Induction of superoxide dismutase by decidualization in human endometrial stromal cells

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The present study was undertaken to investigate the effect of decidualization on superoxide dismutase (SOD) expression in human endometrial stromal cells (ESC). To induce decidualization, isolated ESC were incubated with medroxyprogesterone acetate (MPA, 10⁻⁶ mol/l) and oestradiol (10⁻⁸ mol/l) for 23 days. Insulin-like growth factor-binding protein-1 (IGFBP-1) was used as a marker of decidualization. SOD mRNA in ESC was significantly increased on day 12 of the hormone treatment (P < 0.01), which was concomitant with the onset of IGFBP-1 mRNA expression, and further increased until day 23 of the treatment in a manner similar to the change in IGFBP-1 expression. To examine the synergistic effect of human chorionic gonadotrophin (HCG) with MPA and oestradiol on SOD and IGFBP-1 expression, ESC were incubated with HCG in the presence or absence of MPA and oestradiol. HCG had no synergistic effect on SOD and IGFBP-1 expression. SOD activities in the decidualized endometrial tissue obtained from patients given oestradiol and progesterone for 7–10 days were significantly higher than those in the non-decidualized endometrial tissue from patients without the hormone treatment (P < 0.01).

In conclusion, SOD expression in ESC was induced by MPA and oestradiol accompanied by decidualization, suggesting that SOD may play important roles in decidualization of ESC.

Key words: decidualization/endometrial stromal cell/human/superoxide dismutase/superoxide radical

Introduction

It is well known that superoxide radicals cause tissue damage, whereas superoxide dismutase (SOD) works protectively by scavenging superoxide radicals. Recent evidence has shown that superoxide radicals and its scavenging system play important roles in reproductive function (Riley and Behrman, 1991; Sugino et al., 1996a; Kato et al., 1997). In the human uterus, reactive oxygen species including superoxide radicals are generated in the endometrium (Benedetto et al., 1981; Sugino et al., 1996b). They are increased in the late secretory phase endometrium, just before menstruation, and decreased in the decidua in early pregnancy (Sugino et al., 1996b). In contrast, the human endometrium has specific enzymes to scavenge superoxide radicals: copper-zinc SOD (Cu,Zn-SOD), located in the cytosol, and manganese SOD (Mn-SOD), located in the mitochondria (Sugino et al., 1996b). Both SODs belong to a first enzymatic step that protects cells against toxic oxygen radicals. Cyclic changes in SOD expression have also been reported in the endometrium and decidua (Narimoto et al., 1990; Sugino et al., 1996b). It therefore seems that the superoxide radical and SOD system play an important role in human endometrial function.

We have recently shown immunohistochemically that expression of both Cu,Zn-SOD and Mn-SOD was observed in the predecidual cell in the late secretory phase and increased in the decidual cell (Sugino et al., 1996b), suggesting that SOD expression in the endometrial stromal cell is associated with decidualization. Decidualization of endometrial stromal cells is essential for the establishment and maintenance of pregnancy and also stimulates the synthesis of various hormones, cytokines and growth factors (Daly et al., 1983; Huang et al., 1987; Hatayama et al., 1994; Sakakibara et al., 1994). This increase in metabolism stimulates the generation of superoxide radicals since superoxide radicals are normally generated during normal metabolic activity. In addition, decidual cells produce a cytokine such as tumour necrosis factor (Tabibzadeh, 1991; Hunt et al., 1992). Cytokines have been reported to damage cells through superoxide radical generation (Zimmerman et al., 1989). It is, therefore, important to know how SOD in the endometrial stromal cell changes during decidualization.

It is well known that the process of decidualization is controlled most effectively by oestrogen and progesterone, and it has also been reported that human chorionic gonadotrophin (HCG) promotes decidualization of endometrial stromal cells (Tang and Gurpide, 1993; Han et al., 1996). It is, therefore, possible that HCG promotes the expression of SOD in the endometrial stromal cell undergoing decidualization. In this study, we examined the effect of decidualization on SOD expression in human endometrial stromal cells, and the effect of HCG on SOD expression in endometrial stromal cells undergoing decidualization, using an in-vitro model of decidualization.

Materials and methods

This project was reviewed and approved by the committee of investigations involving human subjects of Yamaguchi University School of Medicine, Japan. Informed consent from the patient was obtained before collection of any tissue samples for this study.

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SOD induction by decidualization

Figure 1. In-vitro decidualization of human endometrial stromal cells induced by medroxyprogesterone acetate (MPA) and oestradiol. (A) Time-course of insulin-like growth factor-binding protein-1 (IGFBP-1) mRNA expression in endometrial stromal cells treated with MPA and oestradiol. mRNA expression was analysed by reverse transcription–polymerase chain reaction (RT–PCR). The quantification data are relative intensities of IGFBP-1 signals measured by densitometry and represent the mean ± SEM of three different experiments. *P < 0.01 versus control. (B) Control (C) Morphology of human endometrial stromal cells treated with oestradiol + MPA for 17 days (original magnification ×90).

Materials
Phenol Red-free Dulbecco’s modified Eagle’s medium (DMEM) and glutamine were purchased from ICN Biomedicals Inc (Aurora, OH, USA). Streptomycin, penicillin, 1× trypsin–EDTA, deoxynucleotide triphosphates and Moloney murine leukaemia virus reverse-transcriptase were from Life Technologies Inc (Grand Island, NY, USA). Collagenase, medroxyprogesterone acetate (MPA), oestradiol and HCG were obtained from Sigma Chemical Co (St Louis, MO, USA). Tissue flasks and nylon mesh were from Becton Dickinson Co (Franklin Lakes, NJ, USA). Random hexamer and Taq DNA polymerase were from Perkin-Elmer Co (Foster City, CA, USA). [α-32P]-deoxyctydine triphosphate (dCTP) was from Amersham (Arlington Heights, IL, USA). Isogen was from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Endometrial stromal cell isolation and cell culture
Human endometrium was obtained at hysterectomy from normally cycling pre-menopausal women, aged 43–49 years, who underwent surgery for myoma uteri. Endometrial samples were histologically diagnosed as late proliferative phase or early secretory phase according to the criteria of Noyes et al. (1950). Tissue samples were washed from patients undergoing hysterectomy for myoma uteri. Seven women, aged 39–49 years, were given ethinyloestradiol orally (0.05 mg) plus norgestrel (0.5 mg) (Planovar, Weis-Ezai Co Ltd, Japan) daily for 7–10 days from the mid-luteal phase to avoid the onset of menstruation at surgery. It was confirmed histologically that the specimen showed decidual changes of endometrial stroma. Endometrial tissues from seven women, aged 40–48 years, at the late secretory phase which was diagnosed histologically,
Figure 2. mRNA expression of copper-zinc superoxide dismutase (Cu,Zn-SOD) and manganese SOD (Mn-SOD) during in-vitro decidualization of human endometrial stromal cells. Time course of (A) Cu,Zn-SOD and (B) Mn-SOD mRNA expression in endometrial stromal cells treated with medroxyprogesterone acetate (MPA) and oestradiol (E2). mRNA expression was analysed by reverse transcription–polymerase chain reaction (RT–PCR). The quantification data are relative intensities of Cu,Zn-SOD or Mn-SOD signals measured by densitometry and represent the mean ± SEM of three different experiments. *P < 0.01 and **P < 0.05 versus control.

Table 1. Effects of treatment with oestrogen and progesterone on Cu,Zn-SOD activity, Mn-SOD activity and lipid peroxide (LPO) concentrations in the human endometrium. Values are given as means ± SE

<table>
<thead>
<tr>
<th></th>
<th>Cu,Zn-SOD</th>
<th>Mn-SOD</th>
<th>LPO (nmol MDA/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.0 ± 1.6</td>
<td>7.7 ± 2.7</td>
<td>104.4 ± 16.7</td>
</tr>
<tr>
<td>Ethinyloestradiol + norgesterel</td>
<td>42.9 ± 5.0a</td>
<td>22.9 ± 3.2a</td>
<td>34.1 ± 7.5a</td>
</tr>
</tbody>
</table>

Endometrial samples were obtained from patients given ethinyloestradiol (0.05 mg) and norgesterel (0.5 mg) for 7–10 days from the mid-luteal phase. Late secretory phase endometrial tissues without the hormone treatment were used as controls. SOD = superoxide dismutase; NU = nitrite unit.

*S < 0.01 versus control.

SOD assay and lipid peroxide assay

After cell incubation, the cells were washed twice with PBS, and resuspended in Tris–HCl buffer (0.1 mol/l, pH 7.4) and sonicated for the SOD assay. Endometrial tissues were homogenized in Tris–HCl buffer cleared by centrifugation and the supernatant was used for SOD assay. Cu,Zn–SOD activity and Mn–SOD activity were determined as reported previously (Sugino et al., 1993a). The amount of protein required for 50% inhibition in the absorbance at 550 nm was defined as one unit (nitrite unit = NU) of SOD activity. All data were expressed in NU of SOD activity per mg protein. Protein concentrations were determined by the method described by Lowry et al. (1951). The intra- and inter-assay coefficients of variation were 3.8 and 9.6%, for the Cu,Zn-SOD assay, and 4.7 and 6.4% for the Mn–SOD assay respectively. Concentrations of lipid peroxides in the endometrial tissue were measured by the thiobarbituric acid method as reported previously (Sugino et al., 1993a). The result was expressed as nmol of malondialdehyde (MDA) per g wet weight.

Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was isolated from the cultured cell with Isogen by the method provided by the manufacturer. For mRNA analysis, RT–PCR was performed as reported previously (Sugino et al., 1998a). The oligonucleotide primers for Cu,Zn-SOD (5’-CGAGCAGAAGGAAAGTAAATG-3’ and 5’-TAGCACGGATACAGATGAGT-3’) and for Mn-SOD (5’-AGTTCAATGGTGTTGTATCA-3’ and 5’-CAATCCACCGATGGAATTA-3’) were designed on the basis of the human Cu,Zn-SOD (Hallewell et al., 1985) and Mn-SOD cDNA sequences (Gene Bank, accession NO. E01408). The oligonucleotide
primers for insulin-like growth factor-binding protein-1 (IGFBP-1) (5′-TGCTGAGGCGAGGACC-3′) and 5′-AGGGATCCCTTCCCATCAG-3′), were used as a marker of decidualization (Kim et al., 1998). Two oligonucleotide primers (5′-CTAGAAGGCTACGCGATGTTGGTGAGCAGGTCTTGAGTCTC-3′) were also used to amplify ribosomal protein L19 as an internal control as reported previously (Chan et al., 1987). Briefly, 3 μg of total RNA was reverse transcribed at 42°C in a reaction mixture (single strength PCR buffer, 2.5 mmol/l deoxy nucleotide triphosphates, 5 μmol/l random hexamer primer, 1.5 mmol/l MgCl2, and 200 IU Moloney murine leukemia virus reverse transcriptase). The RT product was divided into two equal aliquots (one tube was for L19 primers). For PCR amplification, a mixture containing oligonucleotide primers (50 pmol), [α-32P]-dCTP (2 μCi at 3000 Ci/mmol), and Taq DNA polymerase (2.5 IU) was added to each reaction. Amplification was carried out for 25 cycles consisting of 95°C (1 min), 52°C (1 min) and 72°C (1 min) for Cu,Zn-SOD, 25 cycles consisting of Cu,Zn-SOD (1 min), 54°C (1 min) and 72°C (1 min) for Mn-SOD, and 24 cycles consisting of 94°C (1 min), 60°C (2 min) and 72°C (3 min) for IGFBP-1 followed by 10 min of final extension at 72°C in a programmed temperature control system PC-800 (ASTEC, Fukuoka, Japan). The predicted sizes of the PCR-amplified products were 455 bp for Cu,Zn-SOD, 282 bp for Mn-SOD, 379 bp for IGFBP-1 and 194 bp for L19. A linear curve was plotted using number of cycles of amplification versus densitometric values of the PCR products, measured with a BAS2000 (Fuji Photo Film Co, Tokyo, Japan). The optimal number of cycles for amplification within the linear range was chosen for each set of primers of SODs and L19 (data not shown). Reaction products were electrophoresed on an 8% polyacrylamide non-denaturing gel. After autoradiography, RNA bands were visualized with the aid of x-ray film. Reaction products were electrophoresed on an 8% polyacrylamide non-denaturing gel. After autoradiography, RNA bands were visualized with the aid of x-ray film.

**Statistical analysis**

Data were examined by analysis of variance and Duncan’s new multiple range test. Where appropriate, Student’s t-test was employed. P < 0.05 was considered to be significant.

**Results**

In order to induce decidualization in vitro in human endometrial stromal cells, cells were treated with MPA and oestradiol for 23 days. Decidualization of endometrial stromal cells was determined by morphology and IGFBP-1 expression. IGFBP-1 expression is a specific marker of decidualization and it is reported that changes in IGFBP-1 production during decidualization parallel those of prolactin production in human endometrial stromal cells (Giudice et al., 1992; Kim et al., 1998). IGFBP-1 mRNA expression was clearly observed under our culture conditions on day 12 of the treatment and increased thereafter (Figure 1A), accompanied by morphological changes characteristic of decidualization as described previously (Irwin et al., 1989) (Figure 1B,C).

The expression of both Cu,Zn-SOD and Mn-SOD mRNA in the endometrial stromal cell was significantly increased on day 12 of the treatment compared with the control (P < 0.01 and P < 0.05 respectively; Figure 2), which was concomitant with the onset of IGFBP-1 mRNA expression shown in Figure 1A, and further increased thereafter in a manner similar to the change in IGFBP-1 mRNA expression shown in Figures 1A and 2.

Activities of both Cu,Zn-SOD and Mn-SOD in the decidualized endometrial tissue obtained from patients given oestrogen plus progesterone were significantly higher than those in the non-decidualized endometrial tissue from patients without the hormone treatment (Table I). In contrast, lipid peroxide concentrations in the endometrial tissue obtained from patients given oestrogen plus progesterone were significantly lower than those in the control (Table I).

It has been reported that HCG promotes the decidualization of endometrial stromal cells (Tang and Gurpide, 1993; Han et al., 1996). We, therefore, examined the effect of HCG on the expression of IGFBP-1 and SOD in endometrial stromal cells in the presence or absence of MPA and oestradiol. The treatment with MPA plus oestradiol for 18 days remarkably induced the IGFBP-1 mRNA expression in the endometrial stromal cells, but the simultaneous treatment with HCG had no additional effect on IGFBP-1 mRNA expression (Figure 3A). HCG alone caused no effect on IGFBP-1 mRNA expression (Figure 3A). Both activities and mRNA levels of Cu,Zn-SOD and Mn-SOD in the endometrial stromal cell were significantly increased by MPA plus oestradiol, but HCG had no significant effect on the stimulatory effect of MPA plus oestradiol (Figure 3B and 3C). HCG alone also had no effect on the activities and mRNA levels of Cu,Zn-SOD and Mn-SOD (Figure 3B, 3C).

**Discussion**

The present study showed that SOD expression in the human endometrial stromal cell was stimulated by MPA and oestradiol. The SOD induction by MPA and oestradiol occurred concomitantly with the onset of in vitro decidualization, and SOD expression changed in a manner similar to IGFBP-1. The SOD induction accompanied by decidualization is consistent with our previous immunohistochemical observation that SOD is preferentially expressed in (pre) decidual cells (Sugino et al., 1996b). It is, therefore, suggested that SOD induction by MPA and oestradiol is closely related with decidualization. The present study also showed that the activity of Cu,Zn-SOD and Mn-SOD in the decidualized endometrium from patients treated with oestrogen and progesterone was significantly higher than that in patients without the treatment. This effect may be, at least in part, due to the SOD induction in the endometrial stromal cell by decidualization. Recent evidence has also shown that thioredoxin, which is a cellular redox-active protein and protects cells from oxidative stress, was up-regulated by decidualization in the human endometrium (Maruyama et al., 1997, 1999).

The mechanism of SOD induction by decidualization is unclear. It is well known that prolactin and its receptor are detected in the endometrial stromal cell after the late secretory phase (Kauma and Shapiro, 1986; Jabbour et al., 1998) and
produced concomitantly with the onset of in-vitro decidualization (Daly et al., 1983; Huang et al., 1987; Zhu et al., 1990). Since it has recently been found that prolactin induces both Cu,Zn-SOD and Mn-SOD expression in luteal cells (Sugino et al., 1998b), it is possible that the SOD induction in the endometrial stromal cell by decidualization may be mediated by prolactin in an autocrine or paracrine fashion. In contrast, we cannot neglect an alternative possibility that SOD itself is involved in the process of decidualization, because there is a report showing that SOD induces cell differentiation (Allen et al., 1988). Further studies are needed regarding the mechanism of SOD induction by decidualization.

We have reported that SOD plays important roles in maintaining luteal cell integrity and steroidogenic capacity (Sugino et al., 1993a,b, 1998ab, 1999; Sugino and Kato, 1994; Shimamura et al., 1995; Kato et al., 1997). The role of SOD in the human endometrial stromal cell is not clarified yet. Decidualization of endometrial stromal cells stimulates the synthesis of various hormones, cytokines and growth factors (Daly et al., 1983; Huang et al., 1987; Hatayama et al., 1994; Sakakibara et al., 1994). This increase in metabolism stimulates the generation of superoxide radicals since superoxide radicals are normally generated during normal metabolic activity. In addition, decidual cells produce a cytokine such as tumour

Figure 3 parts A, B. For legend see facing page.
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necrosis factor (Tabibzadeh, 1991; Hunt et al., 1992). Cytokines have been reported to damage cells through superoxide radical generation (Zimmerman et al., 1989). We previously suggested that accumulation of reactive oxygen species may be involved in the shedding of the endometrium by causing tissue damage (Sugino et al., 1996b). Thus, the present study may suggest that a possible role of decidualization is to induce the expression of molecules that protect the decidualized stromal cell from toxic oxygen radicals. In addition, it is of interest to note that reactive oxygen species or their product, lipid peroxide, stimulate synthesis of prostaglandin F\(_2\alpha\) (PGF\(_2\alpha\)) that causes uterine contraction (Hemler et al., 1979, 1980; Cherouny et al., 1988). In fact, the increase in PGF\(_2\alpha\) concentrations and lipid peroxide concentrations was observed in the late secretory human endometrium (Ishihara et al., 1986; Sugino et al., 1996b). Taken together, SOD in the decidualized endometrial stromal cell may contribute to the establishment and maintenance of pregnancy by preventing the increase in lipid peroxides leading to the tissue damage or the stimulation of PGF\(_2\alpha\) synthesis.

In the present study, HCG had no synergistic effect on the expression of SOD and IGFBP-1 in the endometrial stromal cell undergoing decidualization, although there are some reports that HCG promotes the decidualization of endometrial stromal cells and the function of decidualized cells (Tang and Gurpide, 1993; Han et al., 1996). Thus, the synergistic effect of HCG on the decidualization of endometrial stromal cells seems controversial. Tseng et al. (1987) reported that HCG had no effect on the aromatase activity stimulated by decidualization induced by MPA and oestradiol in human endometrial stromal cells. In addition, Ren et al. (1990) reported that HCG did not stimulate the placental proteins production in human decidualized cells. There is also a report suggesting that effects of gonadotrophin on the expression of IGFBP-1 in the human endometrial stromal cell may be influenced by the period of incubation (Moy et al., 1996).

In conclusion, the present study showed that Cu,Zn-SOD and Mn-SOD expression in human endometrial stromal cells was induced by MPA and oestradiol accompanied by decidualization, suggesting that SOD may play important roles in decidualization of endometrial stromal cells. Taken together with our previous findings that SOD was strongly expressed in the (pre) decidual cell by immunohistochemistry, and SOD activities are high and lipid peroxide concentrations are low in the decidua (Sugino et al., 1996b), SOD may play important roles in the establishment and maintenance of pregnancy.

![Figure 3](image)

**Figure 3.** Effects of human chorionic gonadotrophin (HCG) on insulin-like growth factor-binding protein-1 (IGFBP-1) (A) mRNA expression, (B) activities and (C) mRNA expression of copper-zinc superoxide dismutase (Cu,Zn-SOD) and manganese SOD (Mn-SOD) in human endometrial stromal cells in the presence or absence of medroxyprogesterone acetate (MPA) and oestradiol (E). Isolated endometrial stromal cells were incubated with HCG (1 or 10 IU/ml) for 18 days in the presence or absence of MPA + oestradiol. mRNA expression was analysed by reverse transcription–polymerase chain reaction (RT–PCR). Values are mean ± SEM of three different experiments. \(aP < 0.01\) and \(bP < 0.05\) versus control.
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References


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