). Both inflammation and DCR are tissue remodeling processes, involving changes in the tissue structure and degradation/modification of ECM.

2.2.2. The control of DCR

The induction of DCR, naturally or artificially, requires an appropriate endocrine environment. In rats and mice, three endocrine requirements have been identified for the optimal induction of DCR: (1) estrogen surge associated with ovulation (estrous estrogen); (2) progesterone secreted by the gestational corpora lutea; and (3) estrogen surge prior to the implantation stimulus (nidatory estrogen) (Shelesnyak *et al.*, 1963; Finn, 1977).

Although the requirement for the implantation stimulus and endocrine factors has been demonstrated unequivocally, the molecular mechanisms underlying the actions of these factors are poorly understood. Prostaglandins (PG), particularly the E series, are important mediators for the induction of DCR in the rat (Kennedy, 1986). DCR can be blocked by indomethacin (a PG synthesis inhibitor), even if all the endocrine factors are satisfied. Recent studies suggest that leukaemia inhibitory factor (LIF) is an important

mediator of the nidatory oestrogen. LIF is produced in the mouse endometrium under the influence of nidatory oestrogen (Bhatt *et al.*, 1991). The inactivation of the LIF gene through homologous recombination technology results in the failure of embryo implantation and DCR (Stewart *et al.*, 1992).

Since DCR is a tissue remodelling process that involves rapid turnover of ECM in the decidualizing endometrium (Mulholland *et al.*, 1992; Weitlauf, 1994), enzymes that degrade or modify ECM may play important roles in the induction of DCR.

2.2.3. The function of DCR

Although DCR is a critical process during embryo implantation, its function during pregnancy is not yet clearly understood. It is likely to be involved in many aspects related to the support of embryo development.

The decidual cells in the metrial gland are highly granulated and rich in glycogen. Decidual cells may act to nourish the developing embryo because the glycogen can serve as an important substrate for energy metabolism in the embryo (Finn, 1977). DCR is probably required for the maintenance of luteal function so that sufficient progesterone is available for the maintenance of pregnancy (Hoffman *et al.*, 1973; Sanchez-Criado and Rothchild, 1986). Corpora lutea undergo premature regression in pseudopregnant rats in the absence of DCR. Decidual cells have also been implicated in protecting the embryo from maternal immune attack. Under natural situations, the mammalian embryo is immunologically alien to the mother. However, the embryo is not rejected by the

maternal immune system even though the embryonic tissue is directly exposed to the maternal blood circulation such as in the rodents and human. Many explanations have been proposed, including the decidua as an immunological barrier. Mouse embryos can continue to develop when they are transplanted under the kidney capsule. However, after the recipient mice are immunized against the embryos, the embryos will die soon after they are transplanted under the kidney capsule but they can continue normal development if they are transplanted to a decidualizing uterus (Kirby *et al.*, 1966).

Mouse embryos transplanted under the kidney capsule develop in a un-controlled fashion, resulting in destruction and hemorrhage in the surrounding kidney tissues. (Porter, 1967; Kirby and Cowell, 1968). This has led to another function proposed for the decidua, namely, the decidua may act to protect the maternal tissue from the invasion of the trophoblast cells. The presence of specific proteinase inhibitors in the decidua adjacent to the trophoblast cells (Harvey *et al.*, 1995; Alexander *et al.*, 1996; Wang *et al.*, 1996) suggest that the decidua may help to confine the invasive activity of the trophoblast cells by suppressing the ECM-degrading proteinases from the trophoblast cells.

2.3. Control of extracellular matrix (ECM) turnover

2.3.1. Extracellular matrix (ECM)

ECM is a non-cellular meshwork consisting of a variety of macromolecules. Some of the ECM molecules are constructed exclusively with polysaccharide chains, such as

hyaluronic acid. Other ECM molecules consist of polysaccharide chains built on a protein framework, such as collagens, fibronectin and laminin, etc. (Trelstad and Silver, 1981).

The primary function of ECM is to provide a scaffold for the development of sophisticated multicellular organisms. Basement membrane is an important form of ECM and acts to segregate cells into various compartments according to their origin, morphology and function. Many cell types are anchored onto surrounding ECM via a group of specific membrane-bound receptors, also known as integrins. Recent findings have shown that integrins are also important signal transducers that mediate signals from ECM for such important cellular functions as cell proliferation and differentiation (Hynes, 1992; Mosher *et al.*, 1992).

2.3.2. ECM-degrading enzymes

ECM undergoes constant changes during development. In adult tissues, certain physiological and pathological processes also involve the modification/degradation of ECM. ECM modification/degradation has been associated with physiological processes such as ovulation, implantation and menstruation, and with pathological processes such tumor invasion, tissue inflammation and wound healing (Danø *et al.*, 1985; Matrisian, 1990).

The degradation of ECM requires the action of ECM-degrading enzymes. ECM-degrading enzymes can be mainly classified into two groups according their mode of action: (1) the serine proteases, which require a serine residue in their enzymatic site, and

(2) matrix- metalloproteinases (MMPs), which require a metal atom in their enzymatic site.

The plasminogen activators(PAs)-plasmin system is a well-studied group of serine proteases that have been implicated in the control of ECM turnover (Danø *et al.*, 1985). PAs covert the latent serine protease, plasminogen, into its active form, plasmin. Plasmin has a wide spectrum of substrate specificity. In addition to its ability to directly degrade ECM, it can also activate certain MMPs that can then act on ECM. Two types of PAs have been identified in many mammalian species: tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). The primary function of tPA is its involvement in vascular fibrinolysis as well as being involved in extravascular pericellular proteolytic processes, whereas uPA is localized to the migrating edge of the cells by binding to a cell surface receptor (Vassalli *et al.*, 1985) and more active in the control of ECM turnover (Danø *et al.*, 1985). Increased production of uPA has been associated with ovulation, embryo implantation, tissue inflammatory reaction, and cancer.

The activity of PAs is counter-balanced by a group of specific PA-inhibitors (PAIs), PA inhibitor-1 (PAI-1) and PA inhibitor-2 (PAI-2). The major PA inhibitor, PAI-1, is produced by endothelial cells, fibroblasts and other cell types, whilst PAI-2 is produced by placental cells and monocytes, with concentrations increasing markedly in serum from pregnant women (Kruithof, 1988).

Several MMPs have been identified that may play important roles in ECM turnover. These MMPs are mainly divided into three subclasses based on substrate

specificity: Collagenases, Gelatinases and Stromelysins. Two MMPs are identified for their ability to digest gelatin (hence the term gelatinases). MMP-2 (Gelatinase A) has a molecular weight of 72 kDa. Upon activation, it displays a molecular weight of about 62 kDa due to the cleavage of a N-terminal peptide. MMP-9 (Gelatinase B) has a molecular weight of 95 kDa (in human) or 105 kDa (in mouse). Type IV collagen, among others, is one of the important ECM substrates for both MMP-2 and MMP-9. Stomelysin and matrilysin are some of the more recently identified MMPs involved in ECM turnover (Table 2-1).

Most of the MMPs identified so far are produced in latent form and require activation. The activation of latent MMPs is a limited proteolysis process, mediated by other proteinases or by self-proteolysis. Few specific activators for MMP have been identified. A membrane-type MMP, MT-MMP-1, has been found to be an important activator of MMP-2 (Sato *et al.*, 1994). Additional MT-MMPs have been identified recently (MT-MMP-2, Will and Hinzmann, 1995; MT-MMP-3, Takino *et al.*, 1995; MT-MMP-4, Puente *et al.*, 1996). These MT-MMPs are anchored on the cell surface and produce focalized proteolysis by locally activating MMPs. Although it is recognized that MT-MMP-1 can activate MMP-2, it is less clear what is the substrate specificity for other MT-MMPs and what activates the MT-MMPs (Table 2-1).

Both proteolytic activation and glycosylation occurs with most of the MMPs so that several molecular mass forms of the proteins are frequently observed. MMP activities are regulated by at least three mechanisms: the level of mRNA (transcription and/or

mRNA stability); the post-translational processing (activation); and counter-balancing by their specific inhibitors. Tissue inhibitors of metalloproteinases (TIMPs) are a group of naturally occurring inhibitors of MMPs. Four TIMPs have been cloned and characterized so far from mammalian tissues (Table 2-2). Like MMPs, all the TIMPs are secreted proteins. They are products of separate genes disspite certain shared structural similarities. The N-terminal domain of all the four TIMPs are responsible for their inhibitory activity against MMPs (Apte *et al.*, 1995; Edwards *et al.*, 1996). TIMP-1 and TIMP-2 are freely diffusible proteins, whereas TIMP-3 is unique in that it has a high binding affinity to ECM and is associated with ECM as soon as it is secreted (Leco *et al.*, 1994). Each TIMP appears to have distinct patterns of tissue distribution. Their substrate specificity is not fully understood. However, limited information indicates that TIMP-1 and TIMP-2 have strong inhibitory activities against MMP-9 and MMP-2, but lower for collagenase-1 and stromelysin (Apte *et al.*, 1995).

Table 2-1. Members of matrix metalloproteinases (MMPs) family

Name of Enzymes	MMP No.	Mr (kDa)		Major Substrates	References
		Latent	Active		
A. Collagenases					
Interstitial Collagenase 1	MMP-1	52	42	collagen I, II, III*. gelatin	Goldberg *et al.* 1986
Neutrophil Collagenase 2	MMP-8	75	65	collagen I*, II, III	Macartney *et al.* 1983
Collagenase 3	MMP-13	53.7	nd	collagenase II	Freije *et al.* 1994
B. Gelatinases (Type IV Collagenases)					
72-kDa Metalloproteinase (Gelatinase A)	MMP-2	72	62	gelatin, collagen IV, fibronectin	Collier *et al.* 1988
92-kDa Metalloproteinase (Gelatinase B)	MMP-9	92 (human) 105 (mouse)	84	gelatin, collagen IV, elastin	Wilhelm *et al.* 1989
C. Stromelysins					
Stromelysin-1	MMP-3	56	45 & 28	proteoglycans, laminin, fibronectin collagen III, IV, V	Chin *et al.* 1985
Stromelysin-2	MMP-10	60	55	mainly as MMP-3	Nicholson *et al.* 1989
Matrilysin (PUMP[1])	MMP-7	28	19	proteoglycans*, fibronectin. elastin, laminin, gelatin,	Quantin *et al.* 1989
D. Membrane-type MMPs (MT-MMPs)					
MT-MMP-1	MMP-14	63	nd	pro-MMP-2	Sato *et al.* 1994
MT-MMP-2	MMP-15	72	nd	?	Will *et al.* 1995
MT-MMP-3	MMP-16	64	nd	?	Takino *et al.* 1995
MT-MMP-4	MMP-17	70	nd	?	Puente *et al.* 1996
E. Other MMPs					
Stromelysin-3	MMP-11	54.6	nd	nd	Basset *et al.* 1990
Metalloelastase	MMP-12	54	22	elastin, fibronectin	Shapiro *et al.* 1993

[1]: putative metalloproteinase; *: exceptional good substrates; nd: not determined; ?: unclear.

Table 2-2. Tissue inhibitor of metalloproteinases (TIMPs)

	TIMP-1*	**TIMP-2***	**TIMP-3***	**TIMP-4****
MMP inhibition	all	all	all?	?
Molecular weight (kDa)	28	21	24	22
Glycosylation	yes	no	yes	?
Solubility	soluble	soluble	matrix-bound	soluble?
Major expression sites	bone, ovary	placenta, lung	kidney, lung	heart
Mode of expression	inducible	constitutive	inducible	?
Pro-MMP complex	MMP-9, MMP-1	MMP-2	MMP-9, MT-MMP-1	MMP-2?
References	Carmichael *et al.* 1986	Goldberg *et al.* 1989	Leco *et al.* 1994	Greene *et al.* 1996

*: Edwards *et al.* 1995.

**: Leco *et al.* 1997.

?: unclear.

CHAPTER THREE. OBJECTIVES

From literature review and previous studies, it appears that an important feature of early pregnancy in rat is the extensive modification and degradation of extracellular matrix (ECM) in the endometrium, which is under the influence of steroid hormones and requires an appropriate balance of proteinases and proteinase inhibitors.

However, the expression mapping in the endometrium and mechanism of regulation for these proteinases and proteinase inhibitors in the period of early pregnancy were still unclear.

The aims of this study were:

1. To establish an expression profile for various ECM-degrading enzymes and their inhibitors in the rat endometrium before, during and after the induction of DCR.
2. To determine the effects of the endocrine environment, DCR and embryo/fetus on the expression of various ECM-degrading enzymes and their inhibitors.

CHAPTER FOUR. MATERIALS AND METHODS

Most of the chemicals and reagents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or BDH Inc. (Toronto, Ont) unless indicated otherwise.

4.1. Treatment of animals (Animal models)

Adult Sprague-Dawley (SD) rats, at 200-250g, were purchased from Charles River Canada (St. Constant, PQ) and housed in a standard laboratory animal facility with temperature- and light-controlled conditions (lights-on from 0700 to 1900 h) with free access to food and water. The maintenance and treatment of the animals was in full compliance with standard laboratory animal care protocols approved by the University of Calgary's Animal Care Committee.

4.1.1. Natural pregnancy

To produce natural pregnancies, female rats were paired with adult fertile males and checked every morning for the presence of sperm in vaginal smears as an indication of mating. The day on which sperm were first found in the vaginal smear was designated to Day 1 of pregnancy. This model was used to study possible effects of the presence of live embryos on the expression of proteinases and their inhibitors during early pregnancy.

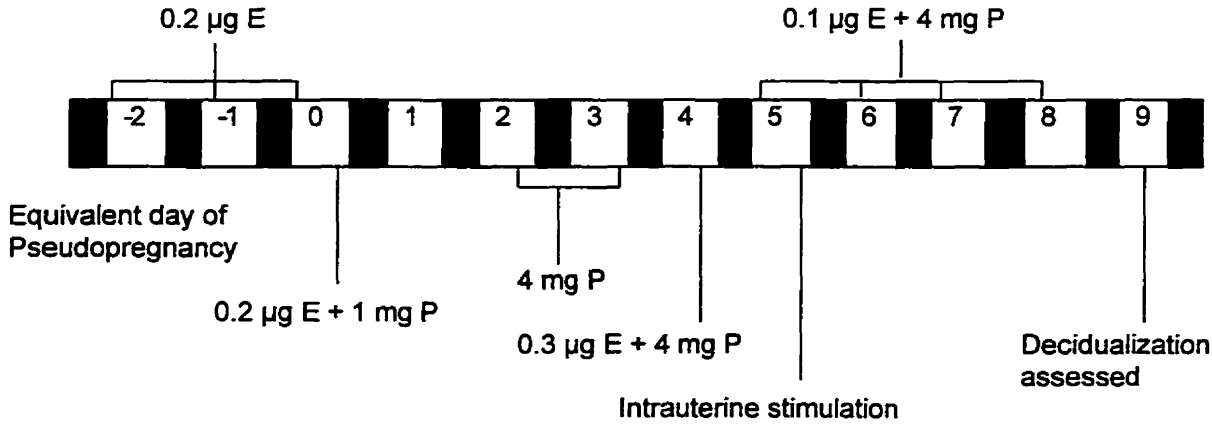
4.1.2. Pseudopregnancy

Two methods were used to establish pseudopregnancies in rats.

A. Ovariectomy and hormonal replacement

Female rats were ovariectomized under anesthesia with an i.p. injection of Averdin (2.5% tribromoethanol in PBS, w/v). The ovariectomy was performed via a dorsal-lateral incision. The ovariectomized rats were allowed at least one week for recovery before the start of hormone injections. A state equivalent to pseudopregnancy was induced by injecting these rats with a series of estradiol and progesterone at the appropriate doses and sequence in different experimental groups, according to Finn and Pope (1984) and Orlando-Mathur and Kennedy (1993) (Figure 4-1 and Table 4-1). The first day on which the progesterone was given was set as Day 0 of pseudopregnancy. All the steroid hormones were dissolved in sesame oil and given to the rats subcutaneously (s.c.) in a volume of 0.2 ml per rat.

Figure 4-1. Schematic representation of the treatment for the induction of decidual cell reaction. This figure shows the schematic representation of the treatment protocol administered to obtain rats sensitized for the decidual cell reaction. The open areas represent lights-on; dark areas lights-off. Steroids were dissolved in sesame oil and administered s.c. The intrauterine stimulation was an injection of 100 µl of sesame oil (modified from Orlando-Mathur and Kennedy, 1993).



E: Estradiol

P: Progesterone

Table 4-1. Experimental groups and treatment on pseudopregnant animals

	Experimental Groups							
Day of Exp.	Day 0	Day 4	Day 5	Day 6	Day 7	Day 7 (oil)	Day 9	Day 9 (oil)
Day -2	Soln. 1	Soln. 1	Soln. 1	Soln. 1	Soln. 1	Soln. 1	Soln. 1	Soln. 1
Day -1	Soln. 1	Soln. 1	Soln. 1	Soln. 1	Soln. 1	Soln. 1	Soln. 1	Soln. 1
Day 0	*Sacrifice*	Soln.1+2	Soln.1+2	Soln.1+2	Soln.1+2	Soln.1+2	Soln.1+2	Soln.1+2
Day 1		None	None	None	None	None	None	None
Day 2		Soln. 3	Soln. 3	Soln. 3	Soln. 3	Soln. 3	Soln. 3	Soln. 3
Day 3		Soln. 3	Soln. 3	Soln. 3	Soln. 3	Soln. 3	Soln. 3	Soln. 3
Day 4		*Sacrifice*	Soln. 4	Soln. 4	Soln. 4	Soln. 4	Soln. 4	Soln. 4
Day 5			*Sacrifice*	Soln. 5	Soln. 5	Soln.5+Oil	Soln. 5	Soln.5+Oil
Day 6				*Sacrifice*	Soln. 5	Soln. 5	Soln. 5	Soln. 5
Day 7					*Sacrifice*	*Sacrifice*	Soln. 5	Soln. 5
Day 8							Soln. 5	Soln. 5
Day 9							*Sacrifice*	*Sacrifice*

Notes:

1. Solutions:

   Soln. 1: Estradiol 1 µg/ml

   Soln. 2: Progesterone 5 mg/ml

   Soln. 3: Progesterone 20 mg/ml

   Soln. 4: Progesterone 20 mg/ml + Estradiol 1.5 µg/ml

   Soln. 5: Progesterone 20 mg/ml + Estradiol 0.5 µg/ml

2. All these steroid hormones were dissolved in sesame oil.
3. The dose of injection is 0.2 ml per 200 g weight. s.c.
4. Solution 1 and 5 are given at 10:00 am; Solution 2, 3 and 4 are given at 4:00 pm.
5. An intrauterine injection of 100 µl of sesame oil is given in the (oil) groups on Day 5.

B. Salpingectomy following fertile mating

Because the early embryos stay in the oviduct for the first 3 days after fertilization (Days 1 - 3 of pregnancy), salpingectomy before Day 3 will prevent the embryos from entering the uterus for implantation. Unilateral salpingectomy would result in a pseudopregnant state in the uterine horn on the same side as salpingectomy, leaving the contralateral uterine horn as a pregnant control.

Female rats were paired with fertile males and checked every morning for mating, as for producing natural pregnancies. In the afternoon on Day 1 of pregnancy, the rats were anesthetized with 2.5% Averdin. The oviduct was exposed via an abdominal incision and a small fragment (1-2 mm) of oviduct was excised near the uterine-oviductal junction. Care was taken not to traumatize the ovary or the remaining reproductive tract.

4.1.3. Induction of decidual cell reaction

Decidual cell reaction (DCR) was artificially induced in some of the pseudopregnant uteri by injecting sesame oil into the uterine lumen. On Day 5 of pseudopregnancy or pregnancy (after unilateral salpingectomy), at 12:00 PM, the rats were anesthetized with 2.5% Averdin and the uterine horn for the induction of DCR was exposed via a abdominal incision. One hundred μl of sesame oil was injected into the uterine lumen from the oviductal tip of the uterine horn, using a 30-gauge needle attached to a 1-ml syringe. DCR was considered to be successfully induced if the decidualized

uterine horn weighed at least three times amount more than that of the control, pseudopregnant uterine horn.

4.1.4. Tissue collection

The rats were killed under euthanasia by an overdose s.c. injection of pentobarbital (Euthanyl™, MTC Pharmaceuticals, Cambridge, Ont) for collecting uterine tissues. Each uterine horn was dissected separately and trimmed free of fat. It was rinsed in cold PBS to remove blood and cut open longitudinally on a glass microscopic slide placed on mashed ice. The endometrial or deciduomal tissues were "scraped" off the myometrium with a spatula as described by Martel and Psychoyos (1978). Decidual tissues on Days 7 and 9 of pregnancy were collected similarly after the embryonic tissues were removed under a stereo microscope. By Day 15 of pregnancy, the decidua has developed into the maternal placenta , which was collected by peeling away from the fetal component with fine forceps.

4.2. Northern blot analysis

The steady-state levels of mRNA for proteinases and proteinase inhibitors in various uterine tissue were determined by Northern blot analysis.

4.2.1. Total RNA isolation

The total RNA from uterine tissues were extracted by a single-step guanidine thiocyanate procedure according to the method of Chomcynski and Sacchi (1987) with modifications described by Arcellana-Panlilio and Schultz (1993).

After removal from the animal, the uterine tissues were minced on ice and immediately homogenized in cold GIT solution (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.5, 0.5% sarkosyl, w/v, and 0.1 M β-mercaptoethanol), using about 1 ml GIT per 100 mg of tissue. While the glass conical tube containing GIT solution was held on ice , the tissues were homogenized for about 20 strokes with a pestle matching the size of the tube (Kontes Scientific Glassware, Vineland, NJ). The following component were then added to the homogenate: 1/10 volume of 2 M sodium acetate, pH 4.0, an equal volume of water-saturated phenol, and equal volume of Sevag's solution (chloroform / isoamyl alcohol, 24 : 1, v/v). The preparation was mixed after each addition, and vortexed vigorously for two bursts of 10 sec each after addition of the Sevag's solution. The mixture was incubated on ice for 15 min followed by centrifugation at 14,000 × g for 15 min at 4 °C. The upper aqueous phase was re-extracted with an equal volume of Sevag's solution. After centrifugation, the upper phase which containing the RNA was collected and transferred to a fresh 1.5 ml tube. An equal volume of isopropanol was added to precipitate RNA at -20 °C for at least 1 hr. After centrifugation at 14,000 × g for 20 min at 4 °C, the pellet of RNA precipitate was resuspended in 1/2 volume GIT solution and 1 volume of isopropanol. RNA was re-precipitated at -20 °C for at least 1 hr and recovered by centrifugation in a micro-centrifuge for 10 min at 4 °C. The RNA pellet was washed in

70% cold ethanol, dried in air and dissolved in DEPC-treated water, and stored at -70 °C until just before use.

A small aliquot of RNA solution was diluted to estimate the yield and the purity by measuring absorbency at 260 and 280 nm, using a spectrophotometer (Hitachi U-200, Japan). Typically, 50 to 100 μg of total RNA was obtained per 200 mg of tissue.

4.2.2. Preparation of cDNA probes

The following cDNA probes have been made and used for Northern blot Hybridization:

Rat: MMP-2 (Gelatinase A), MMP-9 (Gelatinase B), uPA, tPA, PAI-1

Mouse: TIMP-1, TIMP-2, TIMP-3, MT-MMP-1, 18s rRNA

The rat probes for MMP-2 and MMP-9. uPA and tPA were prepared by reverse transcript and polymerase chain reaction (RT-PCR), with total RNA from rat uterus and embryos as templates. The PCR primers for rat MMP-2 and MMP-9 were designated from mouse cDNA sequences referring to the relative references. The primers for rat uPA and tPA were obtained in our laboratory (Zhang *et al.*, 1994) (Table 4-2). The RT-PCR products were cloned into plasmid vectors for sequencing to verify their identity. The rat PAI-1 cDNA plasmid was constructed by Zeheb and Gelehrter (1988) and kindly provided by Dr. Gelehrter of University Michigan, Ann Arbor, MI. The mouse TIMP-1, -2, -3 and MT-MMP-1 cDNA plasmids were kindly provided by Dr. Edwards of University of Calgary. The mouse 18s rRNA plasmid used to standardize the amount of

total RNA samples loading was a gift from Dr. Hammond of University of Western Ontario, London, Ontario.

Table 4-2. Primers of RT-PCR for rat MMP-2, MMP-9, uPA and tPA

Gene	Sequences of PCR primers (from mouse)	Source of total RNA	Fragment size
MMP-2* (Gelatinase A)	5'Primer = 5'-GCTGATGGCGAGTACTGCAA-3' 3'Primer = 5'-TAGTCCTCGGTGGTGCCACA-3'	Rat uterus	286 bp
MMP-9** (Gelatinase B)	5'Primer = 5'-TTGAGTCCGGCAGACAATCC-3' 3'Primer = 5'-CCTTATCCACGCGAATGACG-3'	Rat uterus	433 bp
uPA***	5'Primer = 5'-GTGGAGAACCAGCCCTGGT-3' 3'Primer = 3'-TATGTCTGGTAGACGGACGG-5'	Rat embryos	348 bp
tPA***	5'Primer = 5'-TCCACCTGCGGCCTGAGGCAAT-3' 3'Primer = 3'-GAGGGACTGACCTGTCTCACAC-5'	Rat embryos	445 bp

References:

* Reponen *et al.* 1992.

** Tanaka *et al.* 1993.

*** Zhang *et al.* 1994.

A. RT-PCR

To clone the rat cDNA fragments of MMP-2, MMP-9, uPA and tPA, total RNA from rat uterus tissues or embryos was prepared, as described in section 4.2.1, and reverse transcribed to obtain single strand cDNA. Specifically, total RNA (1 μg) was reverse transcribed at 42 °C for 2 hr in a final volume of 20 μl containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM mixed dNTPs, 0.5 μg $Oligo(dT)_{12-18}$ primer (Pharmacia Biotech Inc., Baie d'Urfé, QC)), and 200 U (1μl) reverse transcriptase (Superscript™ II, GIBCO-BRL Life Technologies, Gaithersburg, MD). The reaction was heated at 95 °C for 5 min and then quenched in ice. One tenth of RT product was used in a polymerase chain reaction (PCR) with relevant primers corresponding to the sequences of Gelatinase A, Gelatinase B, uPA and tPA (Table 4-2). The PCR reaction was proceeded for 35 cycles in a DNA thermal cycle (Perkin Elmer-Cetus, Norwalk, CT) in 100 μl reaction vol containing 1 μM of each primer, 2.5 mM $MgCl_2$, and 0.25 mM dNTPs. Each amplification cycle consisted of denaturing at 94 °C for 1 min, annealing primer to target sequences at 55 °C for 1 min and primer extension at 72 °C for 1 min. The PCR reaction was ended with a final 7 min extension step at 72 °C. An aliquot (15-20 μl) of the PCR products was analyzed by electrophoresis on 1.2 % agarose gels (w/v) along with 100 bp DNA ladder (GIBCO-BRL) and stained with ethidium bromide to visualize PCR products on a UV transluminator (BIO/CAN Scientific, Mississauga, Ont). The PCR product with the anticipated size were excised from gel and subcloned into the plasmid vectors for further application.

B. Subcloning of PCR fragment into plasmid vector

To confirm the identity of the PCR fragment, PCR products were cloned into PCR™ II plasmid vectors with a T-A PCR cloning kit (Invitrogen Co., San Diego, CA) following manufacturer's procedure. Briefly, the appropriate DNA fragment isolated from the gel was ligated with PCR™ II vector in the presence of T4 DNA ligase at 14 °C for 16 hr. The ligation product then was used to transfect into competent *E. coli* cells. The transfected cells were plated on a LB plate with 50 μg/ml ampicillin and X-Gal. The plate was incubated at 37 °C for 18 hr. The white colonies of transformants were picked up and amplified. The nucleotide sequences of the cloned PCR fragment were determined by the DNA Sequencing Laboratory of the University of Calgary, using the dideoxynucleoside terminator method and aided by an automated DNA sequencing system (Applied Biosystems, Foster City, CA).

C. Probe DNA isolation

For use as cDNA probes, the DNA fragments in the plasmid vectors corresponding to the target cDNA sequences were retrieved from plasmid amplification and cut off from vectors by appropriate restriction enzymes (Table 4-3). The fragments were gel isolated and recovered from agarose gel using QIAquick gel extraction kit (Qiagen Inc., Santa Clarita, CA) according to the supplier's instruction.

Table 4-3. cDNA probes used for Northern blot hybridization

Gene	Subcloning into vector*	Restrict digestion Enzyme(s)**	Size of probe	References
Rat				
MMP-2	PCR II	*Eco*R I	300 bp	Reponen *et al.* 1992
MMP-9	PCR II	*Eco*R I	450 bp	Tanaka *et al.* 1993
uPA	pBS M13(SK-)	*Eco*R I / *Pst* I	350 bp	Zhang *et al.* 1994
tPA	pBS M13(SK-)	*Eco*R I / *Hind* III	500 bp	Zhang *et al.* 1994
PAI-1	pBS M13(SK-)	*Hind* III / *Kpn* I	1.7 kb	Zeheb *et al.* 1988
Mouse				
TIMP-1	pBS (KS-)	*Eco*R I / *Xho* I	500 bp	Edwards *et al.* 1986
TIMP-2	pBS (KS-)	*Eco*R I / *Xho* I	700 bp	Leco *et al.* 1992
TIMP-3	pBS (KS-)	*Pst* I	750 bp	Leco *et al.* 1994
MT-MMP-1	PT7Blue	*Eco*R I	550 bp	Sato *et al.* 1994
18s rRNA	PGEM-1	*Pst* I	209 bp	Pikó *et al.* 1982

* PCR II, pBS M13 (SK-) and pBS (KS-) were from Stratagene (La Jolla, CA); PT7Blue was from Novagen Inc.(Madison, WI); PGEM was from Promega Co.(Madison, WI).

** All the restrict enzymes were from Pharmacia Biotech.

4.2.3. Northern blot hybridization

RNA samples were size fractionated by 1 % agarose / formaldehyde gel electrophoresis, transferred to nylon membranes, and the filter hybridized with rat / mouse α-^{32}P-labeled cDNA probes.

A. Formaldehyde RNA gel and membrane transfer

In preparation for running a gel, aliquots containing 10 µg total RNA were mixed with 2 µl of 10 × MOPS buffer (0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.2), 3.5 µl of formaldehyde and 10 µl of formamide. The samples were denatured at 65 °C for 15 min then quenched on ice. The loading buffer (50 µl 10 × MOPS, 80 µl of 37% formaldehyde, 250 µl formamide, 25% glycerol and 0.25% bromophenol blue, w/v, in a total 500 µl) was freshly prepared. The formamide was deionized by stirring 10 ml with 1 g AG-501-X8 resins (Bio-Rad Laboratories, Hercules, CA) for 1 hr at room temperature. The deionized formamide was filtered and stored at -20 °C in single-use aliquots.

The denatured RNA samples were combined with 5 µl of loading buffer and loaded on the prepared 1.0% agarose gel (w/v) containing 6% formaldehyde (v/v). Electrophoresis was carried out in the presence of 1 × MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.2). After electrophoresis, the RNA was blotted by capillary transfer onto positively charged nylon membranes (Hybond N^+; Amersham Canada, Oakville, Ont) and cross-linked by exposing the membranes to a UV light

(Stratalinker TM 1800; Stratagene, La Jolla, CA) for 1 min at an intensity of 12,000 joules/cm^2.

B. cDNA probes labeling

The rat / mouse cDNA probes were labeled with α-^{32}P-dCTP (Amersham Canada) by the random priming technique using an oligo-labeling kit (Ready-To-Go™ DNA labeling kit, Pharmacia Biotech) to a specific activity of approximately 2-5 $\times$ 10^8 cpm/μg following the manufacturer's protocol, and purified on G-50 micro-column (Pharmacia Biotech).

C. Hybridization

The membranes were pre-hybridized for 15 min and hybridization was carried out with α-^{32}P-labeled cDNA probes at 65 °C for 1 hr in Hyb-oven (HYBAID™, Inter Sciences Inc., Markham, Ont) using Rapid-Hyb™ buffer (Amersham Canada).

After hybridization, the membranes were washed (30 min each time) in double-strength SSC (saline-sodium citrate buffer, including 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0)-0.1% SDS (w/v) at room temperature, and twice (30 min each time) at 65 °C in 0.2-strength SSC-0.1% SDS. The membranes were then subjected to autoradiography by exposing the membranes to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) at -70 °C with intensifying screens.

After hybridization with each target cDNA probe, the blots were stripped by immersion in boiling 0.5% SDS and allowed to cool to room temperature. The membranes were then hybridized with mouse 18s rRNA probe to measure the equivalence of loading of RNA samples.

4.3. Zymography and reverse zymography

4.3.1. Protein preparation

Uterine tissues were obtained as for RNA extraction. The tissues were placed in cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% NP-40, 0.5% sodium deoxycholate, 1 μg/ml aprotinin, 0.01 mM E-64, 0.1 mM leupeptin, 5 μM pepstatin A, 100 μg/ml PMSF, 0.1% SDS, w/v) and homogenized immediately. The homogenate was transferred into 1.5 ml microfuge tubes and centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant was collected and the pellet was extracted with the lysis buffer once more. The supernatants from the two centrifugations were pooled. After taking 2 × 50 μl for determining protein concentration, the protein sample was stored at -70 °C until further analysis.

4.3.2. Determination of protein concentration

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard protein. Protein samples (5 μl) were placed

into polystyrene cuvets and brought to a final volume of 800 µl with dH_2O. Duplicates of BSA standards containing 0, 2.5, 5, 10, 15, 20 and 30 µg of protein, respectively, in 800 µl dH_2O were also prepared. To all standards and samples, 200 µl of protein assay dye reagent (Bio-Rad) was added and mixed well. After incubation in the dark for 30 min, spectrophotometric measurements were taken at 595 nm. Using the standards, a standard curve (OD595 vs. µg/ml) was constructed. From the curve and the sample reading, the protein concentration of each sample was determined.

4.3.3. Zymography

To detect the enzymatic activities of PAs and MMPs in zymography, SDS-PAGE gels were prepared with the in-corporation of appropriate substrate (casein for uPA and tPA, and gelatin for gelatinases) into the acrylamide matrix of the gel. All the zymography analyses were repeated at least three times.

A. Casein zymography (for PAs)

The PAs (uPA and tPA) proteolytic activities in the protein samples were visualized by casein zymography as described before (Zhang *et al.*, 1996). Aliquots of samples containing the same amount of total protein were subjected to non-reducing polyacrylamide gel (PAGE) electrophoresis. The separating gel contained 10% acrylamide, 0.05% *N,N'*methylene-bis-acrylamide (w/v), 2 mg/ml casein, 1 µg/ml plasminogen (Sigma Chemical Co. St. Louis MO), 2.5% of 1.5 M Tris buffer (v/v), pH

8.8, 0.4% of SDS, 0.3% ammonium peroxodisulfate (w/v), and 0.1% TEMED (v/v). A stacking gel was layered on the top of the separating gel and had the same composition as separating gel except that 2% acrylamide and Tris buffer (pH 6.8) were used. The total dimensions of the gel were 80 × 85 × 0.8 mm.

Protein samples were not denatured with heat or reducing agents. The sample was mixed with an equal volume of sample buffer (17.4% SDS, 7% sucrose, w/v, and 10 μg/ml bromophenol in water) before loaded on the gel. Protein standards for molecular weight (14 - 200 kDa molecular weight range, GIBCO-BRL) were co-electrophoresed with the tissue samples to estimate the molecular weight of the PAs / MMPs activity.

Electrophoresis ran for approximately 1 hr at 30 mA (150 V) at room temperature in electrophoresis buffer, containing 25 mM Tris-HCl, 0.2 M glycine and 0.1% SDS, pH 8.6. After electrophoresis, the gels were soaked 2 hr in 2.5 % Triton X-100 (v/v) on a moving platform and incubated to allow proteolysis for a further 16-24 hr at 37 °C in PBS. The gels were then fixed in a mixture of methanol : acetic acid : water (45 : 10 : 45) and stained with 0.5% Coomassie brilliant blue dye (0.5% in 45% methanol with 10% acetic acid in water, v/v) for 2 hr, and de-stained in 45% methanol with 10% acetic acid in water.

The uPA activity was detected as a cleared, single band at a position corresponding to a molecular weight of approximately 40 kDa, and tPA of 70-72 kDa (Zhang *et al.*, 1996).

B. Gelatin zymography (for MMP-2 and MMP-9)

Gelatin zymography for MMP-2 and MMP-9 was performed according to Fisher *et al* (1989) and Behrendtsen *et al* (1992), and was similar to the PA zymography. The protein samples were subjected to PAGE in 10% acrylamide gel containing 1 mg/ml gelatin. After electrophoresis, the gel was incubated in 2.5% Triton X-100 for 2 hr at room temperature on a moving platform, and then incubated in Tris-HCl buffer (pH 7.5), containing $CaCl_2$ (1 mM) and $MgCl_2$ (0.5 mM) at 37 °C, to allow gelatinolysis for 16-48 hr. The gels then were stained with Coomassie brilliant blue for 2 hr, and de-stained in 45% methanol with 10% acetic acid in water.

The proteolytic activity of gelatinase was revealed by a cleared zone (bands) on a blue gelatin background in the gel, and its molecular size was estimated by pre-stained protein MW standards(14 - 200 kDa range; GIBCO-BRL) that were co-electrophoresed with the samples.

4.3.4. Reverse zymography

The reverse zymography method described by Erickson *et al* (1984) and Staskus *et al* (1991) was used to measure the activities of PAIs and TIMPs. The conditions of sample preparation, electrophoresis, incubation and staining were similar to zymography except the addition of uPA (for PAI) or MMP (for TIMP). Each reverse zymography was repeated at least three times.

A. PAI reverse zymography

For reverse zymography to detect the PAI, SDS-12% PAGE gels (w/v) were prepared with the incorporation of uPA (0.012 IU/ml), casein (2 mg/ml) and plasminogen (1 μg/ml) into the gel. The conditions for electrophoresis and development of enzymatic activities were the same as for casein zymography. Inhibitor activity in the electrophoretic gel appeared after 18-36 hr of incubation, as lysis-resistant areas (blue band) in the cleared background of the gel, and the molecular weight was estimated by comparing with protein standards (GIBCO-BRL) that were co-electrophoresized.

B. TIMPs reverse zymography

The activity of TIMPs was analyzed by PAGE in 12% acrylamide gel containing 1 mg/ml gelatin. The gel also contained MMP-2, which is a part of a kit from Dr. Edwards of University of Calgary (Leco *et al.*, 1994). Other components in the kit that were used in this study included standards for TIMP-1, -2, and -3. These standards were subjected to PAGE in concurrence with protein samples from uterine tissues. Inhibitor activities were revealed as dark bands against the partially cleared background.

4.4. Data analysis and statistics

4.4.1. Northern Blots

All the Northern blot analyses were repeated three times on RNA samples collected on different occasions. The mRNA signals on autoradiograms were partially quantified by scanning densitometry using the MasterScan Interpretive Densitometer (CSPI Inc., Billerica, MA) and the RFLP Scanalytics software.

The value of relative density for each target mRNA was expressed and normalized as a ratio of signals to 18s rRNA in the same sample, and the ratios were pooled between the three Northern blot analyses to calculate the mean and SE, with the ratio adjusted to "1" for Day 0 or Day 5. The level of significance of differences between the means of the groups was determined by Excel 5.0 software and the Dunce's Multiple Range test, with $P < 0.05$ considered significant.

4.4.2. Zymography and reverse zymography

The scanning and densitometry for zymography and reverse zymography were similar to that for Northern blot analysis. But the relative density of band for zymography was expressed and quantified as negative values.

CHAPTER FIVE. RESULTS

As described in Section 4-1, two methods were used to induce pseudopregnancy in the rat uterus: (1) hormone injections following ovariectomy, and (2) unilateral salpingectomy following natural mating in pregnant rats. The success rate for the induction of DCR and the weight of the deciduoma (the difference between decidualized uterine horn and non-decidualized horn) were the same between the two types of pseudopregnancies. Initially, uterine tissues from these two types of pseudopregnancies were analyzed separately for the levels of specific mRNAs and enzymatic activities. The results indicated that there was no difference between the two types of pseudopregnancy inductions on the corresponding days of pseudopregnancy, in either non-decidualized or decidualized endometrial tissues. Therefore, all the results for endometrial and deciduomal tissues are pooled between the two types of pseudopregnancies.

5.1. Plasminogen activators (PAs) and plasminogen activator inhibitors (PAIs)

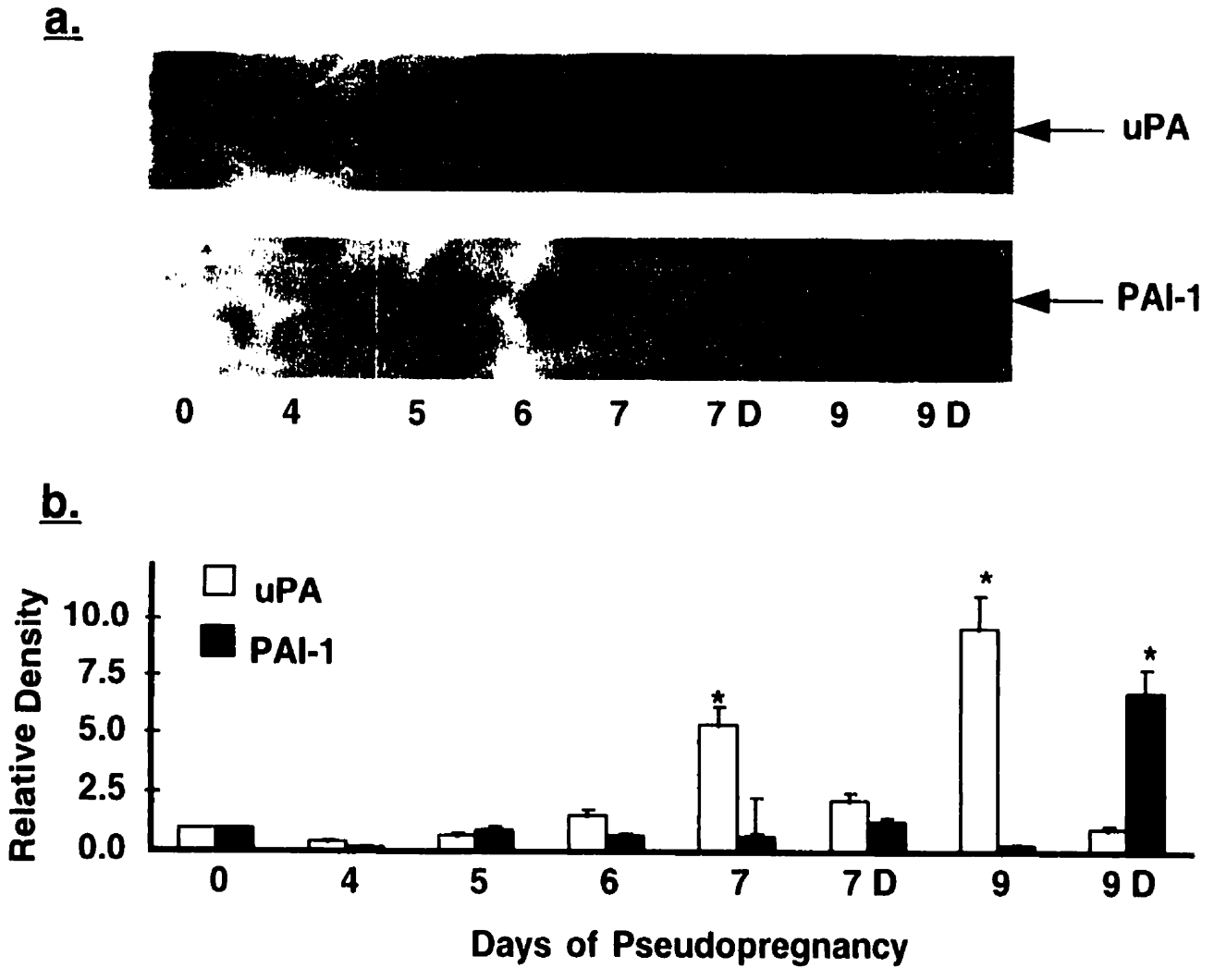
5.1.1. Northern blot analysis

The level of uPA mRNA was low during the first five days of pseudopregnancy, and increased steadily from Day 6 onwards in the non-decidualized endometrium. There was a small increase in the deciduomal tissue, compared with endometrium during the first 5 days of pseudopregnancy, but the level was much lower than in the non-

decidualized endometrium on the same days of pseudopregnancy. The mRNA encoding PAI-1 remained low during most of the pseudopregnant period until Day 9 when its abundance increased significantly (Figure 5-1). The level of tPA mRNA was low in all the uterine tissues examined during the first 9 days of pseudopregnancy, and the results were not presented. The mRNA for PAI-2 was not detected in any of the uterine tissues.

Earlier studies (Wang *et al,* 1996) examined the steady-state levels of mRNA for uPA and PAI-1 in decidua tissues from uteri containing embryos. The pattern and amplitude of changes for uPA mRNA were similar to those in the deciduoma on the corresponding days of pregnancy or pseudopregnancy. However, the PAI-1 mRNA was more abundant in the decidua than in the deciduoma on Day 9 of pregnancy or pseudopregnancy.

Figure 5-1. Northern blot analysis of uPA and PAI-1 mRNA. Endometrial or deciduomal (D) tissues were collected on different days (as indicated) of pseudopregnancy for isolation of total RNA. Total RNA was subjected to Northern blot analysis using specific cDNA probes. Typical autoradiograms are presented (a). The analysis was repeated at least three times and the densitometry data were pooled. The relative density (mean ± SE) was calculated as the ratio of the specific signal to that of the 18s rRNA with the data for Day 0 set for "1" (b). *: denotes significantly different from other tissues.

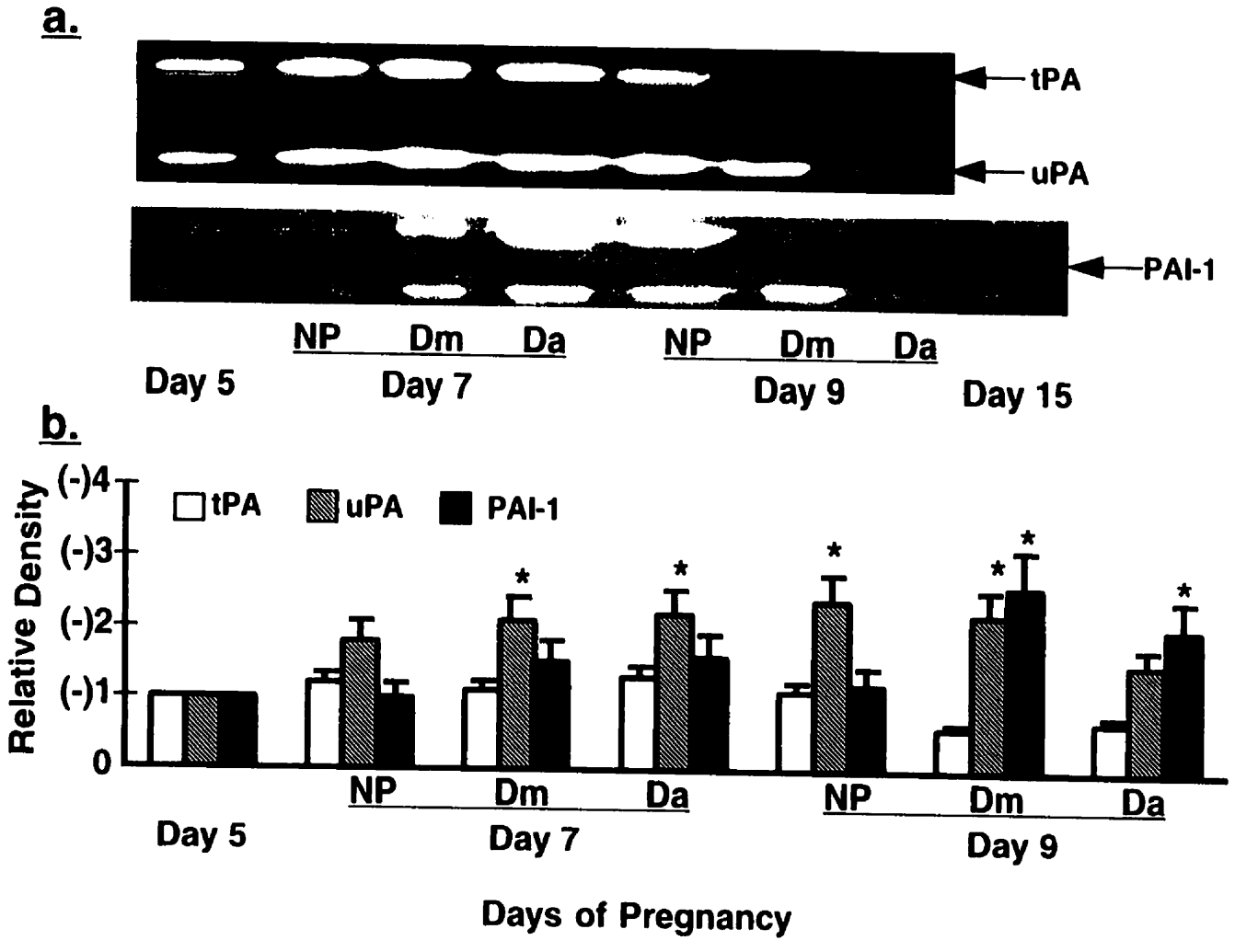

a.
uPA
PAI-1
0
4
5
6
7
7 D
9
9 D
b.
uPA
PAI-1
Relative Density
10.0
7.5
5.0
2.5
0.0
*
*
*
0
4
5
6
7
7 D
9
9 D
Days of Pseudopregnancy

5.1.2. Zymography and reverse zymography

The enzymatic activities of PAs were analyzed by zymography. Two PAs activities were detected (Figure 5-2a). The PA activity represented by the upper band was probably tPA since its MW was approximately 70-72 kDa, similar to that of tPA (Danø *et al.*, 1985). The PA in the lower band was likely uPA, based on its MW (approximately 40-45 kDa, similar to uPA). The level of tPA activity was relatively unchanged in most of the uterine tissues studied, except in the decidualized tissues from Day 9 of pregnancy or pseudopregnancy (Figure 5-2a and 5-2b). The activity of uPA appeared to be elevated significantly in most of the uterine tissues on Days 7 and 9 of pregnancy or pseudopregnancy.

Reverse zymography detected a single PAI activity in some of the uterine tissues (Figure 5-2). This PAI was probably PAI-1 because its MW of approximately 48 kDa was similar to the 48 kDa MW reported for PAI-1 (Tranque *et al.*, 1992). This putative PAI-1 activity was always higher in the decidual or deciduomal tissues than in the non-decidualized endometrial tissues.

Figure 5-2. Zymography/reverse zymography for PAs and PAI-1. Endometrial (NP), deciduomal (Dm) or decidual (Da) tissues were collected on different days (as indicated) of pregnancy to obtain tissue protein. The protein samples were subjected for zymography (for PAs activity) or reverse zymography (for PAI-1 activity). Typical zymograms are presented (a). The analysis was repeated at least three times and the densitometry data were pooled. The relative density (mean ± SE) was calculated as the ratio of the specific signal to the data for Day 5 set for "-1" for PAs activity, or "1" for PAI-1 activity (b). *: denotes significantly different from other tissues.


a.
tPA
uPA
PAI-1
NP
Dm
Da
NP
Dm
Da
Day 5
Day 7
Day 9
Day 15
b.
Relative Density
(-)4
(-)3
(-)2
(-)1
0
tPA
uPA
PAI-1
*
NP
Dm
Da
NP
Dm
Da
Day 5
Day 7
Day 9
Days of Pregnancy

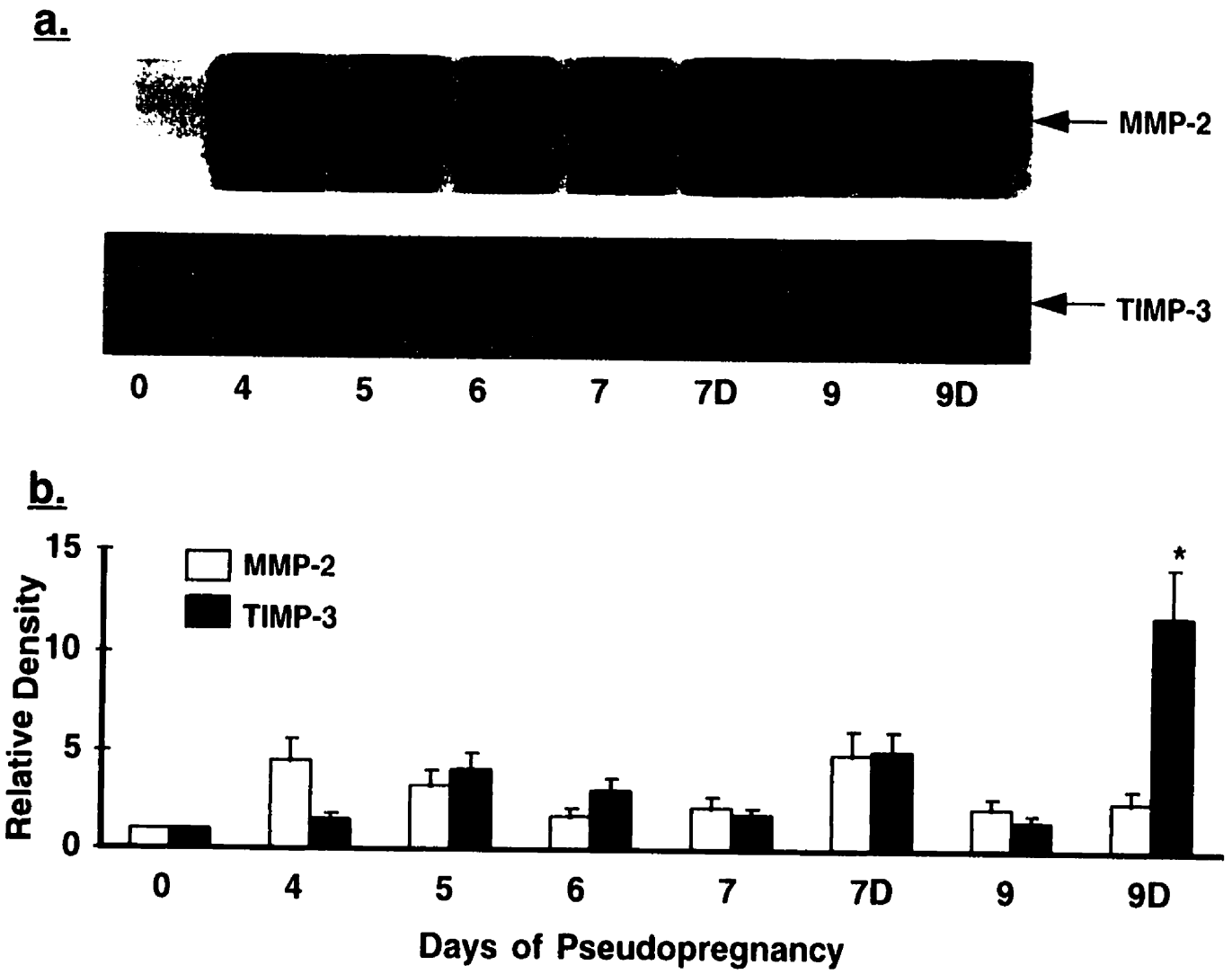
5.2. MMPs and TIMPs

5.2.1. Northern blot analysis

MMP-2 mRNA was low on Day 0 and high on Day 4 of pseudopregnancy (Figure 5-3). Thereafter, MMP-2 mRNA declined slightly, except on Day 7 in the deciduoma that contained greater amount of MMP-2 mRNA than in the other tissues from other stages of pseudopregnancy. However, the difference did not reach a statistical significance between any of the uterine tissues studied. The level of mRNA for MMP-9 was barely detectable in any of the uterine tissues and the results were not presented.

The mRNA levels for TIMP-1 and TIMP-2 were low in all the endometrial /deciduomal tissues throughout of the pseudopregnant period examined in this study. The results are not presented. For TIMP-3 mRNA, there was a small increase on Days 5 and 6 (Figure 5-3). Its abundance was always greater in the deciduoma than in the non-decidualized endometrium and was the highest in the deciduoma on Day 9 of pseudopregnancy.

Figure 5-3. Northern blot analysis for MMP-2 and TIMP-3 mRNA. Endometrial or deciduomal (D) tissues were collected on different days (as indicated) of pseudopregnancy for isolation of total RNA. Total RNA was subjected to Northern blot analysis using specific cDNA probes. Typical autoradiograms are presented (a). The analysis was repeated at least three times and the densitometry data were pooled. The relative density (mean ± SE) was calculated as the ratio of the specific signal to that of the 18s rRNA with the data for Day 0 set for "1" (b). *: denotes significantly different from other tissues.


a.
MMP-2
TIMP-3
0
4
5
6
7
7D
9
9D
b.
MMP-2
TIMP-3
Relative Density
15
10
5
0
*
0
4
5
6
7
7D
9
9D
Days of Pseudopregnancy

Previous studies have shown that the presence of embryo has no effect on uPA mRNA but increased the level of PAI-1 mRNA in the decidualized endometrium (Wang *et al.*, 1996). Here, the levels of mRNA for MMP-2 and TIMP-3 between the oil-induced deciduoma and embryo-induced decidua, were compared in order to determine possible effect of the presence of embryos/fetus in the uterus (Figure 5-4). There was no difference in the level of MMP-2 mRNA between deciduomal and decidual tissues, but TIMP-3 mRNA was always higher in the deciduoma than in the decidua.

MT-MMP-1 was the first membrane-bound MMP identified as a specific activator of MMP-2. Its mRNA level was analyzed in some the uterine tissues from pregnant or pseudopregnant rats (Figure 5-5). The level was relatively low in most tissues studied, except a significant increase in the deciduomal tissues from Day 7 of pseudopregnancy.

Figure 5-4. Comparison of mRNA levels between decidua and deciduoma. Endometrial, deciduomal (Dm) or decidual (Da) tissues were collected on different days (as indicated) pregnancy for isolation of total RNA and Northern blot analysis. The level of mRNA, expressed as "Relative Density (mean ± SE)" with Day 5 set for "1", was compared between different tissues. *: denotes significantly different from other tissues ($p < 0.05$).

Comparison of mRNA levels between dicidua (Da) and deciduoma (Dm) for MMP-2 and TIMP-3

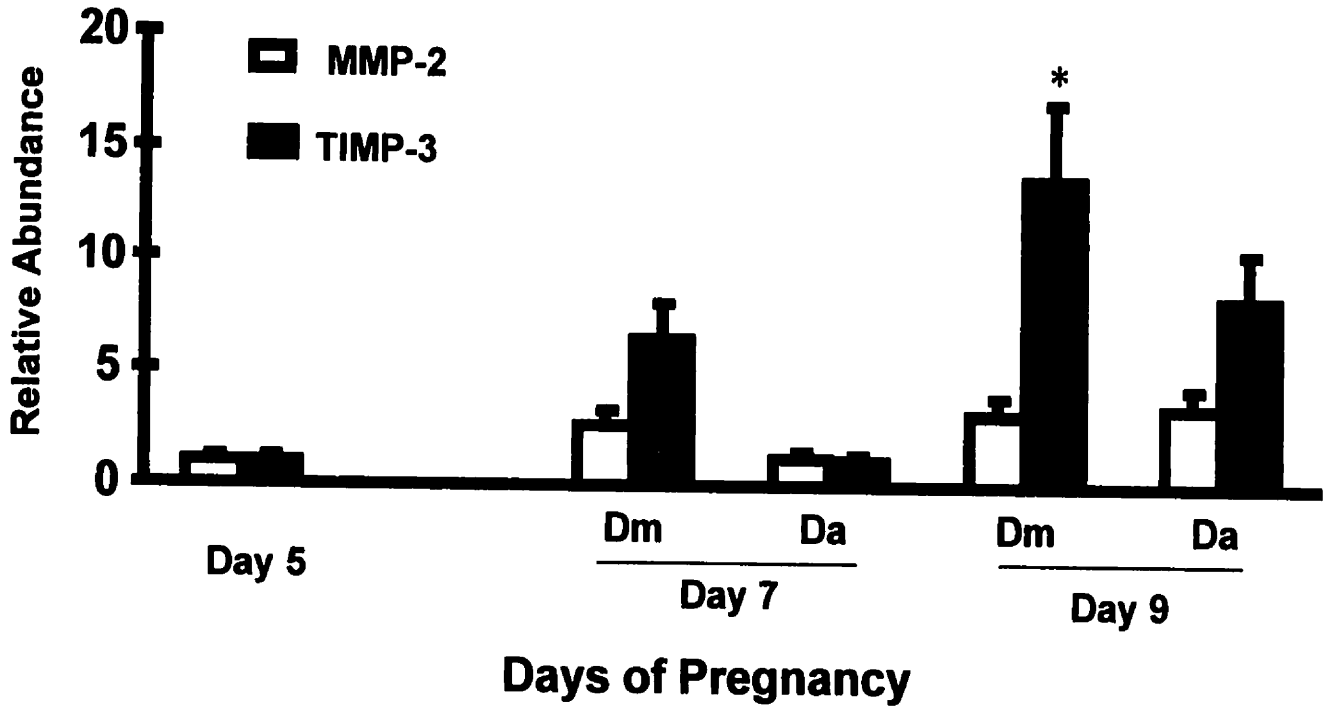
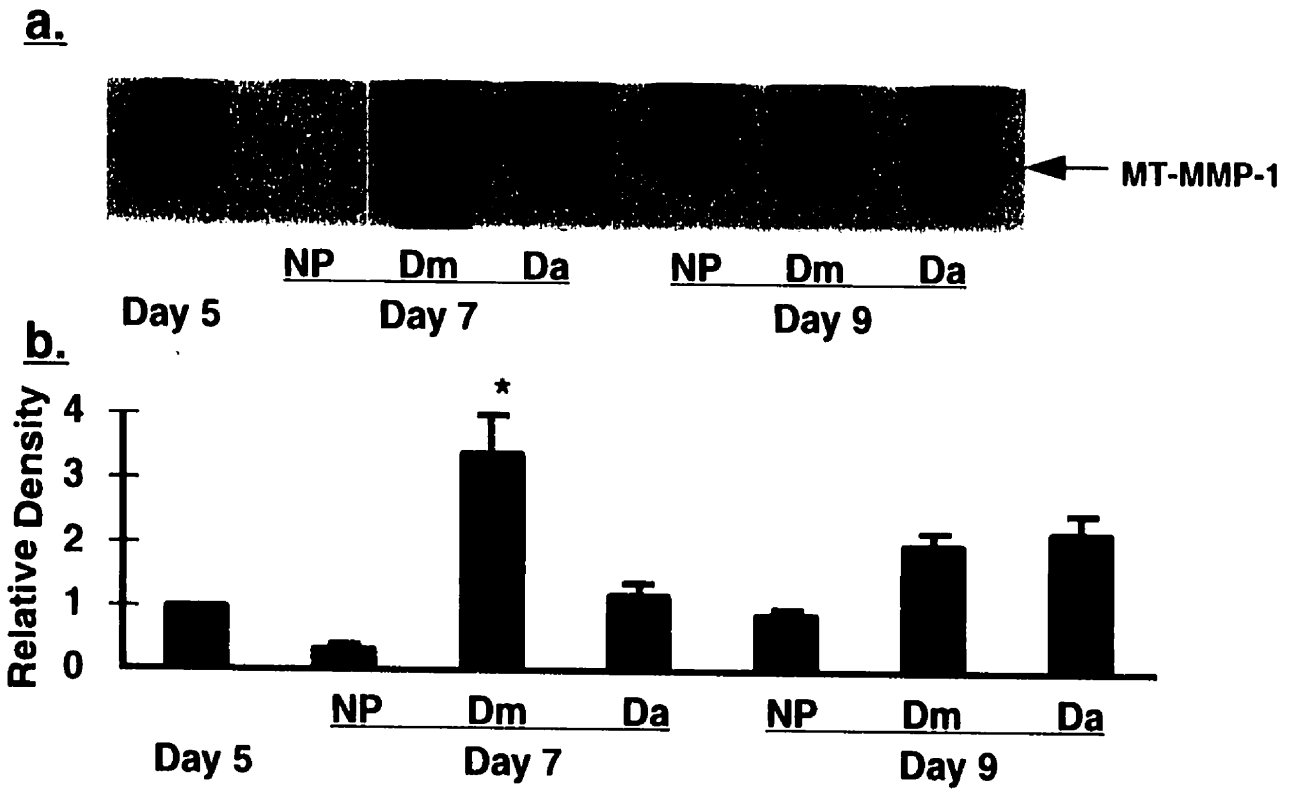


Figure 5-5. Northern blot analysis for MT-MMP-1 mRNA. Endometrial (NP), deciduomal (Dm) or decidual (Da) tissues were collected on different days (as indicated) of pregnancy for isolation of total RNA. Total RNA was subjected to Northern blot analysis using a specific cDNA probe. A typical autoradiogram is presented (a). The analysis was repeated at least three times and the densitometry data were pooled. The relative density (mean ± SE) was calculated as the ratio of the specific signal to that of the 18s rRNA with the data for Day 0 set for "1" (b). *: denotes significantly different from other tissues.


a.
MT-MMP-1
NP
Dm
Da
NP
Dm
Da
Day 5
Day 7
Day 9
b.
Relative Density
0
1
2
3
4
*
NP
Dm
Da
NP
Dm
Da
Day 5
Day 7
Day 9

5.2.2. Zymography and reverse zymography:

Zymography and reverse-zymography techniques were used to analyze enzymatic activities of the proteinases and their inhibitors in the endometrial and decidual tissues.

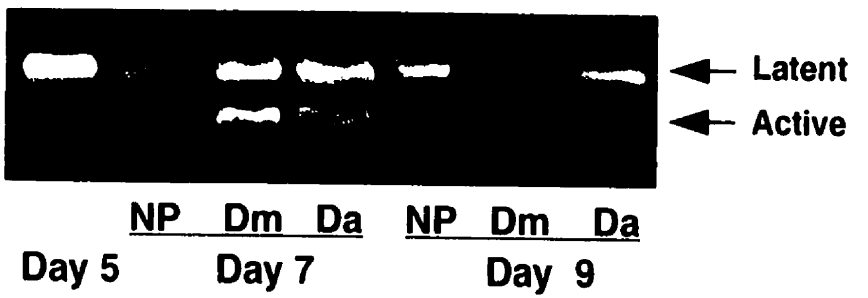
Two bands were identified by gelatin-zymography at positions near that of MMP-2 (Figure 5-6). One had a molecular weight equivalent to 72 kDa, probably representing the latent form of MMP-2. The other band with a slightly lower molecular weight was presumably the activated form of MMP-2. Both the latent and activated forms of MMP-2 were low in most of the tissues studied, except that on Day 7, the level of activated MMP-2 was significantly increased in the deciduoma and decidua.

A double band with a molecular weight close to 100 kDa was visible in some tissue samples (Figure 5-7). This probably represented the latent (upper band) and activated (lower band) forms of MMP-9. The latent and activated forms of MMP-9 in decidualized tissues were either similar to or slightly less than the non-decidualized endometrium on Day 5 of pseudopregnancy (Figure 5-7b).

The activities of TIMP-1, TIMP-2 and TIMP-3 were all visible in the uterine tissues studied (Figure 5-8). However, only the level of TIMP-1 and TIMP-3 activities was increased significantly in deciduomal and decidual tissues on Day 9, in comparison with other tissues.

Figure 5-6. Zymography for MMP-2. Endometrial (NP), deciduomal (Dm) or decidual (Da) tissues were collected on different days (as indicated) of pregnancy to obtain tissue protein. The protein samples were subjected for zymography. A typical zymogram is presented (a). The analysis was repeated at least three times and the densitometry data were pooled. The relative density (mean ± SE) was calculated as the ratio of the specific signal to the data for Day 5 set for "-1" (b). *: denotes significantly different from other tissues.

a.


Latent
Active
NP Dm Da NP Dm Da
Day 5
Day 7
Day 9

b.

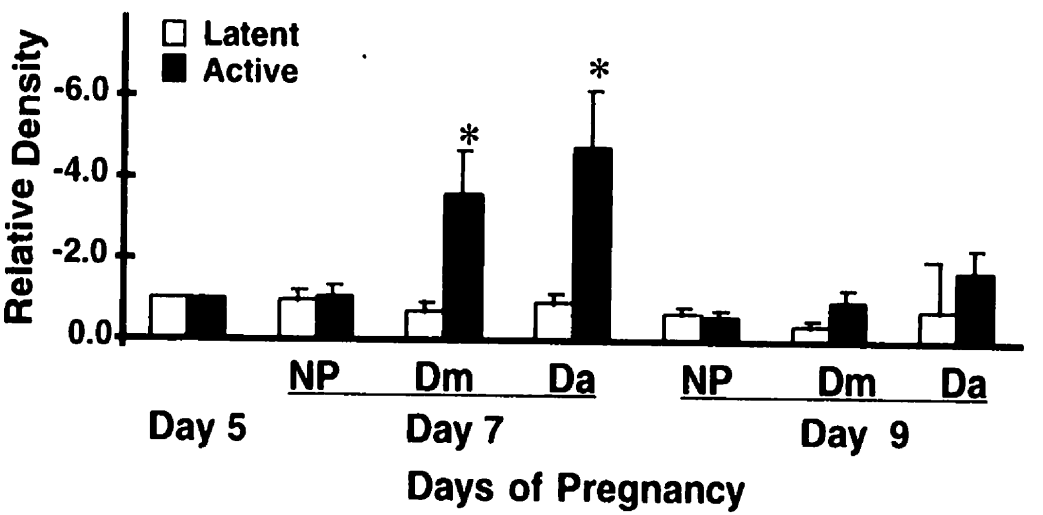

Latent
Active
Relative Density
-6.0
-4.0
-2.0
0.0
*
*
NP Dm Da NP Dm Da
Day 5
Day 7
Day 9
Days of Pregnancy

Figure 5-7. Zymography for MMP-9. Endometrial (NP), deciduomal (Dm) or decidual (Da) tissues were collected on different days (as indicated) of pregnancy to obtain tissue protein. The protein samples were subjected for zymography. A typical zymogram is presented (a). The analysis was repeated at least three times and the densitometry data were pooled. The relative density (mean ± SE) was calculated as the ratio of the specific signal to the data for Day 5 set for "-1" (b).

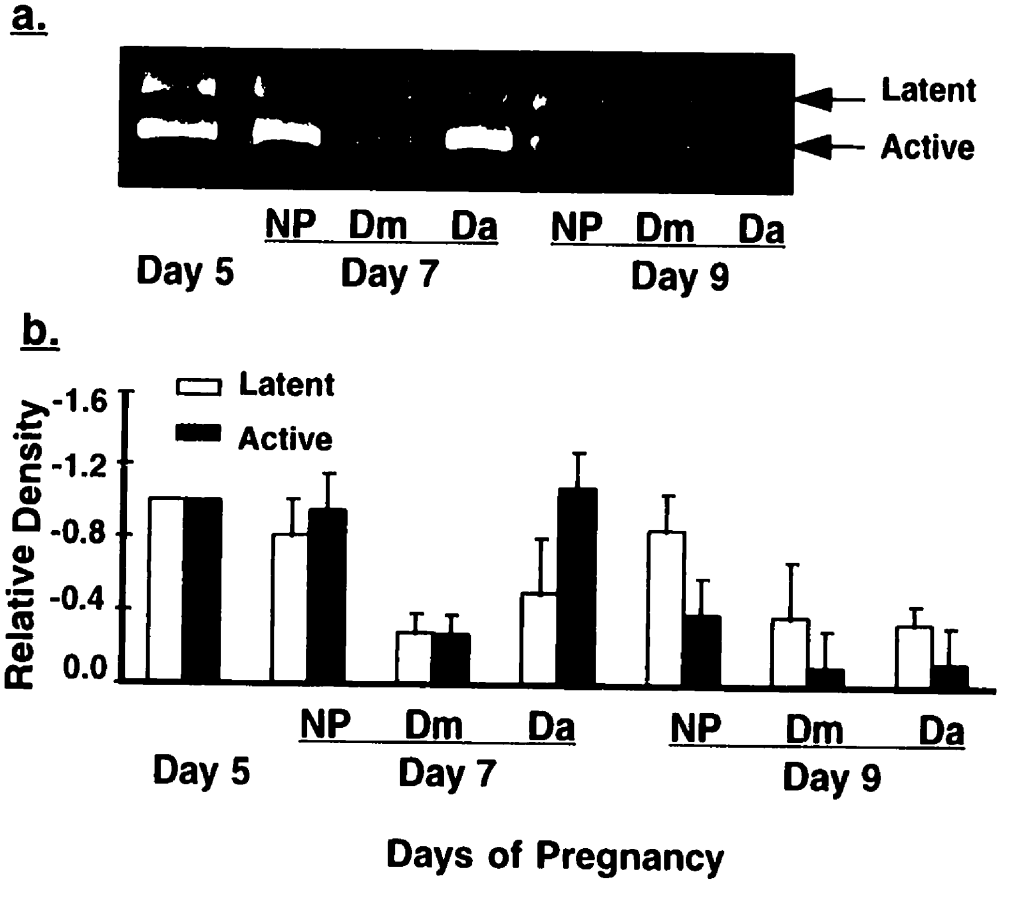
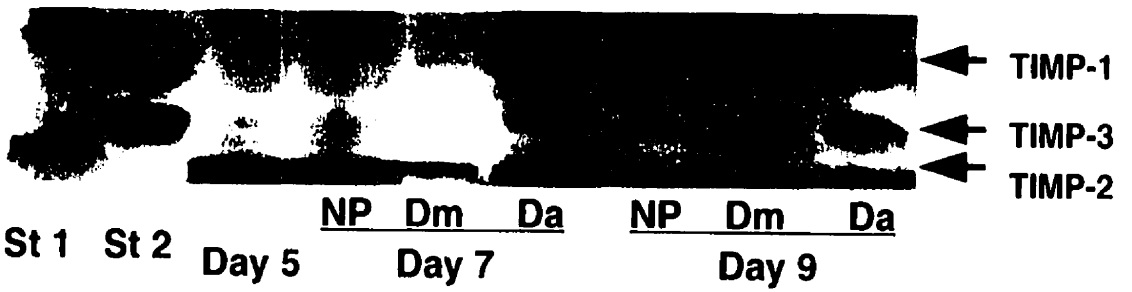
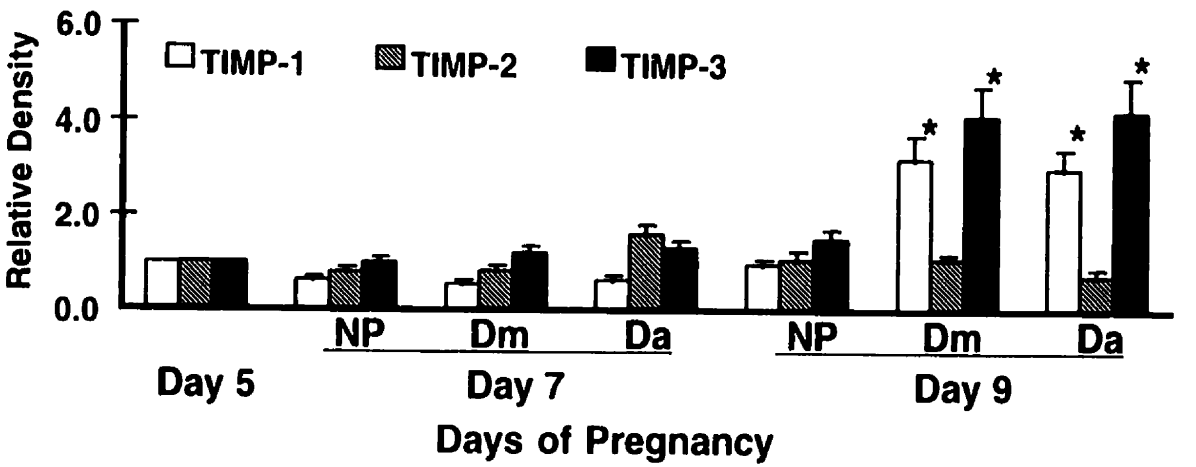

a.
Latent
Active
NP Dm Da NP Dm Da
Day 5
Day 7
Day 9
b.
Latent
Active
Relative Density
-1.6
-1.2
-0.8
-0.4
0.0
NP Dm Da NP Dm Da
Day 5
Day 7
Day 9
Days of Pregnancy

Figure 5-8. Reverse zymography for TIMPs. Endometrial (NP), deciduomal (Dm) or decidual (Da) tissues were collected on different days (as indicated) of pregnancy to obtain tissue protein. The protein samples were subjected to reverse zymography. A typical reverse zymogram is presented (a). The analysis was repeated at least three times and the densitometry data were pooled. The relative density (mean ± SE) was calculated as the ratio of the specific signal to the data for Day 5 set for "1" (b). *: denotes significantly different from other tissues.

a.


TIMP-1
TIMP-3
TIMP-2
St 1
St 2
Day 5
NP
Dm
Da
Day 7
NP
Dm
Da
Day 9

b.


Relative Density
6.0
4.0
2.0
0.0
TIMP-1
TIMP-2
TIMP-3
*
*
*
*
NP
Dm
Da
NP
Dm
Da
Day 5
Day 7
Day 9
Days of Pregnancy

CHAPTER SIX. DISCUSSION AND CONCLUSION

6.1. PAs and PAIs

Previous studies from our laboratory have shown that rat uterine stromal cells secrete uPA in vitro and this secretion is regulated by prostaglandin E_2, an important mediator for the induction of DCR (Zhang *et al.*, 1996). The presence of uPA mRNA in the decidua was later confirmed by in situ hybridization (Wang *et al.*, 1996). In the present study, the level of uPA mRNA was analyzed in uterine tissues collected throughout the first half of gestation. Comparisons were also made between the decidualized and non-decidualized tissues on corresponding days of pregnancy or pseudopregnancy. The level of uPA mRNA was low during the first five days of pseudopregnancy and increased steadily from Day 5 onwards in the non-decidualized endometrium. In comparison, this increase was much less in the decidual than deciduomal tissues on the corresponding days of pregnancy or pseudopregnancy.

The difference in mRNA levels between the decidualized and non-decidualized tissues was not reflected in the enzymatic activities of uPA. The uPA activity was increased from Day 7 onwards in both decidualized and non-decidualized endometrial tissues. This discrepancy could be caused by a number of factors. For example, the control of the translation of the uPA mRNA is dissociated from that of the mRNA transcription and/or stabilization. Alternatively, the level of uPA activity in the uterine

tissues was not accurately reflected because the uterine tissues were inevitably contaminated with blood which is known to contain uPA. Similar explanations may apply for the discrepancy between the levels of tPA mRNA and enzymatic activity. Very low levels of tPA mRNA were found in the uterine tissues studied, but the tPA activity was present at relatively high levels in most of the tissues.

The finding that the level of uPA mRNA in non-decidualized endometrium was higher than in the decidualized tissues indicates that the abundance of uPA mRNA is increased by factors that are not specific to the process of decidualization. This agrees with recent studies that "disqualified" uPA as a marker for decidualization (Kennedy and Ross, 1997). A small dose of estradiol (1 μg estradiol per rat) prior to the induction of decidualization is essential for decidualization to occur. However, an "overdose" of estradiol (10 μg) inhibits decidualization. In the recent study (Kennedy and Ross, 1997), the levels of uPA secretion *in vitro* were similar between non-decidualizing uterine cells from rats overdosed with the pre-decidualization estradiol and the decidualizing cells from rats that were properly sensitized for decidualization.

It is likely that uPA mRNA is regulated by the endocrine environment during early pregnancy and the process of decidualization reduces the enhancing effect of the endocrine factors on uPA mRNA. By Day 5, the endometrium is primed with progesterone and nidatory estrogen and is therefore fully sensitized for decidualization. Thereafter, continuous exposure to increasing levels of progesterone and estrogen is essential for maintaining decidualization (Psychoyos, 1973; Finn, 1977). Both

progesterone and estradiol can regulate uPA expression (Danø *et al.*, 1985). In the rat endometrium, these two steroids may have a synergistic effect on uPA mRNA expression. Thus, from Day 5 of pregnancy or pseudopregnancy onwards, the uterus is exposed to increasing amounts of both progesterone and estradiol, which may result in the steady increase in the abundance of uPA mRNA.

PAs has been implicated in various physiological and pathological processes that involve ECM degradation or modification (Danø *et al.*, 1985). PAs activity is increased in ovarian follicles just prior to ovulation (Beers *et al.*, 1975). Secretion of uPA by early embryos coincides with the beginning of implantation (Strickland *et al.*, 1976; Zhang *et al.*, 1994). Some invasive tumor cells have increased amount of PA activity (Danø *et al.*, 1985). In the mouse, PA from the embryos has been thought to be involved in the break-down of endometrial ECM during embryo implantation (Strickland and Richards, 1992). However, ECM degradation occurs in the endometrium prior to implantation and independent of the embryos. The increased vascular permeability in the uterus of the mouse and rat, which involves the ECM degradation in the vascular wall, occurs before any signs of DCR begins to appear, and can be induced artificially without the embryo (Psychoyos, 1973; Finn, 1977). uPA is likely one of the important mediators in this increased vascular permeability since uPA has been found to be associated with vasculature in the non-decidualized endometrium but not in the decidua in the rat (Bacharach *et al.*, 1992).

The loss of ECM components, such as collagen and hyaluronic acid, occurs in the decidualizing endometrium of the mouse or rat, independent of the embryos (Brown *et al.*, 1992; Mulholland *et al.*, 1992). Histological studies also found that the break-down of the ECM precedes embryo implantation (Abrahamson and Zorn, 1993). The increased uPA expression in the rat uterus on Days 7 and Day 9 of pregnancy or pseudopregnancy may be required, at least partially, for the increased ECM break-down during early pregnancy in the decidualizing and non-decidualizing endometrial tissues.

PA activity is counter-balanced by some naturally occurring inhibitors, including PAI-1 and PAI-2. The present study confirmed previous findings that the decidual and deciduomal tissues increase their production of PAI, particularly PAI-1. This increased PAI-1 may act to limit the invasive activity of the trophoblast cells during later stages of embryo implantation. That is, one of the roles of the decidua is in limiting embryo invasion as noted earlier (Porter, 1967; Kirby and Cowell, 1968), may be mediated, at least partially, by PAI-1.

6.2. MMPs and TIMPs

The importance of MMPs in the control of ECM remodelling is reflected by the rapid expansion of newly identified members in this family of proteinases (Matrisian, 1990; Basbaum and Werb, 1996). The presence of MMPs and TIMPs in the mouse uterus has been the subject of a number of studies (e.g. Waterhouse *et al.*, 1993; Harvey *et al.*,

1995; Alexander *et al.*, 1996). While this study was underway, a comprehensive study was published by Alexander *et al* (1996) which established an "expression map" for various MMPs and TIMPs in the mouse decidua during early pregnancy. Using the techniques of in situ hybridization, Northern blot analysis and zymography, Alexander *et al* (1996) found that the most prominent MMP in the decidua is MMP-2, which is localized in the interface between the decidua and the underlying myometrium. The most important TIMP in the mouse decidua is probably TIMP-3. It is localized in the decidua adjacent to the implanting embryo (Harvey *et al.*, 1995; Alexander *et al.*, 1996). Its expression is associated with decidualization but is independent of viable embryos (Leco *et al.*, 1996). The present study confirmed and expanded these earlier findings. The present study demonstrated that MMP-2 mRNA and enzyme activity are present in both decidualized and non-decidualized endometrium in pregnant or pseudopregnant rats. Further, the present study found that the activated, not the latent form, of MMP-2 is significantly increased in the deciduomal and decidual tissues. This increase in the activated MMP-2 coincides with an increase in the level of mRNA for MT-MMP-1, a specific activator of MMP-2. However, a parallel increase in MT-MMP-1 mRNA was not observed in the embryo-induced decidua. It is possible that in the pregnant uterus the embryo may produce MT-MMP-1 that is responsible for the activation of MMP-2. The increased MT-MMP-1 expression in the deciduoma may compensate for the lack of the embryonic activator. Consistent with the findings of Alexander *et al* (1996), the present study found little mRNA for MMP-9 in the uterine tissues. However, both studies

observed the enzymatic activity of MMP-9 in the endometrium and decidualized tissues. This may be due to either contamination of uterine tissues with blood-borne MMP-9 or an unusually high turn-over rate for its mRNA. In comparison, a high level of MMP-9 mRNA was found in the trophoblast cells of implanting embryos (Harvey *et al.*, 1995; Alexander *et al.*, 1996).

The decidualization process involves cell migration and tissue remodeling (Weitlauf, 1994). MMP-2 is probably one of several important proteinases that controls the ECM during this tissue remodeling process. Because it is localized close to the underlying myometrium, the decidual MMP-2 may also play an important role in preparing the decidua/maternal placenta for dissociation from the uterus at parturition (Alexander *et al.*, 1996).

Consistent with previous findings, the present study demonstrated that the expression of TIMP-3 is associated with the decidualization process. In addition, the present study showed that the abundance of TIMP-3 mRNA is greater in the deciduoma than in the decidua. This difference is probably explained by the differences in the development of embryo-induced and artificially induced DCR. The embryo-induced DCR is localized adjacent to the embryo. The tissues from the pregnant uteri might be contaminated with non-decidualized endometrial cells. In contrast, artificially induced DCR is relatively non-specific and involves the endometrium in the entire uterine horn that is injected with oil. Therefore, tissue samples collected from uteri injected with oil might have a greater proportion of truly decidualized cells than those from pregnant uteri.

Alternatively, the embryo may play a role in modulating the expression of TIMP-3. This is logical considering that the primary function of TIMP-3 is probably to restrict the invasive activity of trophoblast by suppressing MMP-9 activity from the trophoblast. The embryo must overcome this restriction in order to invade successfully. Obviously, this modulating effect of embryo is absent in the deciduoma that may result in a greater expression of this proteinase inhibitor.

Alexander *et al* (1996) suggested that another possible function of TIMP-3 is to protect the integrity the decidua by controlling apoptosis in the decidua. The decidual cells undergo active apoptosis that is probably necessary for the restructuring of the decidua. Although DCR is initiated in the anti-mesometrial region, the decidual cells in this region soon degenerate and newly transformed decidual cells "re-colonize" in the mesometrial region (Finn, 1977; Weitlauf, 1994). Proteinases are involved in this apoptosis process (Gu *et al.*, 1994). TIMP-3 may act to prevent the decidual cells surrounding the embryo from undergoing apoptosis by counter-acting the activity of the proteinases that can cause apoptosis.

6.3. Conclusion

The present study confirmed and extended previous findings that certain proteinases and their inhibitors, including uPA, PAI-1, MMP-2, MT-MMP-1, and TIMP-3 are expressed in the endometrium during pregnancy. These proteinases and inhibitors

are probably controlled by different mechanism. For example, uPA appears to be up-regulated by the endocrine environment during early pregnancy, whereas TIMP-3 expression is tightly associated with the process of decidualization. The level of control of expression also differs between these enzymes. For example, the control of MMP-2 expression appears to be at the level of post-translation (activation of the enzyme). In contrast, the expression of TIMP-3 mRNA is closely correlated to its inhibitory activity. A lack of correlation between mRNA levels and enzymatic activities was observed for tPA and MMP-9. The presence of embryo influenced the mRNA levels for PAI-1 (Wang *et al.*, 1996), TIMP-3 and MT-MMP-1, but appeared to have little effect on the expression of other proteinases.

These findings reinforce earlier suggestions that ECM degradation/modification plays an important role during embryo implantation and decidualization in the uterus, although different enzymes may participate in different aspects the tissue remodeling process during decidualization. However, it is beyond the scope of this study to establish an unequivocal role for each of the proteinases or their inhibitors during embryo implantation. Although functional studies are necessary, it may prove very difficult to establish a clear role for each of the enzymes and their inhibitors. This is because many of the proteinases and their inhibitors overlap in their expression patterns and in their functions, and may compensate for each other in gene knockout animal models.

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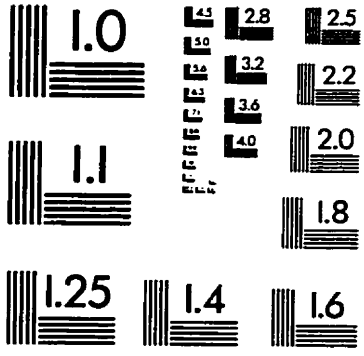
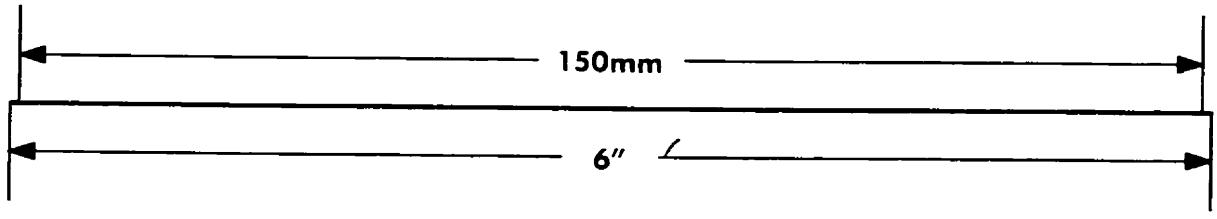
TEST TARGET (QA-3)





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