Expression Profiles of Secretory Leucocyte Protease Inhibitor, MMP9, and Neutrophil Elastase in the Mouse Uterus

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ABSTRACT : The tremendous changes of uterine endometrium are observed during early pregnancy and protease and their inhibitors are involved in regulation of cell proliferation and remodeling of the tissues through remodeling the extracellular matrix (ECM). Some of the proteases and protease inhibitors have been suspected to a factor in endometrial changes but many parts of their expression profiles and the physiological roles are not uncovered. To evaluate the functional roles of them, in this study the expression profiles of proteases and protease inhibitors were analyzed using real-time quantitative PCR analysis. *Mmp9* (matrix metalloproteinase 9) mRNA levels peaked on day 4 at the time of implantation. On the other hand, *Ela2* (neutrophil elastase, NE) mRNA levels were peaked on day 2 of pregnancy. Its expression were decreased until day 4 of pregnancy but increased rapidly until day 7 of pregnancy and decreased again. NE inhibitor *Slpi* (secretory leukocyte protease inhibitor, SLPI) mRNA levels were related with the implantation stage and with the levels of *Ela2*. At the time of implantation the expression levels of *Slpi* mRNA were about 5 times higher than the *Ela2* mRNA in the uterus. In the implantation stage embryos, *Mmp9* specific mRNA was only detected at the blastocyst. On the other hand, the expression level of SLPI was higher than that of the *Ela2* mRNA at blastocyst and 4.5 day p.c. embryos. Based on these results it is suggested that MMP9, SLPI, and NE have important physiological role in embryo implantation both in uterus and embryos.

Key words : SLPI, NE, MMP9, Implantation, Uterus.

INTRODUCTION

Uterine endometrial differentiation is essential step for successful ontogeny in mammals. For that in mammals the embryos have to adhere and to implant in the uterine endometrium which is reorganized to accept the heterogeneous embryo. Endometrium is constructed with the epithelium and stroma. Epithelium is ciliated and covered with mucose with many glands (Ferenczy & Richart, 1973), and stroma is a connective tissue with mesenchymal cell and amorphous extracellular matrix (ECM) (Brenner & Slayden, 1994). It has been known that the endometrial differentiation is under the control of sex steroid hormones (Cheon et al., 2002). Endometrial changes are accomplished through proliferation and type changes of these cells, and remodeling of ECM to prepare the implantation and response to the embryo.

ECM is responsible for the uterine preparation and continuous modulation during pregnancy (Cheon, 2007). Collagens and laminin are deposited in the basement layer and stroma and are rearranged for embryo invasion. Also, for the decidual response ECM components are expressed at a high level in implanting areas (Iwahashi et al., 1996; Kim & Cheon, 2006; Mizuno et al., 1999; Tanaka et al., 2008). The changes of these components and structures can regulate the proliferation and differentiation of trophectoderm and endometrial itself (Chrobak et al., 2004; Qin et al., 2003). In addition ECM composition can be cause of infertility from unknown causes (Bilalis et al., 1996).

Proteases (peptidase or proteinases) and protease inhibitors are key molecules for degradation or keeping the stability of ECM. A few of proteases and protease inhibitors have been detected in the pregnant uterus (Chen et

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al., 2010; Daimon & Wada, 2005; Skrzypczak et al., 2007). MMP9 degrades the ECM to degrade decidua and have great role in the embryo invasion and endometrial transformation (Plaisier et al., 2008; Zambuzzi et al., 2009). Zhao and colleagues (2002) suggested that *Mmp9* mRNA was barely detectable in endometrium during the implantation period, but one of the controversial studies has demonstrated MMP9 activity and *Mmp9* mRNA in the endometrium just before implantation (Sharkey et al., 1996).

Secretory leukocyte protease inhibitor inhibits the activity of serine proteases (Stetler et al., 1990; Wright et al., 1999). It is highly expressed in the pregnant uterine endometrium and is suggested an important role in implantation and maintenance of an intact utero-placental interface (Badinga et al., 1994; Chen et al., 2004). However, so far, the expression profile is controversy between the studies (Chen et al., 2004; Sharma et al., 2008). One of the target protease of SLPI is NE. In uterus, it has been suggested that NE is secreted from neutrophils which migrate into the stroma. Secreted NE degrades type III collagen in the endometrial stroma and result in generating spaces (Okada et al., 2001). Although NE is a suspected protease in the pregnant uterus, the expression is not fully understood.

Taken together, these studies indicate important role for MMP9, SLPI, NE in the process of endometrial differentiation. However, MMP9, SLPI, NE during the early preimplantation period has not been thoroughly studied regarding pregnant stages. In the present study, we found that *Mmp9*, *Slpi*, *Ela2* mRNA expressions are dramatically changed spatio-temporally during early pregnant stage and SLPI may work thoroughly at the time of implantation.

MATERIALS AND METHODS

1. Animals

All methods used in this study were approved by the Animal Care Committee at Sungshin Women's University. In addition, all studies were conducted according to the NIH Guidelines for the Care and Use of Experimental Animals. Outbred female mice (CD1) were maintained in 14 hr light and 10 hr dark. Female mice were superovulated by 5 IU pregnant mares serum gonadotropin (PMSG, Sigma, St. Louis) followed by human chorionic gonadotropin (hCG) 48 hr later. Following the hCG injection, females were placed in cages with males and checked the presence of vaginal plugs at next day morning and designated as day 1 of pregnancy.

2. Preparation of Culture Media and Uterus and Embryo Collection

BWW medium was used in this experiment. The medium was prepared fresh and used to collect the embryos and wash the uterus. Bovine serum albumin (BSA, fraction V, Sigma, St. Louis) was prepared at a concentration of 4 mg/ml. Mice were sacrificed by cervical dislocation at 96 hr and 120 hr post hCG injection to collect blastocysts and 4.5d p.c embryos, respectively. The early pregnant uteri were collected at 24 (day 1), 48 (day 2), 72 (day 3), 96 (day 4), 120 (day 5), 144 (day 6), 168 (day 7), and 192 hr (day 8) post hCG injection.

3. RT-PCR and Quantitative PCR

Total RNA was extracted from embryo and uterus using RNAeasy mini kit (Qiagen, Valencia, CA, USA) and TRYzol, respectively. Real-time quantitative PCR was performed in a Thermal Cycle Dice™ Real Time system (TP800; TakaRa Bio, Inc., Shiga, Japan) using SYBR Green diction according to the manufacturer's instructions. Primer sequences were as follows: mouse *Slpi* mRNA (GeneBank accession number: NM_011414), 5'-TGAATCCTGTTCC CATTCG-3' and 5'-GCAGGGAAGTAG TTTCAG-3'; mouse *Mmp9* mRNA (GeneBank accession number: NM_013599), 5'-TGTACGGACCCGAAGCGGACATT-3' and 5'-GGTA CAGGAAGAGTACTGCTTG-3'; mouse *Ela2* mRNA (GeneBank accession number: NM_015779), 5'-CATGCTAC TGGCATTGTTCCTGG-3' and 5'-CACCTGCACGTTGG CGTTAAT-3'; mouse *Actb* mRNA (GeneBank accession number: NM_007393), 5'-GTGGGCCGCTCTAGGCAC

CAA-3' and 5'-CTCTTTGATGTC ACGCACGATTTC-3'; mouse *Arbp* mRNA (GeneBank accession number: NM_007475), 5'-CGACCTGGAAGTCCAACTACTTCCT-3' AND 5'-GCACCTTATTGGCCAACAGCA-3'. The amplification reaction was performed under the following conditions: 40 cycles of denaturation at 94℃, annealing 59℃, and extension 72℃. Dissociation curved were generated after each PCR run to ensure that a single product of the appropriate length was amplified. The mean threshold cycle (Ct) and standard error were calculated from individual Ct values obtained from three replicates per stage. The normalized mean Ct was computed as ΔCt by subtracting the mean Ct of PPIA from the Ct of a MMP9, NE, and SLPI for each stage embryo. In the case of uterus, 36B4 mRNA was used as control. ΔΔCt was then calculated as the difference between the ΔCT values of a control and each stage. The n-fold change in gene expression relative to a control (PN stage) was computerized as $2^{-\Delta\Delta CT}$. The error bars indicate $2^{-\Delta\Delta CT}\pm$ the standard deviation. All experiments were conducted in triplicate.

4. Statistical Analysis

Data are represented mean±S.D. from 3 independent measurements. Data were analyzed by Student's *t*-test. In all tests, $p<0.05$ was considered to indicate statistical significance.

RESULTS

1. Profiles of *Mmp9* mRNA Expression in the Pregnant Uterus

Expression of *Mmp9* is vital for normal uterine function. MMP 9 degrades the ECM of uterine endometrium and help to invasion of the trophoblast. *Mmp9* mRNA was detected from day 1 of pregnancy and showed fluctuation in the expression levels. The expression peaked on day 4 of pregnancy but decreased continuously until day 6 of pregnancy. After then it was increased continuously (Fig. 1).

When compared with the level of *Ela2* mRNA, the level of *Mmp9* mRNA was high at least 11 times on day 1 of pregnancy. On day 4 of pregnancy, the expression level of *Mmp9* was high at least 21 times compared with the *Ela2* ($p<0.05$). *Mmp9* RNA expression level was always high at these stages (Fig. 2).

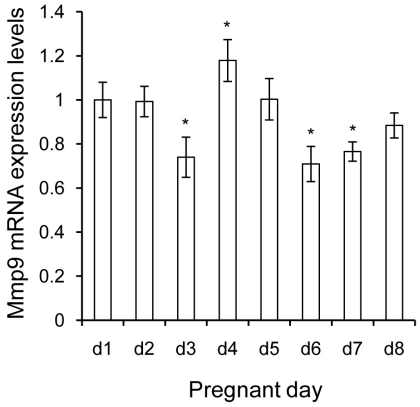

Fig. 1. Expression profiles of *Mmp9* mRNA in the pregnant uterus. The pregnant mouse uterus was collected at day 1 (d1), day2 (d2), day 3 (d3), day 4 (d4), day 5 (d5), day 6 (d6), day 7(d7), and day 8 (d8). Using the uteri of each physiological stages total RNA was extracted and used for the real-time quantitative RT-PCR. The asterisks indicate significant differences from day 1 values (*$p<0.05$).

2. Profiles of *Ela2* mRNA Expression in the Pregnant Uterus

Ela2 mRNA was detected from day 1 and showed peak at day 2 of pregnancy. It was decreased until day 4 and showed lowest level at this stage of pregnancy, and then increased. On day 7 of pregnancy, it showed peak again (Fig. 3). Compared with the *Mmp9*, the expression level was very low (Fig. 2). It shows the possible that role of NE is different from the MMP9 at these stages.

3. Profiles of *Slpi* mRNA Expression in the Pregnant Uterus

According to Chen et al. (2004), *Slpi* mRNA expression in rat is under the control of estrogen. In the mouse, it is not clear whether estrogen control the expression of *Slpi* in uterus or not. The expression level of *Mmp9* mRNA

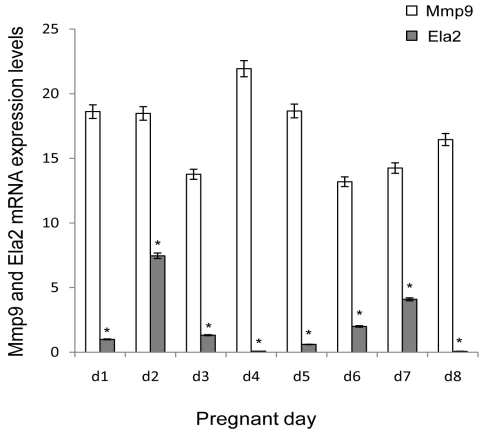

Fig. 2. Relative expression levels of *Mmp9* mRNA and *Ela2* mRNA in the pregnant uterus. The pregnant mouse uterus was collected at day 1 (d1), day2 (d2), day 3 (d3), day 4 (d4), day 5 (d5), day 6 (d6), day 7(d7), and day 8 (d8). Using the uteri of each physiological stages total RNA was extracted and used for the real-time quantitative RT-PCR. The asterisks indicate significant differences from *Mmp9* mRNA of same day values (*p<0.05).

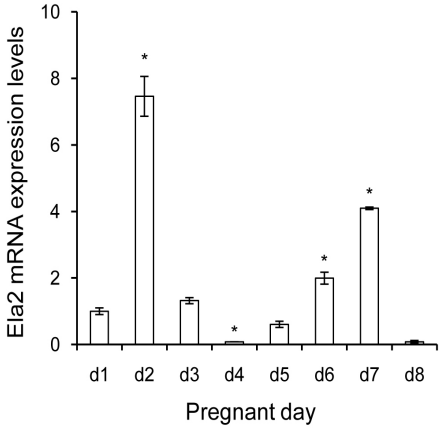

Fig. 3. Expression of *Ela2* mRNA in the pregnant uterus. The pregnant mouse uterus was collected at day 1 (d1), day2 (d2), day 3 (d3), day 4 (d4), day 5 (d5), day 6 (d6), day 7(d7), and day 8 (d8). Using the uteri of each physiological stages total RNA was extracted and used for the real-time quantitative RT-PCR. The asterisks indicate significant differences from day 1 values (*p<0.05).

was highest on day 1 of pregnancy. It was decreased continuously until day 3 but it increased on day 4 of pregnancy.

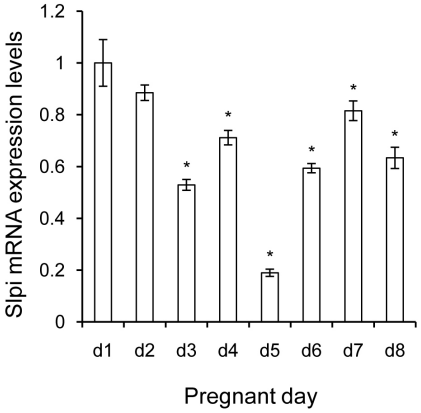

Fig. 4. Expression profiles of *Slpi* mRNA in the pregnant uterus. The pregnant mouse uterus was collected at day 1 (d1), day2 (d2), day 3 (d3), day 4 (d4), day 5 (d5), day 6 (d6), day 7(d7), and day 8 (d8). Using the uteri of each physiological stages total RNA was extracted and used for the real-time quantitative RT-PCR. The asterisks indicate significant differences from day 1 values (*p< 0.05).

On day 5, its level was lowest but increased again until day 7. These expression patterns suggest that estrogen may control the expression of *Slpi* in mouse (Fig. 4).

Activity of proteases is dependent on their inhibitors. SLPI inhibits the activity of NE and the role of NE is dependent on the concentration of SLPI. Interestingly a *Slpi* mRNA level is high on day 4 of gestation but the *Ela2* mRNA level is lowest on day 4 of pregnancy. After then, however, on day 5, 6, and 7 the expression level of *Slpi* mRNA was continuously increased concurrently with that of *Ela2* mRNA (Fig. 5).

4. Expression of *Mmp9*, *Ela2*, and *Slpi* mRNA in the Embryos of Periimplantation Stages

At the time of implantation the expression patterns of *Ela2* and *Slpi* mRNAs suggest a pleiotropic role of SLPI. On the other hand, the pattern of *Mmp9* mRNA on day 4 of pregnancy suggests the dramatic remodeling of ECM of endometrium. For the further evaluation, the expression patterns of them were examined in blastocyst and 4.5 day

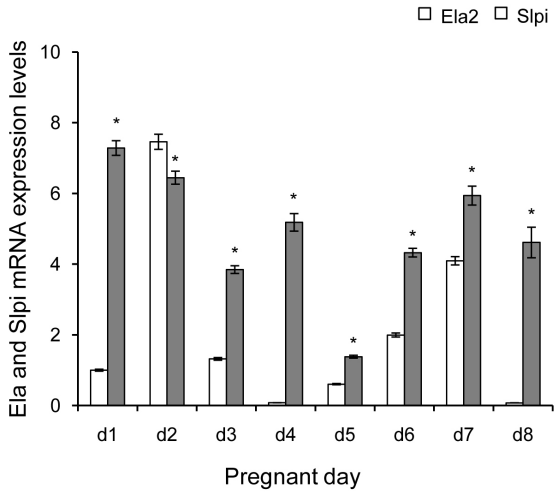

Fig. 5. Relative expression levels of *Ela2* mRNA and *Slpi* mRNa in the pregnant uterus. The pregnant mouse uterus was collected at day 1 (d1), day2 (d2), day 3 (d3), day 4 (d4), day 5 (d5), day 6 (d6), day 7 (d7), and day 8 (d8). Using the uteri of each physiological stages total RNA was extracted and used for the real-time quantitative RT-PCR. The asterisks indicate significant differences from *Ela2* mRNA of same day values (*p<0.05).

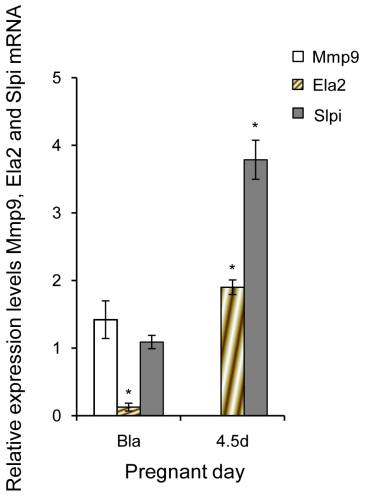

Fig. 6. Expression of *Mmp9*, *Ela2*, and *Slpi* mRNA in the blastocyst and 4.5 day p.c. embryo. Blastocysts and 4.5 day p.c. embryos were collected at 96 hr and 120 hr post hCG injection, respectively. From 10 embryos total RNA was extracted and used for the real-time quantitative RT-PCR. The asterisks indicate significant differences from *Mmp9* mRNA of each stage (*p< 0.05).

p.c. embryo. *Mmp9* specific mRNA was detected in blastocyst but not in 4.5day p.c. embryos. *Ela2* specific mRNA was barely detected on blastocyst but it was increased at least 5 times on 4.5day p.c. embryos. The level of *Slpi* specific mRNA was relatively high on day 4 compared with the other genes and it was increased at least 3.5 times on day 4.5 day p.c. embryos. These expression profiles showed the possible physiological role of SLPI at the time of implantation (Fig. 5).

DISCUSSION

Uterine preparation for embryo implantation is accomplished through complex interaction between progesterone, estrogen, and local communications. In the molecular levels, that regulates the cellular response and the ECM modulation. Sex steroid hormones control the MMPs expression through extracellular matrix metalloproteinase inducer (EMMPRIN) (Braundmeier et al., 2006; 2010). MMPs are a family of zinc-dependent enzyme and have combined specificities for all components of ECM (Curry, 2003). The physiological roles of MMPs are including the enhancement of synaptic plasticity, implantation and wound healing (Kean et al., 2009).

MMP9 is secreted form of MMP and known as gelatinase, and beta-dystroglycan, gelatin type Ⅰ and Ⅴ, fibronectin, and type Ⅳ and type Ⅴ collagen are inclued in its target molecules (Michaluk et al., 2007; Stellas et al., 2010). Collagens are involved in endometrial ECM and the remodeling of ECM is indispensable in embryos development. Distribution and expression of collagens have been described thoroughly in the uterus of rodent during pregnancy (Cheon, 2007; Hurst et al., 1994; Stellas et al., 2010). Although the MMPs expression is under the control of sex steroid hormones through EMMPRIN in the uterus, the kinds of MMPs should be controlled by the stage and tissue specificity in endometrium during pregnancy. Through this examination, the patterns of *Mmp9*

mRNA expression were explored. *Mmp9* mRNA was detected from day 1 of pregnancy with high level at least 10 times. Its expression levels decreased at day 3 and showed peak at day 4 of pregnancy. According to Finn and Martin (1967) and Goodger and Rogers (1993), the uterine cells proliferate in a stage and tissue specificity during pregnancy. On day 1 of pregnancy, there is a moderate degree of proliferative activity in the luminal epithelium, and very little proliferative activity in the glandular epithelium, endometrial stroma. On day 2 of pregnancy, there was a high degree of proliferative activity in the luminal and glandular epithelium, and virtually none elsewhere. On day 3 of pregnancy, the degree of proliferative activity in the epithelium declined substantially, but increased in the endothelial cell of the endometrial stroma. On day 4 of pregnancy, proliferative activity was minimal in the epithelium, but was increased throughout the stroma generally. Based on them it is suggested that the MMP9 at these stages are involved in ECM remodeling for epithelial or stroma cell proliferation to prepare the implantation.

On day 5, 6 and 7 of pregnancy, the epithelial cell was similar to that seen on day 4, although more proliferating cells were seen in the stroma especially the decidualizing site. (Finn & Martin, 1967; Goodger & Rogers, 1993). At these stages, the cells near the implanted embryos undergo dramatic changes such as decidual differentiation, degradation of the differentiated cell, and placental formation. Interestingly, the expression of *Mmp9* mRNA was increased continuously from day 5. Although other MMPs will be involved in these changes, from these date it is confirmed again that *MMP9* is involved in differentiation of endometrial cells in the implanting area.

In the case of NE, previously it is reported that it degrades type Ⅲ collagen and supports implantation (Okada et al., 2001), but the functional role is not clear. The first step to do evaluation the functional role, the expression profile of *Ela2* mRNA was examined. Relative expression level was very low on day 1 but it peaked on day 2 of pregnancy. After then it was decreased and on day 4 of pregnancy it was not detected. However, it increased again continuously on day 7. When compared the expression levels of its specific inhibitor, SLPI, it seems like that NE does not inhibit the work of SLPI in the uterus or embryo as a competitor.

SLPI has various physiological roles as a paracrine and autocrine factor. In uterus, the role of steroids in SLPI expression is controversy (Chen et al., 2004; King et al., 2003). SLPI can mediate proliferation of endometrial epithelial cell and other cells (Zhang et al., 2002). In the present study, the expression profile showed that its expression is under the control of estrogen, because its expression level was high on day 1 of pregnancy at least 7.5 times. In addition, the level of Slpi mRNA was increased again on day 4 of pregnancy. Usually the genes which are regulated by estrogen are highly expressed on day 1 and 4 of pregnancy. Such an expression pattern showed that SLPI may work at the time of implantation. *Ela2* mRNA was not detecting on day 4 of pregnancy and then it increased continuously on day 7 of pregnancy with the increase of the expression level of *Slpi* mRNA. On day 8 of pregnancy, *Ela2* mRNA was not detected but the level of *Slpi* mRNA was high. On the other hand, the *Mmp9*, *Ela2*, and *Slpi* mRNA expressions were detected. *Mmp9* mRNA was only detected in blastocyt (embryos were got at 96 hr post hCG injection) but was not in 4.5 day p.c. embryos. In the case of *Slpi*, it was expressed but *Ela2* was not expressed. Even though, the functional roles of SLPI and NE are not clearly evaluated in the pregnant uterus, these results suggested that SLPI may work as paracrine or autocrine regulator in addition with their work as a protease inhibitor during these stages

At early pregnant stages, there are many biological events especially preparation of embryo and the uterus for implantation. MMPs and plasminogen activators are thought an important factor of successful implantation but it has been suspected that many other protease and protease inhibitors will be involved in those processes. The physio-

logical means of the expression profiles of *Mmp9*, *Ela2*, and *Slpi* mRNAs are not well evaluated so far. We need further study to evaluate the meaning of the profiles of these genes during early pregnancy.

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