Generation of peroxynitrite and apoptosis in placenta of patients with chorioamnionitis: possible implications in placental abruption

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The reaction of nitric oxide (NO) and superoxide results in the formation of peroxynitrite, a potent and relatively long-lived oxidant. In infectious diseases, these molecules are not only bactericidal but also toxic to host cells. Chorioamnionitis is often complicated by premature rupture of membranes and can be associated with placental abruption. These diseases are significant causes of premature low-birth-weight deliveries and consequently the morbidity and mortality of neonates. Lipopolysaccharide, bacterial endotoxin, is known to be elevated in the amniotic fluid of patients with chorioamnionitis. Lipopolysaccharide is known to induce the formation of NO and superoxide. We report here that nitrite/nitrate, stable metabolites of NO, were increased in serum from patients with chorioamnionitis. Immunohistochemical studies demonstrated enhanced expression of inducible NO synthase and formation of nitrotyrosine, a footprint of peroxynitrite, in the placentae from patients with chorioamnionitis and also in patients with placental abruption. Furthermore, apoptotic cell death was also increased in the placentae from patients with both diseases. These results suggest that chorioamnionitis and a portion of placental abruption may share a common cascade of placental injury. Nitric oxide and its metabolites may play an important role in this cascade.

Key words: apoptosis/chorioamnionitis/interleukin-1β-converting enzyme (ICE)/peroxynitrite/placental abruption

Introduction

Nitric oxide (NO) has been reported to be a bioactive molecule regulating vascular circulation, platelet aggregation, neurotransmission, and immunological function (Moncada et al., 1991). Although NO plays many physiological roles in various organs, it is also known to have harmful effects on cells under pathological conditions, including ischaemia/reperfusion, inflammatory diseases, and autoimmune diseases (Beckman et al., 1996). In these diseases NO and superoxide are generated simultaneously. The reaction of these molecules results in formation of peroxynitrite, which is a potent and relatively long-lived oxidant (Beckman, 1996). In infectious diseases, these molecules are not only bactericidal but also toxic to host cells.

Chorioamnionitis is an infectious disease in the perinatal feto–maternal unit. It is often complicated with premature rupture of membranes and can be a cause of placental abruption (Cunningham et al., 1993). It is reported that 6.3% of the patients with preterm prematurely ruptured membranes develop placental abruption (Cunningham et al., 1993). These diseases lead to premature low-birth-weight deliveries and consequently morbidity and mortality of neonates. Furthermore, preterm infants whose delivery was accompanied by chorioamnionitis are reported to have a higher rate of developmental problems as compared with preterm infants by other causes (Morales, 1987). Approximately one in four mothers delivered prematurely will have positive amniotic fluid cultures (Skoll et al., 1989). Bacterial infection causes uterine contraction, fragility of membranes, and consequent preterm labour and sometimes placental abruption.

Lipopolysaccharide (LPS), an endotoxin found in Gram-negative bacteria, is elevated in the amniotic fluid during chorioamnionitis (Romeo et al., 1987) and is known to induce various cytotoxic reactions (Bradley, 1979). Lipopolysaccharide is a well-known inducer of NO and superoxide in various cells and organs and induces the formation of peroxynitrite (West et al., 1994; Wizemann et al., 1994; Beckman, 1996). Although the formation of these molecules may cause damage to placental tissue, their involvement in this inflammatory disease is not fully elucidated.

In the present study, we investigated the localization of NO synthases (NOSs) and the formation of peroxynitrite in placentae from patients with chorioamnionitis and also from patients with placental abruption. Because it has been reported that both NO and peroxynitrite are known to cause apoptosis in various cells (Sarih et al., 1993; Ankarcrona et al., 1994; Kitajima et al., 1994; Blanco et al., 1995; Lin et al., 1995; Sato et al., 1995), we also examined apoptosis occurring in placentae during chorioamnionitis and placental abruption.

Chorioamnionitis, pre-eclamptic change, or even subclinical infection or ischaemia in the placenta may induce hypoxia and production of various bioactive mediators, including cytokines, eicosanoids and reactive oxygen species. In a portion of these patients, overproduced NO, superoxide, and peroxynitrite may play an important role in placental cell death and subsequent placental abruption. From these viewpoints, we discuss the similarities in the mechanism of
placental damage between chorioamnionitis and placental abruption, which are clinically complicated with each other.

Materials and methods

Sample collection
Informed consents were obtained from all patients involved in this study. The protocol of this study was approved by the local institutional review board. Blood samples were taken from the peripheral vein of normal pregnant women \( n = 30 \) and patients with chorioamnionitis \( n = 16 \). To minimize the influence of nitrate in food, samples were collected in the morning after overnight fasting. The serum fraction was obtained by centrifugation and stored at \(-70^\circ\text{C}\) until analysis.

Placentae were obtained from the patients of chorioamnionitis \( n = 10 \) and placental abruption \( n = 6 \). Placentae were also obtained from normal pregnant women at term \( n = 10 \) and from patients with preterm delivery due to maternal heart disease or placenta praevia \( n = 8 \). Immediately after vaginal delivery or Caesarean section, the placenta was incised at 2–3 cm from the margin and specimens were collected. In cases of placental abruption, specimens were collected from the separated part. These samples were stored at \(-70^\circ\text{C}\), or fixed in 10% buffered formalin and then embedded in paraffin. Sections of 4 \( \mu \text{m} \) were prepared for further examinations. Chorioamnionitis was diagnosed by clinical features such as fever, elevation of c-reactive protein in serum, and/or leukocytosis in peripheral venous blood. It was also confirmed by histological examination (Blanc, 1981) after haematoxylin–eosin staining. Placental abruption was diagnosed by clinical features such as uterine tenderness, fetal distress, coagulopathy, and confirmed by existence of a retroplacental haematoma.

Portions of placenta were also removed, washed with ice-cold physiological saline, and cut into 1–2 mm pieces and homogenized in ice-cold homogenization buffer (10 mM HEPES, pH 7.5, containing 320 mM sucrose, 100 \( \mu \text{M} \) EDTA, 1.5 mM dithiothreitol, 10 \( \mu \text{g} / \text{ml} \) trypsin inhibitor, 10 \( \mu \text{g} / \text{ml} \) leupeptin, 2 \( \mu \text{g} / \text{ml} \) aprotinin, 1 \( \mu \text{g} / \text{ml} \) phenylmethanesulphonyl fluoride) (Knowles et al., 1990) with the use of a Teflon homogenizer. Homogenates were stored at \(-70^\circ\text{C}\) for later Western blotting. Protein concentrations of these samples were determined by the method of Bradford (Bio-Rad, Osaka, Japan) with the use of bovine serum albumin as a standard.

Measurement of nitrite/nitrate in serum
The serum was deproteinized by a filter (Ultrafree-MC 10 000 NMWL Filter Unit, Millipore, Bedford, MA, USA), which was thoroughly washed by ultrapure water (Milli-Q water purification system, Millipore) in advance. Nitrate in the sample was reduced to nitrite by nitrate reductase prepared from Escherichia coli (Boehringer Mannheim GmbH, Mannheim, Germany) in 20 mM Tris–HCl buffer containing 40 \( \mu \text{M} \) NADPH (Sigma Chemicals, St Louis, MO, USA) as described previously (Misko et al., 1993). The Griess reagents were used to determine the nitrite concentration (Green et al., 1982). Sodium nitrate was also used as the standard to ensure the efficacy of the nitrate reductase. These reactions were performed in a 96-well microplate and optical density at 540 nm was measured by Microplate reader (Model 3550, Bio-Rad).

Immunohistochemistry
Immunohistochemical detection was performed using DAKO LSAB kit and DAB chromogen (DAKO Corporation, Carpinteria, CA, USA) following the manufacturer’s instructions. To determine the type of NO synthase (NOS) in placenta, polyclonal antibodies to endothelial NOS (eNOS), or inducible NOS (iNOS) purchased from Affinity Bioreagents (Golden, CO, USA) were used as primary antibodies. These antibodies were raised against a synthetic peptide of a sequence derived from bovine eNOS or mouse macrophage iNOS by immunization of rabbits. The concentrations used were 1:250 and 1:200 respectively. We prepared heart and large vascular vessels from normal rats, and liver from LPS-treated rats as positive control slides for eNOS and iNOS respectively. As a negative control, preimmune rabbit serum was used instead of the primary antibodies to NOS.

To determine the formation of peroxynitrite, nitrotyrosine was detected by immunohistochemical techniques (Beckman, 1996). A rabbit polyclonal antibody raised against nitrated KLH (Upstate Biotechnology Incorporated, Lake Placid, NY, USA) was used as a primary antibody with 1:500 dilution. As a positive control slide, we used the liver from LPS-treated rats.

Expression of interleukin-1\( \beta \)-converting enzyme (ICE), a cysteine protease involved in the cascade of apoptosis (Yuan et al., 1993), was also examined in placenta by a specific polyclonal antibody by immunization of rabbit with a synthetic peptide of a sequence derived from human ICE (Upstate Biotechnology) as a primary antibody with 1:250 dilution.

Western blot analysis of inducible NOS
Western blot analysis was performed as described previously (Nakatsuka et al., 1998) with a slight modification. The placental homogenate (80 \( \mu \text{g} \) of protein) was analysed with the use of SDS–polyacrylamide gel electrophoresis (7% gel). The gels were blotted onto a nitrocellulose membrane, blocked with 0.2 mg/ml thimerosal in blocking buffer and probed (1:1000) with a rabbit polyclonal antibody against inducible NOS (Affinity Bioreagents). An anti-rabbit IgG antibody (1:5000) conjugated to peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) was used as a secondary antibody. An ECL reagent (Amersham International, Buckinghamshire, UK) and X-OMAT Blue film (NEN Life Science Products, Boston, MA, USA) was used to detect the peroxidase conjugate as described by the manufacturer. Intensity of the bands was evaluated by a laser densitometer (Scanning Imager 300XS, Molecular Dynamics, Sunnyvale, CA, USA). Differing amounts of homogenate prepared from placenta with chorioamnionitis were analysed to ensure that density was linearly dependent on the amount of NOS over the relevant concentration range.

Detection of apoptotic cell death
Apoptosis in placental tissues was detected by in-situ nick end labelling technique using ApopTag Plus kit (Oncor, Gaithersburg, MD, USA) following the manufacturer’s instructions. Methyl green dye was used for counterstaining.
Apoptosis in placenta of chorioamnionitis

Figure 2. Immunohistochemical staining for inducible NOS protein in placentae. (A) Control placenta from a patient with placenta praevia (30 weeks gestation). (B) Placenta from a patient with chorioamnionitis (28 weeks gestation). (C) Placenta from a patient with placental abruption (29 weeks gestation). (D) Negative control: placenta from a patient with chorioamnionitis (28 weeks gestation). Brown staining by DAB chromagen in cytoplasm demonstrated immunoreactivity. Mayer’s haematoxylin was used for counterstaining. Scale bar is 50 µm.

Figure 3. Immunohistochemical staining for endothelial NOS protein in placentae. (A) Control placenta from a patient with placenta praevia (30 weeks gestation). (B) Placenta from a patient with chorioamnionitis (28 weeks gestation). (C) Placenta from a patient with placental abruption (29 weeks gestation). (D) Negative control: placenta from a patient with chorioamnionitis (28 weeks gestation). Brown staining by DAB chromagen in cytoplasm demonstrated immunoreactivity. Mayer’s haematoxylin was used for counterstaining. Scale bar is 50 µm.

Figure 4. Immunohistochemical staining for nitrotyrosine in placentae. Nitrination of tyrosine residues in protein are specific footprints of peroxynitrite. (A) Control placenta from a patient with placenta praevia (30 weeks gestation). (B) Placenta from a patient with chorioamnionitis (28 weeks gestation). (C) Placenta from a patient with placental abruption (29 weeks gestation). (D) Negative control: placenta from a patient with chorioamnionitis (28 weeks gestation). Brown staining by DAB chromagen in cytoplasm demonstrated immunoreactivity. Mayer’s haematoxylin was used for counterstaining. Scale bar is 50 µm.

Statistics
Statistical significance was determined by two-factor factorial analysis of variance (ANOVA) or Welch’s t-test. Data are presented as mean ± SD and a P value < 0.05 was considered statistically significant.

Results

Clinical features of patients with chorioamnionitis
The age of the patients with chorioamnionitis was 27.9 ± 4.6 years (mean ± SD). The gestational age of delivery was 28.9 ± 3.0 weeks and ranged from 24 to 34 weeks. Premature rupture of membranes was detected in 81.3% of the patients. The mean highest concentration of c-reactive protein in serum and the mean highest count of white blood cells in peripheral blood before delivery were 4.6 ± 3.8 mg/ml and 16013 ± 5385 cells/µl respectively. Positive vaginal cultures were obtained from all patients. In the culture of vaginal discharge 1.8 ± 1.1 species of bacteria were detected. The histological investigation revealed all patients had chorioamnionitis stage II or III (Blanc, 1981).

Nitrite/nitrate concentration in serum
Serum nitrite/nitrate in normal pregnant women during 24–27 (n = 10), 28–31 (n = 10), and 32–35 weeks (n = 10) were 48.6 ± 25.8, 58.2 ± 23.9, and 37.6 ± 24.8 (mean ± SD) µmol/l respectively (Figure 1). There may be a tendency for concentrations of nitrite/nitrate in serum to vary depending on
Figure 5. Levels of immunodetectable iNOS in homogenate from placenta. The values represent the mean ± SD. The bands demonstrated are the representatives of immunoblot analysis of six control placentae and of eight placentae with chorioamnionitis. (A) 1–4: control, (B) 1–4: chorioamnionitis. Dense 130 kDa bands corresponding to iNOS protein were detected in samples from patients with chorioamnionitis (B 1–4), while fine bands were detected in samples from control patients (A 1–4).

Figure 6. Apoptotic cells in placentae were detected by in-situ nick end labelling technique. (A) Control placenta from a patient with placenta praevia (30 weeks gestation). (B) Placenta from a patient with chorioamnionitis (28 weeks gestation). (C) Placenta from a patient with placental abruption (29 weeks gestation). Brown staining by DAB chromagen in cytoplasm demonstrated immunoreactivity. Mayer’s haematoxylin was used for counterstaining. Scale bar is 50 µm.

gestational weeks. Serum nitrite/nitrate in pregnant women with chorioamnionitis during 24–27 (n = 5), 28–31 (n = 7), and 32–35 weeks (n = 4) were 82.5 ± 24.9, 84.0 ± 20.1, and 84.7 ± 23.0 (mean ± SD) µmol/l respectively. It is significantly higher in patients with chorioamnionitis as compared with normal pregnant women (P < 0.0001, two-factor factorial ANOVA).

Localization of nitric oxide synthases and nitrotyrosine in placenta

The immunoreactivities to iNOS were obviously increased in syncytiotrophoblasts and Hofbauer cells in placentae of chorioamnionitis or placental abruption as compared with those of normal pregnancy (Figure 2). The immunoreactivities to eNOS were detected in syncytiotrophoblasts of control placentae (Figure 3). No significant difference in intensity of staining was observed among the placentae of normal pregnancy, chorioamnionitis and placental abruption (Figure 3). Intensive immunoreactivities to nitrotyrosine were detected in syncytiotrophoblasts and Hofbauer cells in placentae of chorioamnionitis or placental abruption while little immunoreactivity was detected in those of normal pregnancy (Figure 4).

Inducible NOS protein in placenta

Western blot analysis revealed that iNOS existed in placenta of normal pregnant women (Figure 5). However, the levels of immunodetectable iNOS protein in the homogenate of placentae from patients with chorioamnionitis were higher, ~3.2-fold of those from normal pregnant women. This difference was significant (Welch’s t-test, P < 0.01).

Apoptosis in placenta

Although apoptotic cells were detected in the placentae of normal pregnancy, they were increased significantly in the placentae from the patients of chorioamnionitis or placental abruption (Figure 6). Apoptosis was localized mainly in syncytiotrophoblasts and Hofbauer cells. Interleukin-1β-converting enzyme, one of the molecules involved in the apoptotic cascade (Yuan et al., 1993), was also detected in syncytiotrophoblasts of control placentae. However, more intense expression was detected immunohistochemically in placentae from patients with chorioamnionitis or placental abruption (Figure 7).
Discussion
Nitrite/nitrate concentrations in serum, which represented production of NO, were higher in patients with chorioamnionitis than in normal pregnant women. There is a similar report that nitrite/nitrate in plasma is increased in the patients of preterm labour or premature rupture of membranes than in normal pregnant women (Jaeckle et al., 1994). In their report, the authors postulated that the source of this elevation of nitrite/nitrate was the synthesis of NO by macrophages and polymorphonuclear leukocytes activated by bacterial infection. However, it is known that LPS can induce expression of iNOS and production of NO in endothelial cells, smooth muscle cells, and hepatocytes. Furthermore, our study indicates that trophoblasts and Hofbauer cells can contribute to the elevation of NO found in patients with chorioamnionitis.

Endothelial NOS has been reported to exist in human placenta (Myatt et al., 1993, 1997a; Garvey et al., 1994; Eis et al., 1995; Zarlingo et al., 1997). We also detected the immunoreactivities of eNOS in syncytiotrophoblasts of the placentae from normal pregnant women. Neuronal NOS has been also reported to be expressed in normal placenta (Thompson et al., 1997). This suggests that NOS is expressed constitutively and NO plays a bioregulatory role in placenta under physiological conditions. Trophoblast-derived NO may prevent both platelet and leukocyte adhesion to the syncytiotrophoblast surface in the intervillus space and maintain microcirculation. It may also affect the arachidonic acid metabolism (Nakatsuka and Osawa, 1994) and P450 cytochromes (Osawa, 1995) and may regulate placental functions.

We detected iNOS in the placentae of control preterm deliveries by immunoblot analysis. Furthermore, iNOS protein was significantly induced in the placentae from patients with chorioamnionitis. We observed that iNOS protein was localized in syncytiotrophoblasts and Hofbauer cells in the placentae from patients suffering from chorioamnionitis or placental abruption while little was detected in normal placentae. The localization of iNOS in human placenta was reported previously (Schonfelder et al., 1996; Myatt et al., 1997b; Zarlingo et al., 1997). These reports suggest that in normal placenta iNOS is absent (Garvey et al., 1994; Myatt et al., 1997b) or expressed in a low degree (Schonfelder et al., 1996; Zarlingo et al., 1997). Hofbauer cells in villous stroma are reported to express iNOS (Schonfelder et al., 1996; Zarlingo et al., 1997) while syncytiotrophoblasts express this type of NOS in placenta from some normal pregnant women (Myatt et al., 1997b) or from the patients with gestational diabetes (Schonfelder et al., 1996).

We also demonstrated that the induction of iNOS was accompanied by the generation of peroxynitrite, expression of ICE, and apoptotic cell death in placentae from these patients. It has been reported that the concentrations of LPS (Romeo et al., 1987), interleukin-1β (IL-1β) (Romeo et al., 1989a), and tumour necrosis factor-α (TNF-α) (Romeo et al., 1989b) in amniotic fluid were higher in chorioamnionitis as compared with normal pregnancy. These molecules are known to induce iNOS (Moncada et al., 1991).

It is possible that constitutively expressed iNOS plays a beneficial role in the placenta by releasing low amounts of NO. However, it is likely that in chorioamnionitis iNOS may cause marked increase of NO and may result in placental dysfunction. Furthermore, we observed that the formation of peroxynitrite, a potent oxidant, was enhanced in the placentae of chorioamnionitis or placental abruption. It has been reported that peroxynitrite causes protein modification, lipid peroxidation, and DNA damage (Beckman, 1996). One of the characteristic features of cell damage by peroxynitrite is known to be apoptosis (Lin et al., 1995).

We detected apoptotic cells in the placentae of normal pregnancy and this is consistent with previous reports (Nelson, 1996; Smith et al., 1997). However, a larger number of apoptotic cells was observed in placenta from patients of chorioamnionitis or placental abruption. Apoptosis was localized mainly in syncytiotrophoblasts and Hofbauer cells, in which increased formation of peroxynitrite is detected.

Interleukin-1β converting enzyme which cleaves proIL-1β and makes it active IL-1β (Thornberry, 1992). This molecule is also called caspase-1 and is one of the members of the apoptotic cascade. It exists in syncytiotrophoblasts of placentae from normal pregnant women. However, immunoreactivities of ICE were increased in trophoblasts and Hofbauer cells of placentae from the patients of chorioamnionitis or placental abruption. These results suggest that ICE may be involved in this trophoblastic apoptosis.

Chorioamnionitis is known to be a significant cause of placental abruption. In the present report, placentae of placental abruption demonstrated similarities to those of chorioamnionitis at least from the viewpoints of localization of NOS, induction of peroxynitrite, apoptosis and expression of ICE. Our results suggest that a common cascade of cell death and tissue injury might exist in both chorioamnionitis and placental abruption. Because various molecules can enhance formation of NO and peroxynitrite, the initial trigger of this cell death cascade may be multifarious. Chorioamnionitis, pre-eclamptic change, or even subclinical infection or ischaemia in the placenta may induce production of various bioactive mediators like cytokines, eicosanoids, reactive oxygen species. Some of these molecules are known to enhance formation of NO and peroxynitrite. Overproduced NO or peroxynitrite may cause placental cell death and sequential placental abruption. Because NO can activate matrix metalloproteinases (Murrell et al., 1995), these proteolytic enzymes may also be involved in this cascade. Further investigation is in progress.

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