Inhibition of proteases involved in embryo implantation by cholesterol sulfate

Minako Koizumi, Mikio Momoeda1, Hisahiko Hiroi, Fumiko Nakazawa, Hanako Nakae, Tomoko Ohno, Tetsu Yano, and Yuji Taketani

Obstetrics and Gynaecology, The University of Tokyo, Tokyo, Japan

1Correspondence address. 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan. Fax: +81-3-3816-2017; E-mail momoeda-tky@umin.ac.jp

Background: Matrix metalloproteinases (MMPs) and the plasminogen activator (PA)/plasmin system are two major groups of proteases involved in the matrix degradation required for embryo implantation. We previously showed that the content of cholesterol sulfate (CS) in rabbit endometrium increases characteristically during the implantation period. Furthermore, CS has been reported to inhibit serine proteases. In this study, we investigated whether CS can regulate the activity of proteases in cultured human endometrial stromal cells.

Methods and results: CS (1–30 μM) and plasminogen (precursor of plasmin) were added to the culture media of human endometrial stromal cells and incubated for 24 h. Culture media were collected for analysis of plasmin and MMP-2, -3 and -9 enzyme activities using fluorescence assays. Plasmin and MMP-3 activities were significantly reduced by CS in a dose-dependent manner (P < 0.001). Western blot analysis of the culture media revealed that CS inhibited the conversion by plasmin of MMP-3 from the precursor form to the active form. Fluorescence assay using a common substrate of MMP-2 and MMP-9 showed that enzymatic activity remains at >50%, even at 30 μM CS. Gelatin zymography demonstrated that CS inhibited the activation of MMP-9 but not MMP-2 from the precursor, suggesting that the activation of MMP-2 may be independent of plasmin.

Conclusions: CS inhibits not only plasmin activity but also MMP activities indirectly by inhibiting the plasmin-mediated process. These findings suggest that CS may be an important regulator of proteolysis during trophoblast invasion.

Key words: matrix metalloproteinases / plasmin / cholesterol sulfate / endometrium / implantation

Introduction

Implantation of the embryo into maternal endometrium is a crucial step in the establishment of pregnancy. During trophoblast penetration of the basement membrane of the epithelium and invasion into stroma, proteases play a role in the degradation of the extracellular matrix (ECM). Two major groups of proteases, matrix metalloproteinases (MMPs) and the plasminogen activator (PA)/plasmin system, are involved in this degradation process (Duc-Goiran et al., 1999). Human trophoblast cells appear to have a high degree of invasive capacity, similar to that of malignant cells. However, the trophoblast invasion is tightly and locally controlled, whereas tumor cells invade inappropriately (Salamonsen, 1999).

The MMPs are a family of more than 20 related enzymes that degrade a number of ECM components and are considered as the most important proteases in numerous biological processes. The MMP system controls the following aspects of reproductive function: follicular development, ovulation, menstruation and implantation. According to numerous studies using animal models, multiple subtypes of MMPs are expressed not only in invading trophoblast cells but also in maternal endometrium during the implantation process (Curry and Osteen, 2003). Regulation of the local expression and activity of MMPs could be crucial for the establishment of implantation. At the transcription level, the expression of MMPs is induced by inflammatory cytokines, such as interleukin-1 and tumor necrosis factor α (Rawdanowicz et al., 1994), but inhibited by progesterone (Osteen et al., 1994). Most MMPs are secreted as inactive propeptides requiring activation in the extracellular space by proteases, such as plasmin or other MMPs. Tissue inhibitors of metalloproteinases (TIMPs) are locally produced and inhibit specifically active forms of MMPs in extracellular space. The expression of TIMP-3 was observed in the early phase of pregnancy and induced by progesterone (Higuchi et al., 1995), suggesting that TIMP-3 could be an important regulator inhibiting trophoblast invasion.
The PA/plasmin system also has a broad spectrum of substrates and is involved in tissue remodeling. PA, that cleaves plasminogen to plasmin, is expressed in the endometrium throughout the menstrual cycle (Nordengren et al., 2004) and regulates proliferation, maturation, decidualization and menstruation (Littlefield, 1991). The PA/plasmin system exerts its action on matrix degradation, in part indirectly, by proteolytic activation of pro-MMPs. Among proteases that can mediate the activation of pro-MMPs in extracellular space, plasmin is considered as the most significant activator of pro-MMPs physiologically (Murphy et al., 2000).

Cholesterol sulfate (CS) is the most important known sterol sulfate and is widely distributed in human tissues and cells. CS has been recognized as a regulator of steroid synthesis and is involved in a variety of enzyme activities and membrane stabilization. One of the most investigated physiological roles for CS is keratinocyte differentiation and the development of the epidermal barrier (Strott and Higashi, 2003). In reproductive organs, CS has been reported to be involved in the regulation of steroid hormone synthesis (Tuckey et al., 1994; Sugawara et al., 2007; Tsutsumi et al., 2008). However, the role of CS in endometrium remains unclear.

Our previous reports showed that CS in rabbit endometrium increased specifically during the implantation period (Momoeda et al., 1991, 1994). Moreover, the expression of cholesterol sulfotransferase, SULT2B1b, the key enzyme for synthesis of CS, was induced during the mid-luteal phase of the menstrual cycle in human endometrium (Koizumi et al., 2009). CS has also been reported to inhibit the activity of serine proteases, including plasmin (Iwamori et al., 1999). We have examined the role of CS as a possible candidate for the regulator of proteases during the implantation period.

The purpose of the present study was to determine whether CS regulated the activities of two major proteases, MMPs and plasmin, involved in ECM degradation during the implantation process, using cultured human endometrial stromal cells (ESCs).

**Materials and Methods**

**Isolation and culture of ESCs and treatments**

Endometrial tissues were obtained from women undergoing hysterectomy for benign disease unrelated to endometrial pathology. All patients had regular menstrual cycles and none of them had received hormonal treatment for at least two cycles before surgery. The specimens were dated according to the criteria of Noyes et al. (1950) and referring to the patients’ menstrual history. The experimental procedure was approved by the institutional review board of the University of Tokyo, and all patients gave written consent for tissue collection. ESCs were separated and maintained in monolayer culture, as previously described (Koga et al., 2001). Briefly, endometrial tissue was minced to small pieces and digested by incubation in Dulbecco's minimum essential medium (DMEM)/F-12 (Gibco, Grand Island, NY, USA), which contained 0.25% type I collagenase (Sigma, St. Louis, MO, USA) and deoxyribonuclease I (60 U/ml; Invitrogen, Carlsbad, CA, USA). The dispersed endometrial cells were separated by filtration through a 40-μm pore size nylon cell strainer (Becton Dickinson and Co, Franklin Lakes, NJ, USA). ESCs were filtered and endometrial epithelial cells were caught by a strainer. Filtered ESCs were centrifuged and plated in phenol-red free DMEM/F-12 containing 10% charcoal stripped fetal bovine serum (HyClone, Logan, UT, USA) in 100-mm culture dishes. At the first passage, cells were plated in 48-well dishes (Becton Dickinson) at a density of 2.5 × 10^5 cells per well. Cells were washed two times with phosphate-buffered saline and changed to a serum-free condition at 24 h with or without 1 μg/ml plasminogen (Wako, Osaka, Japan) and various concentrations of CS (1–30 μM; Sigma), and then cultured for an additional 24 h. The medium was harvested, centrifuged at 1700 g for 10 min to remove cellular debris and stored at −20 °C for subsequent analysis. CS was added from stock solution in dimethylsulfoxide (DMSO) to the appropriate final concentration. Control cells received the same concentration (0.05%) of DMSO. The effect of CS on cell viability was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Tokyo, Japan). The purity of both the stromal and epithelial cell preparations was more than 95%, as judged by positive cellular staining for vimentin or cytokeratin, and negative staining for CD45 (which identifies leukocytes) (Koga et al., 2001; Hirota et al., 2005).

**Plasmin activity assay**

The fluorogenic plasmin substrate Boc-Glu-Lys-Lys-MCA (Peptide Institute, Inc., Osaka, Japan) was dissolved at 10 mM in DMSO and stored at −20 °C in the dark to avoid photo-oxidation. To measure plasmin activity, the substrate was diluted to 0.25 mM in assay buffer containing 50 mM Tris–HCl, 150 mM NaCl, pH 7.5, as described previously (Iwamori et al., 1999). The reaction was initiated by adding 100 μl of cell-conditioned medium to 100 μl of assay buffer containing substrate at 37 °C in the dark for 1 h. At the end of incubation, the reaction was stopped by adding 800 μl of 10% acetic acid. The rate of substrate cleavage was measured in triplicate for each CS concentration examined, using a microplate filter (Spectroan FL-2575; Towa scientific Co., Ltd., Tokyo, Japan) with fluorescence excitation and emission wavelengths of 380 and 460 nm, respectively.

**MMP activity assay**

The fluorogenic MMP-3 substrate MOCAc-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH2 and MMP-2/MMP-9 substrate MOCAc-Pro-Leu-Gly-Leu-A2pr(Dnp)-Ala-Arg-NH2 (Peptide Institute, Inc.) were prepared as 1 mM stock solutions in DMSO and stored at −20 °C in the dark to avoid photo-oxidation. The substrate was diluted to 20 μM in assay buffer containing 50 mM Tris–HCl, 150 mM NaCl, 10 mM CaCl2, pH 7.5 for MMP-3, and 50 mM Tris–HCl, 200 mM NaCl, 20 mM CaCl2, pH 7.5 for MMP-2/MMP-9, as described in the literature (Nagase et al., 1994; Monea et al., 2002). The reaction was initiated by adding 100 μl of cell-conditioned medium to 100 μl of assay buffer containing substrate for 15 min. At the end of incubation, the reaction was blocked by adding 800 μl of 10% acetic acid and the rate of substrate cleavage was measured with fluorescence excitation and emission wavelengths of 325 and 393 nm, respectively. To confirm that CS did not interfere in the substrate assay, we performed the same assay with substrate of plasmin, MMP-3 and MMP-2/MMP-9 but without cell-conditioned medium.

**Western blot analysis**

MMP-3 antibody (clone 55-2A4), which recognizes both latent and active forms of MMP-3, was purchased from Cosmo Bio (Tokyo, Japan). Cell-conditioned medium was separated electrophoretically by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis using a 5–20% gradient gel (Atto, Tokyo, Japan) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk in 20 mM Tris base, 150 mM NaCl with 0.2% Tween-20, pH 7.6 (TBST-T) for 2 h and incubated overnight with anti-MMP-3 (2.5 μg/ml), (clone 55-2A4) at 1:500 dilution. The blots were then washed and incubated for 1 h with peroxidase-labeled...
anti-mouse immunoglobulin G (Amersham Biosciences, Piscataway, NJ, USA) at 1:10 000 dilutions. Each step was followed by extensive washing in TBS-T. The blots were developed using the enhanced chemiluminescence plus western blotting detection system (Amersham Biosciences). Immunodetected bands were scanned using the Storm 860 phosphorimager (Molecular Dynamics, Tokyo, Japan).

**Gelatin zymography**

Gelatinase activity was measured using a Gelatin Zymo electrophoresis kit (Yagi, Yamagata, Japan) according to the manufacturer’s recommendations. Cell-conditioned medium was mixed with the same volume of sample buffer (50 mM Tris–HCl, 2% SDS, 10% glycerol and 0.01% Bromphenol blue, pH 6.8) and loaded onto SDS-7.5% polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, the gels were washed with 200 ml of 2.5% Triton X-100 at room temperature and additionally washed with 200 ml of 50 mM Tris–HCl, 150 mM NaCl, pH 7.5. For the enzyme reaction, the gels were incubated with 50 ml of 50 mM Tris–HCl, 200 mM NaCl and 5 mM CaCl2, pH 7.5 at 37°C for 40 h, stained 1 h with Coomassie Blue G-25 and destained in a solution containing 5% (v/v) acetic acid, 30% (v/v) methanol and 65% (v/v) water. The experiment was repeated four times. The stained digestion area was scanned and measured by densitometry. The intensity of active form of MMP-2 and MMP-9 was compared with control (plasminogen). Values are presented as mean ± SEM of triplicate cultures.

**Statistical analysis**

The measurements of fluorescence assay were analyzed by analysis of variance. If there were significant differences, the Tukey–Kramer multiple comparisons test was then applied for post hoc analysis. The measurements of densitometry of zymography were analyzed by the paired Student’s t-test. *P* < 0.05 was considered to be statistically significant. Values are presented as mean ± SEM of triplicate cultures.

**Results**

**Inhibition of plasmin activity by CS in culture media of ESCs**

To determine the effect of CS on plasmin activity in cultured endometrial cells, CS in various concentrations (1–30 μM) and plasminogen, a precursor of plasmin, was added to the culture media of ESCs. By adding 1, 5, 10, 15, 20 and 30 μM CS, the activity of plasmin in culture media was significantly reduced to 75.7, 41.3, 36.5, 27.2, 20.8 and 13.9% of control (with plasminogen, no CS), respectively (*P* < 0.001) (Fig. 1). Tukey–Kramer test showed significant reduction from control level at 5, 10, 15, 20 and 30 μM CS (*P* < 0.05). Cell viability was not significantly different with each concentration of CS.

**Effect of CS on the conversion of MMP-3 from latent form to active form**

To investigate whether CS inhibits MMP-3 activity in cultured endometrial cells, we performed a fluorescence assay using a specific substrate of MMP-3. The activity of MMP-3 in culture media was significantly reduced to 75.7, 41.3, 36.5, 27.2, 20.8 and 13.9% of control (with plasminogen, no CS), respectively (*P* < 0.001) (Fig. 2). Tukey–Kramer test showed significant reduction from control level at 5, 10, 15, 20 and 30 μM CS (*P* < 0.05). Western blot analysis demonstrated that CS inhibited the conversion of MMP-3 from the latent form (pro-MMP-3, Mr 59/57 × 10^3) to the active form (Mr 45 and 28 × 10^3) by plasmin in a dose-dependent manner (Fig. 3). Concomitant addition of the plasminogen inhibitor aprotinin with plasminogen nearly eliminated the conversion of the latent to the active form, which suggested that activation of MMP-3 is mainly due to plasmin activity.

**Effect of CS on the conversion of MMP-2 and MMP-9 from latent form to active form**

We next examined whether CS inhibited MMP-2 and MMP-9 activities in culture media from ESCs by fluorogenic assay using a common substrate of MMP-2 and MMP-9. Figure 4 demonstrates that the activities of MMP-2 and MMP-9, which were increased in the presence of plasminogen, were partially reduced but remained at about 50% of control activity.
Discussion

Human trophoblast cells appear to have a high degree of invasive capacity and can penetrate as far as the first third of the myometrium (Ramsey et al., 1976). ECM proteolysis is a crucial step during embryo implantation into maternal endometrium. MMPs and the PA/plasmin system are known to be the two major groups of proteases involved during trophoblast invasion (Salamonsen, 1999). In this process, protection of the endometrial structure is required for the establishment of pregnancy. In this study, we showed that CS could act as a novel regulator of proteases involved in trophoblast invasion. We first demonstrated that CS inhibited the activity of plasmin, and second that CS also inhibited the activities of MMP-3 and MMP-9 secreted from ESCs.

CS inhibited the transition of MMPs from latent to active forms, whereas the expression of latent forms of MMPs remained at the same levels. Moreover, conversion from the latent to active form of MMP-3 was nearly eliminated by adding aprotinin with plasminogen. This finding suggests that the inhibition of MMP activities by CS occurred indirectly through the inhibition of plasmin activity. As plasmin is the activator of several MMP subtypes, CS could inhibit the activities of other subtypes of MMPs in ESCs as well.

MMP-3 acts on a diverse array of ECM substrates and activates other MMPs, and it seems to play a major role in proteolysis. The expression of MMP-3 in decidual cells has been reported (Schatz et al., 1994). We observed remarkable inhibition of the MMP-3 activity (plasminogen, no CS) even at higher doses of CS ($P = 0.0137$). Tukey–Kramer test showed a significant reduction from control level only at 15 $\mu$M CS ($P < 0.05$). To examine the effect of CS on the activities of MMP-2 and MMP-9 separately, we performed gelatin zymography (Fig. 5). Conversion from pro-MMP-9 to active MMP-9 was observed by adding plasminogen and this was inhibited significantly by 10 $\mu$M CS (Table I), whereas active MMP-2 was detected even in the absence of plasminogen and the activity of MMP-2 was not inhibited by CS. We observed no signals of activity for plasmin, MMP-3 and MMP-2/MMP-9 in the control experiments, which had no cell-conditioned medium in the substrate assay (data not shown).

**Figure 3** Western blot analysis of MMP-3 in conditioned medium of human ESCs.

The cells were incubated for 24 h in serum-free medium in the absence or presence of plasminogen (1 $\mu$g/ml) and increasing doses of CS. Aprotinin (plasminogen inhibitor, 0.3 mg/ml) was added as indicated. The band at 59/57 kDa represents the latent form of MMP-3 and that at 45 and 28 kDa, the active form. The experiment was repeated three times with comparable results.

**Figure 4** Effect of CS on MMP-2 and MMP-9 activities of human ESCs.

The cells were incubated for 24 h in serum-free medium in the absence or presence of 1 $\mu$g/ml of plasminogen and increasing doses of CS for 24 h. Fluorescent assay using a common substrate of MMP-2 and MMP-9 was performed to evaluate both enzymatic activities simultaneously. Values are the mean ± SEM of triplicate cultures. *$P < 0.05$, Tukey–Kramer test.

**Figure 5** Gelatin zymography of conditioned medium of human ESCs.

The cells were incubated for 24 h in serum-free medium in the absence or presence of 1 $\mu$g/ml of plasminogen and 10 $\mu$M of CS. The mixture of recombinant pro-MMP-9, pro-MMP-2 and active MMP-2 was used as a marker for specific enzyme. The experiment was repeated three times with comparable results.

**Table I** Gelatin zymography of conditioned medium of human endometrial stromal cells

<table>
<thead>
<tr>
<th>% Of control (mean ± SE)</th>
<th>Plasminogen + CS−</th>
<th>Plasminogen + CS+</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>49.1 ± 7.3</td>
<td>15.3 ± 1.2*</td>
</tr>
<tr>
<td>MMP-2</td>
<td>36.3 ± 8.4</td>
<td>30.5 ± 3.3</td>
</tr>
</tbody>
</table>

Results of gelatin zymography from four experiments are compared by densitometry and analyzed by paired t-test.

*Plasminogen + CS− versus Plasminogen + CS+, $P < 0.05$. 

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by CS. This is consistent with the results of Nagase et al. (1990), which showed direct activation of extracellular MMP-3 by plasmin.

The involvement of MMP-9 in the implantation process in human endometrium has been suggested by Jeziorska et al. (1996). Their study reported that MMP-9 secretion from glandular epithelium was optimal during the peri-implantation phase. The activation process of MMP-9 is debatable. MMP-9 can be also activated by a plasmin-dependent mechanism (Baramova et al., 1997; Mazzieri et al., 1997); however, studies from other laboratories have concluded that activation of pro-MMP-9 occurred through the activation of the MMP-3/plasmin cascade, and not directly by plasmin (Ramos-DeSimone et al., 1999). Rigot et al. (2001) showed that the activation of MMP-9 was dependent on the MMP-3 activity in tissue culture of endometrium. We observed the inhibition of MMP-9 activity by CS; however, we could not determine whether MMP-3 was involved in the inhibition of MMP-9 activity.

It is noteworthy that the activation of MMP-2 was not inhibited by CS. The activation of MMP-2 proceeds independently from that of other MMPs; MMP-2 activation was resistant to the PA/plasmin system and occurred via a novel mechanism involving the membrane-type I MMP (MT1-MMP) and TIMP-2 (Murphy et al., 2000; Curry and Osteen, 2003). The trimolecular complex of MT1-MMP/TIMP-2/pro-MMP-2 is localized to the cell surface and autocleaved, resulting in activation of MMP-2. For that reason, the activation of MMP-2 seemed to occur in absence of plasmin, as seen in Fig. 5, resulting in absence of inhibition of MMP-2 activity at higher doses of CS.

Regulation of protease activities by CS has been reported previously. CS activates coagulation factor XII and kallikrein activities, whereas it inhibits activities of serine proteases such as thrombin, plasmin, trypsin and chymotrypsin (Iwamori et al., 1997, 1999). However, there are few reports that refer to the involvement of CS in MMP activities. Recently, CS has been reported to bind MMP-7 and promote proteolysis on the surface of colon cancer cells, inducing cell aggregation (Yamamoto et al., 2006). This finding indicates that CS could promote acquisition of metastasis characteristics in colon cancer cells, in contrast to our results which showed that CS could inhibit trophoblast invasion. This discrepancy in the effect of CS on MMPs can be explained by differences in the location at which CS binds the MMPs; that is, on the cell surface or extracellular space.

Our previous study showed that the expression of SULT2B1b, the enzyme for CS synthesis, was up-regulated by progesterone and relaxin in cultured human endometrial cells (Koizumi et al., 2009). Progesterone is a well-known inhibitor of MMPs; progesterone suppresses MMP gene transcription in human endometrium (Osteen et al., 1994; Curry and Osteen, 2003). The effect of relaxin on MMP expression in human endometrium varies with cell type; relaxin is a positive regulator of pro-MMP-1 and pro-MMP-3 in cervical fibroblasts (Paleywala et al., 2001), but a negative regulator of pro-MMP-1 in cultured human endometrial cells (Paleywala et al., 2002). In conjunction with our data, these findings suggest that progesterone and relaxin not only directly regulate MMPs production but also increase CS synthesis, resulting in the inhibition of MMP activation in human endometrium.

The present study explored the physiological roles of CS in extracellular space. The secretion of CS from human endometrial epithelial cells has been confirmed (Koizumi et al., 2009). Recent reports have shown that CS is a ligand for the retinoic acid-related receptor α (RORα) (Bitsch et al., 2003; Kallen et al., 2003). RORα is a nuclear receptor involved in the regulation of diverse pathophysiological processes. RORα is known as a hypoxia-inducible factor 1 (HIF-1) target gene (Chauvet et al., 2004). Additionally, HIF-1 is regulated transcriptionally by RORα (Kim et al., 2008). HIF-1 is a nuclear protein that activates gene transcription specifically in response to hypoxic conditions and acts as a marker for hypoxia. Several studies have shown the contribution of HIF-1 to MMP production. For example, MMP expression was up-regulated by HIF-1 in some types of cells (Luo et al., 2006; Fujisawa et al., 2007). Cultured ESCs responded to hypoxia with increased HIF-1, and hypoxia inhibited the production of pro-MMP-1 and pro-MMP-3, and the activation of MMP-2 (Zhang and Salamonsen, 2002). Therefore, CS may also decrease the production of MMPs by binding to RORα in ESCs.

In conclusion, our study has demonstrated the participation of CS in inhibiting the activities of MMPs and plasmin in cultured human ESCs. Our results indicate that CS may act as an important regulator during trophoblast invasion in human endometrium.

References


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