Imbalance between ROS production and elimination results in apoptosis induction in primary smooth chorion trophoblast cells prepared from human fetal membrane tissues

Bo Yuan a,⁎, Kunio Ohyama a, Toshio Bessho b, Noboru Uchide a, Hiroo Toyoda a

a Department of Clinical Molecular Genetics, Faculty of Pharmacy, Tokyo University of Pharmacy & Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0355, Japan
b Yoneyama Maternity Hospital, 2-12 Shin-machi, Hachioji, Tokyo 192-0065, Japan

Received 4 August 2007; accepted 15 December 2007

Abstract

We have previously demonstrated that induction of apoptosis was observed in the smooth chorion trophoblast cells of human fetal membranes prepared at term, and that apoptosis progressed rapidly during in vitro incubation of the tissues. Furthermore, we identified the contribution of ROS production system (e.g., oxidant enzymes, such as iNOS and Cox-2) to the apoptosis induction in the chorion cells, suggesting an important role of the two inducible enzymes in the induction process. In this study, we examined the role of ROS elimination system (e.g., antioxidant enzymes, such as glutathione peroxidase (GPx) and catalase) in the apoptosis induction of the chorion cells, since the apoptosis induction by oxidative stress is a result of imbalance between production and elimination of ROS. Treatment of chorion and amnion cells with mercaptosuccinic acid (MS, GPx inhibitor) and 3-amino-1,2,4-triazole (ATZ, catalase inhibitor) resulted in an inhibition of GPx and catalase activity, respectively. Furthermore, incubation with MS alone induced apoptosis in the chorion cells and apoptosis level was enhanced by the addition of ATZ, while ATZ alone hardly induced apoptosis in the chorion cells. However, none of these reagents induced apoptosis in the amnion cells. Moreover, an increase of the level of hemeoxygenase-1 gene expression was observed only in the amnion cells when both antioxidant enzyme activities were suppressed. Therefore, we concluded that GPx played a more critical role than catalase in the control of the apoptosis induction of the chorion cells, suggesting that the threshold levels of stress tolerance in the chorion cells are much lower than those in the amnion cells.

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Keywords: Apoptosis; Fetal chorion cells; Oxidative stress; ROS; Antioxidative enzyme

Introduction

The rupture of fetal membrane (FM) is an essential part of the delivery process, accompanied by the onset of uterine contractions (Challis, 2000). Although it has been hypothesized that some biochemical events, including degradation of the connective tissue (Stegemann and Stalder, 1967), reduced elasticity (Artal et al., 1976) and decreased thickness (Helming et al., 1991) of FM, which contribute to the rupture at the normal delivery, the mechanisms involved in the FM rupture during the birth process are little known. In our previous studies, we proposed that apoptosis induction in the chorion tissues is one of the mechanisms of FM rupture associated with labor (Ohyama et al., 1998, 2001; Yuan et al., 2006). The induction of apoptosis was observed in the smooth chorion trophoblast layer of human FM prepared at the term. The extent of apoptosis induction in the trophoblast layer progressed rapidly during in vitro incubation of the tissues (Ohyama et al., 1998). We also demonstrated that the progression of apoptosis was suppressed by the presence of antioxidative reagents, suggesting that an intracellular oxidative stress played critical roles in the induction of apoptosis observed in the trophoblast layer at the stage of parturition (Ohyama et al., 2001). Furthermore, we
demonstrated that the expression levels of Bax, Bak and Bad mRNAs increased, while those of Bcl-2 mRNA decreased; the expression levels of pro-caspase-3 and -9, proform-poly (ADP-ribose) polymerase decreased; and a leakage of cytochrome c from mitochondria in the chorion tissues was observed during the in vitro incubation. These results suggested that the apoptosis induction observed in the chorion trophoblasts was induced by oxidative stress resulting in mitochondria damage (Ohyama et al., 2001; Yuan et al., 2006).

Moreover, we demonstrated that an inducible nitric oxide synthase (iNOS) inhibitor, a general and a selective cyclooxygenase (Cox)-2 inhibitors suppressed in vitro progression of the apoptosis, and that an NO donor reagent induced apoptosis in primary cultured chorion cells. These results suggested for the first time that iNOS and Cox-2, both of which are known to play a central role in reactive oxygen species (ROS) production (Payne et al., 1995), participated in the induction of chorion cells apoptosis through production of ROS (Yuan et al., 2006). Oxidative stress, as a result of an imbalance between the production and elimination of ROS within the cells (Margaret and Amanda, 1996), is known to induce apoptosis in various cell types (Payne et al., 1995). Therefore, we have been investigating on the contribution of ROS production system (e.g., oxidant enzymes like iNOS and Cox-2) to the trophoblasts apoptosis in the chorion tissues. However, the study concerning a possible contribution of ROS elimination system (e.g., antioxidant enzymes like glutathione peroxidase (GPx), catalase) to the apoptosis induction in the chorion trophoblasts has not yet performed at cellular level.

In this study, in order to reveal the relationship between ROS elimination system and the chorion trophoblasts apoptosis induction, we investigated the contribution of antioxidant enzymes, such as GPx and catalase, to the apoptosis induction in the primary cultured chorion cells, using known inhibitors for GPx, such as mercaptosuccinic acid (MS) and for catalase, such as 3-amino-1,2,4-triazole (ATZ) (Shiba and Shimamoto, 1999; Ishihara et al., 2005).

Materials and methods

Materials

MS and ATZ were purchased from Sigma Ltd. (St. Louis, MO, USA). Agarose X for DNA fragmentation analysis was purchased from Nippon Gene (Tokyo, Japan). GPx assay kit and catalase assay kit were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Preparation of primary cultured cells from human fetal membrane

Fetal membranes were prepared aseptically from placenta by Cesarean section at the month of normal parturition as described (Ohyama et al., 1998). Primary culture chorion and amnion cells were prepared from freshly prepared fetal membrane tissues according to the methods described previously (Ohyama et al., 1998). This study has been approved by the IRB committee of Tokyo University of Pharmacy and Life Sciences. An informed consent was obtained from each patient at the time of surgery.

Intracellular GPx and catalase activity assay

After treatment with MS and ATZ for a designated time, primary culture chorion and amnion cells were collected by centrifugation at 1500 g for 10 min at 4 °C, followed by homogenization of these cells with a prechilled Potter-type Teflon homogenizer in a 1 mL of cold buffer for GPx activity assay (50 mM potassium phosphate, pH7.5, containing 5 mM EDTA and 1 mM dithiothreitol); for catalase activity assay (50 mM potassium phosphate, pH7.0, containing 1 mM EDTA). Cell homogenate was centrifuged at 10,000 g for 15 min at 4 °C, and the supernatant was subjected to analysis. Protein concentrations of the samples were determined according to the Bradford’s method according to the manufacturer’s instruction (Bradford, 1976). An Enzyme activity assay was performed using GPx assay kit and catalase assay kit, respectively, from Cayman Chemical Co., Inc. (Ann Arbor, MI) in accordance with the manufacturer’s protocol.

Determination of apoptosis

DNA gel electrophoresis

Preparation and agarose gel electrophoresis of DNA were carried out according to the methods reported previously (Ohyama et al., 1998). Extracted DNA was dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA), and the DNA concentration was determined by staining with Hoechst 33258 (Sigma Ltd. St. Louis, MO, USA) as described (Cesarone et al., 1979). The DNA samples (20 μg in 20 μL) and a Read-Load™ 100 bp DNA Ladder (Invitrogen, CA, USA) as a DNA size marker were electrophoresed on a 2% agarose gel (Agarose X, Wako Pure Chemical Industries, Osaka, Japan) using TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). Gels were stained with ethidium bromide (Sigma Ltd. St. Louis, MO, USA) and then viewed under printgraph (ATTO Corp, Tokyo, Japan). Relative amount of DNA was calculated from the density of the gray level on the digitized image using NIH Image 1.60. DNA larger than 2072 bp and DNA ranging in size from 100 to 2072 bp were designated as high molecular weight DNA and fragmented DNA, respectively. The DNA fragmentation rate was calculated as the relative amount of fragmented DNA in the digitized photograph.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL) staining

Primary culture chorion and amnion cells were cultured on a collagen-coated cover glass (Iwaki, Tokyo, Japan). After treatment cells with MS and ATZ for 48 h, cells were washed with PBS (pH7.4) and fixed with 4% formaldehyde/PBS (pH7.4). The TUNEL staining against the cells was performed using ApoAlert DNA fragmentation assay kit (BD Biosciences, Palo Alto, CA, USA) according to the manufacturer’s instructions. Cells were counter-stained with propidium iodide (PI) (Sigma Ltd. St. Louis, MO, USA). After staining, the cover glass was sealed on a glass coverslip with FluorSave™ reagent.
(EMD Biosciences, Inc. La Jolla, CA, USA) and then viewed under fluorescence microscopy Axiovert 200 (Carl Zeiss, Germany). In order to quantify TUNEL-positive cells, photomicrographs were taken through FITC filter followed by viewing through PI filters. TUNEL-positive cells with yellow color were counted and averaged from three different viewing fields. The number of apoptotic cells was expressed as a percentage of the total number of cells counted under viewing fields. Routinely between 150 and 300 cells were counted per viewing field.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA and complementary DNA were prepared as described previously (Yuan et al., 2006). Total RNA was extracted from cells using RNA extraction kit, ISOGEN (Wako Pure Chemical Industries, Osaka, Japan). Complementary DNA was synthesized from 1 μg of RNA using 100 pmol random hexamers and 100 U Moloney murine leukemia virus reverse transcriptase (Invitrogen, CA, USA) in a total volume of 20 μL, according to the manufacturer’s instructions. PCR was performed according to the method described previously (Andus et al., 1993). DNA primers for RT-PCR were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA); sense primer (5’-CCA GCA ACA AAG TGC AAG ATT C-3’) and antisense primer (5’-CTG CAG GAA CTG AGG ATG CTG-3’) for hemeoxygenase-1 (HO-1) mRNA; and sense primer (5’-CCT TCC TGG GCA TGG AGT CCT G-3’) and antisense primer (5’-GGA GCA ATG ATC TTG ATC TTG-3’) for β-actin mRNA. PCR was carried out 27 cycles for HO-1 mRNA (1 min at 94 °C for denaturation, 1 min at 60 °C for annealing and 1 min at 72 °C for extension); and 21 cycles for β-actin mRNA (1 min at 94 °C for denaturation, 1 min at 55 °C for annealing and 1 min at 72 °C for extension) using a Takara Thermal Cycler MP (TAKARA SHUMO, Osaka, Japan). The specificity of the HO-1 mRNA PCR product was determined by digesting the product at a restriction site of Sph I within the HO-1 gene. Five microliters of the PCR product and Ready-Load™ 100 bp DNA Ladder (Invitrogen, CA, USA) as a DNA size marker were electrophoresed on a 2.0% agarose gel and visualized by ethidium bromide staining, and then viewed under...
printgraph (ATTO Corp, Tokyo, Japan). The relative expression levels of HO-1/β-actin gene were calculated as the ratios against that at 0-time using NIH image 1.60.

Statistics

Statistical analysis was conducted using ANOVA with a Dunnett’s post test method. A $p$ value less than 0.05 was considered significant.

Results

Effects of MS (GPx inhibitor) and ATZ (catalase inhibitor) on intracellular GPx and catalase activity in primary culture chorion and amnion cells

Primary culture chorion and amnion cells were treated with MS and ATZ at final concentrations of 14 mM and 40 mM, respectively, either for 24 or 48 h. GPx activity observed in untreated chorion cells (ca. 1200 units/mg of protein) was similar to that in untreated amnion cells (Fig. 1A and B). In the chorion cells, GPx activity after 24-h incubation with MS alone or in combination with ATZ decreased to 700 units/mg of protein, and the activity was not changed after 48-h incubation (Fig. 1A). In the amnion cells, GPx activity decreased slightly after the treatment with MS alone or in combination with ATZ for 24- or 48-h incubation (Fig. 1B). The combinational effect of MS and ATZ on GPx activity was not observed either in chorion or amnion cells.

Catalase activity (more than 700 units/mg of protein) in untreated chorion cells was higher than that (about 400 units/mg of protein) in untreated amnion cells (Fig. 1C and D). In these cells, the enzyme activities decreased to 200 units/mg of protein after the treatment with ATZ alone or in combination with MS for 24 h, the activity at which was not changed after 48-h incubation (Fig. 1C and D). No combinational effects of MS with ATZ on the enzyme activity were observed in either chorion or amnion cells.

Apoptosis induction in smooth chorion cells by MS and ATZ

We investigated the effects of antioxidative enzyme inhibitors on the apoptosis induction in the chorion and amnion cells. DNA fragmentation was observed in the chorion cells after the treatment with MS alone for 48 h accompanied with the increase in the number of TUNEL-positive cells (28.2±3.3%) (Fig. 2, Table 1). No significant increase in the number of TUNEL-positive cells treated with ATZ alone for 48 h was
observed as compared with those in the control (control: 9.0±3.4% vs. 40 mM ATZ: 20.9±7.1, p<0.05) (Fig. 2, Table 1). Moreover, the level of TUNEL-positive cells in the presence of MS was enhanced from 28.2±3.3% to 35.1±8.2% by the addition of ATZ (Fig. 2, Table 1). A similar combinational effect of two inhibitors on the extent of DNA fragmentation was observed in the chorion cells (Fig. 3). However, none of these reagents induced apoptosis in the amnion cells (Figs. 2 and 3, Table 1). DNA fragmentation was not detected in the chorion and amnion cells after 24-h treatment with MS and ATZ (data not shown).

The expression level of HO-1 gene in primary culture chorion and amnion cells treated with ATZ and MS

After the treatment of chorion and amnion cells with MS and ATZ at final concentrations of 14 mM and 40 mM, respectively, we analyzed the expression profile of HO-1 mRNA by RT-PCR. After 6-h treatment of the chorion cells with MS alone or a combination of MS with ATZ, a slight increase, but not statistically significant, in the expression level of HO-1 gene was observed (Fig. 4A). The expression level of HO-1 gene did not vary in the amnion cells after the treatment with MS alone (Fig. 4B). However, after 6-h treatment with the combination of MS with ATZ, the level of HO-1 gene expression increased approximately 5-fold in the amnion cells and the increase continued up to 12 h (Fig. 4B). No changes of HO-1

Table 1
Quantitative analysis of TUNEL-positive chorion and amnion cells treated with MS and ATZ

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Treatment</th>
<th>% of TUNEL-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Individual values</td>
<td>Means±SD</td>
</tr>
<tr>
<td>Chorion cells</td>
<td>Control</td>
<td>12.6 5.8 8.5 9.0±3.4</td>
</tr>
<tr>
<td></td>
<td>ATZ 40 (mM)</td>
<td>16.7 16.9 29.0 20.9±7.1</td>
</tr>
<tr>
<td></td>
<td>MS 14 (mM)</td>
<td>31.5 25.0 28.0 28.2±3.3</td>
</tr>
<tr>
<td></td>
<td>ATZ 40 (mM) + MS 14 (mM)</td>
<td>37.1 42.2 26.1 35.1±8.2</td>
</tr>
<tr>
<td>Amnion cells</td>
<td>Control</td>
<td>0.0 0.0 0.0 0.0±0.0</td>
</tr>
<tr>
<td></td>
<td>ATZ 40 (mM)</td>
<td>0.2 1.2 0.5 0.6±0.5</td>
</tr>
<tr>
<td></td>
<td>MS 14 (mM)</td>
<td>0.3 0.5 1.3 0.7±0.5</td>
</tr>
<tr>
<td></td>
<td>ATZ 40 (mM) + MS 14 (mM)</td>
<td>0.4 0.9 1.1 0.8±0.3</td>
</tr>
</tbody>
</table>

†, p<0.05; ††, p<0.01.

To quantify apoptotic cells, the total number of cells and TUNEL-positive cells was counted and averaged from three different viewing fields. The number of apoptotic cells was expressed as a percentage of the total number of cells counted within viewing fields. Routinely between 150 and 300 cells were counted per viewing filed. Data are shown as the means and SD from three independent experiments. Statistical analysis was conducted using ANOVA with a Dunnett’s post test. Significant differences in the number of apoptotic cells of chorion cells between control and treatment with ATZ and MS are shown.

Fig. 3. DNA fragmentation analysis of primary culture chorion and amnion cells treated with MS and ATZ. Panels show DNA electrophoresis pattern of chorion (A) and amnion (B) cells incubated with MS (14 mM), ATZ (40 mM), or both for 48 h respectively. Panel (C) shows the extent of DNA fragmentation estimated from the agarose gel electrophoresis pattern of extracted DNA from chorion cells. Data are shown as the means and SD from three independent experiments. Statistical analysis was conducted using ANOVA with a Dunnett’s post test. Significant differences between control and treatment with MS and ATZ are shown (#, p<0.01). Sizes of DNA markers in lane M are shown as base pairs.
gene expression levels were observed in either chorion or amnion cells after the treatment with ATZ alone (Fig. 4A and B).

Discussion

We have previously demonstrated that the apoptosis induction of trophoblasts in smooth chorion tissues of human fetal membranes (FM) was mediated through the intracellular oxidative stress, resulting in mitochondria damage at the term (Ohyama et al., 1998, 2001; Yuan et al., 2006). We have also demonstrated that an inducible nitric oxide synthase (iNOS) inhibitor, a general and a selective cyclooxygenase (Cox)-2 inhibitors suppressed in vitro progression of the trophoblasts apoptosis; and an NO donor reagent induced apoptosis in primary culture trophoblast cells (Yuan et al., 2006). These two inducible enzymes are known to play a central role in reactive oxygen species (ROS) production (Payne et al., 1995). We demonstrated for the first time that ROS produced by iNOS and Cox-2 played a critical role in the induction of chorion tissue trophoblast apoptosis (Yuan et al., 2006). All of these studies suggest that ROS production system is a key pathway in controlling the apoptosis induction in the chorion cells. Our previous results clearly demonstrated the contribution of ROS production cascade to the apoptosis induction, but the role in ROS elimination cascade to the apoptosis induction is unknown.

In this study, we demonstrated that inhibition of GPx or catalase activity by respective inhibitors resulted in the apoptosis induction in the chorion cells, but not in the amnion cells. GPx and catalase are important components as the primary antioxidants for ROS elimination before they damage target cells (Margaret and Amanda, 1996). It has been reported that endogenous oxidative stress, caused by the inhibition of GPx and catalase by MS and ATZ, respectively, induced apoptosis in primary culture of rat hepatocytes (Shiba and Shimamoto, 1999). Our previous results demonstrated the involvement of activation of caspase-3 and -9 in the apoptosis induction in the smooth chorion trophoblast cells of FM tissues (Yuan et al., 2006), which is different from those previously observed in rat hepatocytes (Ishihara et al., 2005). This discrepancy may be explained by the difference of cell types and the experimental conditions. It has also been reported that the apoptosis, induced by H2O2 in primary mature rat oligodendrocytes, was potentiated by inhibiting GPx and catalase by MS and ATZ, respectively (Baud et al., 2004). Furthermore, results suggested...
that GPx–catalase cooperativity was required for resistance to H$_2$O$_2$ by the oligodendrocytes (Baud et al., 2004). In the present study, we demonstrated that inhibition of GPxs by MS resulted in the apoptosis induction in the chorion cells and the extent of apoptosis induction was enhanced by the inhibition of catalase by ATZ. Although the details of the functional relationship between GPx and catalase in the apoptosis induction is not yet clear, this study provides for the first evidence, to our knowledge, that the status of the activities of GPx and catalase in the chorion cells play a pivotal role in controlling the apoptosis induction.

Physiological significance of an imbalance of ROS production and elimination has been demonstrated during pregnancy. It has been demonstrated that a high metabolic demand during pregnancy results in an increased production of ROS (Spatling et al., 1992; Halliwell and Gutteridge, 1990). Furthermore, an increased rate of ROS production was observed during embryo development as a result of elevated oxygen requirements (Goto et al., 1992; Halliwell and Gutteridge, 1990). Additionally, an increase in the production of ROS has been demonstrated in pregnant rats (Mover-Lev and Ar, 1997). It has been demonstrated that the levels of plasma thiol and red blood cell superoxide dismutase in pregnant women were significantly lower than those in non-pregnant women (Wisdom et al., 1991). Moreover, the concentrations of plasma tocopherol were significantly lower in pregnant women compared to those in non-pregnant women (Ishihara et al., 2004), suggesting that oxidative stress in pregnant women is a result of imbalance between the production and elimination rate of ROS that leads to the accumulation of ROS within the cells of FM.

In addition to ROS, Fas/FasL system (Runic et al., 1996, 1998) was also indicated to be involved in the apoptosis induction in the FM. However, the conventional cell death signal-transducing pathway was not observed in our experiment (Ohyama et al., 2001). Moreover, the fact that inflammatory cytokines (interleukin-$\alpha$ and tumor necrosis factor-$\alpha$) (Menon et al., 2002; Fortunato and Menon, 2003) were observed in the apoptosis induction in the FM, was supported by our data (Ohyama et al., 2001; Yuan et al., 2006). Based on these evidences, we suggested that apoptosis induction in the chorion tissues causes spontaneous FM disruption prior to parturition.

It should be noted that although the GPx and catalase activities were suppressed in the amnion cells, the apoptosis was not observed in the cells, suggesting that a compensatory antioxidant system may be activated to maintain ROS elimination potential. In this regard, it should be noted that an increase of HO-1 gene expression was only observed in the amnion cells when the cells were treated in combination with MS and ATZ. A recent study demonstrated that up-regulated HO-1 expression following stimulation with the pro-oxidant, 15-HPETE (15-hydroperoxyeicosatetraenoic acid), in that the levels of this antioxidant inversely correlated with apoptosis induced by 15-HPETE in primary bovine aortic endothelial cells (Trigona et al., 2006). HO-1 is a rate-limiting enzyme that can degrade heme into equimolar quantities of carbon monoxide, free iron and biliverdin, which is subsequently converted to bilirubin by the enzyme biliverdin reductase (Tenhunen et al., 1968, 1969). Furthermore, accumulating evidence indicates that biliverdin/bilirubin can mediate the protective effects of induced HO-1 in many disease models, such as ischemia/reperfusion injury and organ transplantation, via its antioxidant properties, anti-apoptotic and anti-inflammatory (Ollinger et al., 2007). Taking these previous results and our observations into account, we hypothesize that a threshold level to oxidative stress in the amnion cells is much higher than that in the chorion cells. We also suggest that when the levels of oxidative stress exceed threshold levels for the amnion cells, HO-1 gene expression is quickly induced and compensates for the dysfunction of antioxidant system resulting in the protection for the cells. In fact, we previously reported that pre-stimulation of amnion cells with hemin, a HO-1 inducer, significantly attenuated influenza virus-HA induced apoptosis (Ohyama et al., 2003).

Conclusions

We proposed that inability of ROS elimination system as a result of reducing antioxidant enzyme activities induced apoptosis only in the chorion trophoblast layer when exposed to oxidative stress. Our results suggest that the threshold levels of stress tolerance in the amnion cells are much higher than those in the chorion cells, which results in more vulnerable to apoptosis induction in the chorion cells by oxidative stress.

Acknowledgments

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology and by the Promotion and Mutual Aid Corporation for Private Schools of Japan.

References


