NAD(P)H oxidase in human fetal membrane chorion laeve trophoblasts with or without chorioamnionitis: ultrastructural enzyme histochemical study

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We examined the subcellular localizations of NAD(P)H oxidase, a reactive oxygen species (ROS)-producing enzyme, in fetal membrane chorion laeve trophoblasts from preterm or term pregnant women with or without chorioamnionitis (CAM). Ultrastructural enzyme histochemistry for NAD(P)H oxidase was used. In fetal membranes without CAM, approximately one quarter of the chorion laeve trophoblasts (25.6%) showed NAD(P)H oxidase activity on their surface plasma membranes and microvillous membranes. In mild CAM, the proportion of these NAD(P)H oxidase-positive cells significantly increased, reaching about half (51.0%). Enzyme activity appeared on the plasma and microvillous membranes and also on both phagosomal membranes and intracellular vesico-tubular structures. Appearance of NAD(P)H oxidase on surface plasma membranes, phagosomal membranes, and vesico-tubular structures is strong cytochemical evidence of phagocytic cell activation. These observations indicate that chorion laeve trophoblasts possess NAD(P)H oxidase activity, and therefore that fetal membranes themselves have ROS-generating capacity. Further, in fetal membrane inflammation, chorion laeve trophoblasts exhibited enzyme distribution characteristic of activated professional phagocytes. Similar to phagocytes infiltrating to the intrauterine environment, chorion laeve trophoblast NAD(P)H oxidase may play a role both in the defence of chorioamnion against infection and in the pathogenesis or pathophysiology of CAM-related preterm delivery.

Key words: chorioamnionitis/chorion laeve/NAD(P)H oxidase/phagocytosis/trophoblast

Introduction

Chorioamnionitis (CAM) is thought to be one of the leading causes of preterm delivery (Cunningham et al., 1997). In CAM, professional phagocytes [polymorphonuclear leukocytes (PMN) and macrophages] migrate from the maternal circulation to the fetal membranes where they phagocytose and digest bacteria and other undesirable substances, thus protecting the mother (host) from bacterial infection (Benirschke and Kaufmann, 2000). They also injure the host, however, by releasing a variety of cytotoxic substances including reactive oxygen species (ROS), powerful cytotoxic and thus tissue-destroying agents, in and around the fetal membranes (Weinberg and Athens, 1993). Activated or phagocytosing phagocytes and their discharged cytotoxic substances including ROS may therefore play significant roles both in the maintenance of normal pregnancy and the pathophysiology of CAM. It is not yet clear, however, whether cells constituting the fetal membranes themselves possess ROS-generating capacity. Nor is it known whether this ROS generation, if present in fetal membranes, has biological significance under normal and pathological (CAM) conditions.

NAD(P)H oxidase, primarily belonging to the professional phagocytes, is an important ROS-generating enzyme. This enzyme, using NADH or NADPH as a substrate, produces a superoxide, which is rapidly converted to hydrogen peroxide, both of which are powerful ROS (Robinson and Badwey, 1995). We previously demonstrated that this enzyme is also present in term placental syncytiotrophoblasts, indicating that the presence of NAD(P)H oxidase is not exclusively confined to professional phagocytic cells (Matsubara and Tamada, 1991). Later, biochemical analysis indicated that NAD(P)H oxidase is also present in vascular endothelial cells and smooth muscle cells in various experimental animals and humans. This is known as cardiovascular NAD(P)H oxidase (Griendling et al., 1994, 2000). Attempts have never been made, however, to cytochemically localize this enzyme in cytotrophoblasts in fetal membranes, namely in chorion laeve trophoblasts.

Fetal membranes, lying between the maternal decidua–uterus and the fetal amniotic cavity, may act as a barrier against the entrance of various noxious substances, including bacteria from the maternal to the fetal side. Concerning this, we have recently shown that chorion laeve trophoblasts in
fetal membranes with CAM exhibit structural changes characteristic of stimulated or phagocytosing professional phagocytes (Matsubara et al., 2000). Chorion laeve trophoblasts were therefore considered to be capable of undergoing phagocytosis, as non-professional phagocytic cells (Matsubara et al., 2000). We have concluded that activated or phagocytosing chorion laeve trophoblasts (non-professional phagocytic cells of fetal origin) may play important roles in both the defence against, and progression of, infection-related preterm delivery (Matsubara et al., 2000), similar to activated PMN and macrophages infiltrating to the chorioamnion (Matsubara et al., 1999). Since chorion laeve trophoblasts have the capacity to undergo phagocytosis, and since NAD(P)H oxidase primarily belongs to phagocytic cells, it may be expected that chorion laeve trophoblasts may also possess NAD(P)H oxidase. We have also postulated that changes in the distribution pattern of NAD(P)H oxidase, if this enzyme is present in these cells, may occur in CAM, considering that ROS generation may have a close relationship with the pathogenesis and pathophysiology of this disorder. The present study was an effort to verify these hypotheses. We have examined the subcellular distribution pattern of NAD(P)H oxidase in chorion laeve trophoblasts from pregnant women, and compared it with that from patients with CAM, using ultrastructural enzyme histochemistry for NAD(P)H oxidase (Briggs et al., 1975; Robinson and Badwey, 1995).

Materials and methods

Subjects

Fetal membranes from 15 pregnant Japanese women were studied. They were classified into the following three groups. (i) Five women who delivered preterm or term singleton infants at 32–38 (36.0 ± 2.5, mean ± SD) weeks of gestation without signs of clinical CAM. The gestational age in this group was older than that of the mild CAM group mentioned below, but with no statistical difference (P = 0.28), and thus, this group served as gestational age-matched controls. The preterm infants were delivered abdominally due to maternal complications, and therefore had no evidence of uterine contractions. Routine histological examination confirmed the absence of histological CAM (without CAM group). (ii) Five women who delivered preterm or term singleton infants at 30–39 (33.8 ± 3.3) weeks of gestation and who exhibited clinical signs of infection, such as elevated C-reactive protein (2.3 ± 2.0 mg/dl), leukocytosis (13 600 ± 2810/µl) and low grade fever (37.0–37.5°C), and had regular uterine contractions of at least 10 min intervals. Patients with preterm labour initiation received tocolysis treatment with a β2-agonist and antibiotic therapy without success. Term patients also received antibiotic treatment. Routine histological examination revealed mild CAM, as previously described (Blanc, 1981); leukocyte infiltration was confined beneath the trophoblastic layers of the chorioamnion (mild CAM group). (iii) Five pregnant women who delivered preterm or term infants at 28–37 (31.4 ± 3.8) weeks of gestation, whose fetal membranes were confirmed to exhibit severe CAM by histological examination; leukocytes infiltrated to the amniotic layers (Blanc, 1981) (severe CAM group). Elevated C-reactive protein (3.1 ± 2.1 mg/dl) and leukocytosis (15 700 ± 6750/µl) were also observed, but there were no significant differences in these infection markers between the mild and severe CAM groups.

Enzyme histochemistry

After obtaining informed consent from all women and approval from the Ethics Committee of our institute, pieces of reflected chorionicamnion from three different sites at a distance of 5 cm from the placental margin were taken within 3 min of placental delivery. These pieces were from membranes overlaying the uterine body, not those overlaying the cervix, since the latter membranes exhibit a slight morphological difference from the former ones (McLaren et al., 1999). Fetal membranes were fixed in 0.25% glutaraldehyde in a cacodylate buffer (0.1 mol/l, pH 7.4) for 30 min at 4°C. After being washed in the cacodylate buffer, the pieces were sectioned (40 µm) on either a Vibratome or a freezing microtome. The sections were preincubated for 10 min at 37°C in a 0.1 mol/l Tris–maleate buffer (pH 7.5) with 7% sucrose containing 1 mol/l 3-amino-1,2,4-triazole. They were then incubated in a final reaction medium (Briggs et al., 1975) for 20 min at 37°C. The medium consisted of a 0.1 mol/l Tris–maleate buffer (pH 7.5), 7% sucrose, 10 mmol/l 3-amino-1,2,4-triazole, 1 mmol/l cerium chloride (CeCl3), and 0.8 mmol/l NADH or NADPH. NAD(P)H oxidase, using NADH or NADPH as a substrate, generates superoxide, which is then rapidly converted to hydrogen peroxide by dismutation. Cerium in the reaction medium binds to the hydrogen peroxide generated, leading to the formation of cerium perhydroxide, which forms electron-dense deposits readily visualized by electron microscopy (Briggs et al., 1975; Robinson and Badwey, 1995). As cited in the Results section, laeve trophoblasts in the mild CAM group showed phagosome-like structures in their cytoplasm. To confirm them as phagosomes or phago-lysosomes, acid phosphatase histochemistry was also performed in fetal membranes from patients with mild CAM, using the cerium method (Robinson and Karnovsky, 1983). Sections were postfixed in 1.0% buffered osmium tetroxide, dehydrated and embedded in epoxy resin. Ultrathin sections were prepared, either unstained or lightly stained with uranyl acetate and lead citrate, and observed under a transmission electron microscope (TEM). To ensure specific detection of enzyme activity, a series of cytochemical control experiments was performed as follows: (i) substrate (NADH or NADPH) omission, (ii) replacement of substrate (NADH or NADPH) by NAD or NADP, (iii) omission of 3-amino-1,2,4-triazole with the addition of catalase (1 mg/ml), and (iv) boiling the section (at 100°C for 10 min) before the reaction. Modifications to the reaction medium cited above, or section boiling, theoretically prohibit the formation of superoxide or hydrogen peroxide, and have been used widely in NAD(P)H oxidase enzyme histochemistry of phagocytes to ensure the specific detection of the enzyme activity. These experiments, therefore, can also serve as cytochemical controls in detection of this enzyme in tissues other than phagocytic cells.

Semi-quantitative analysis and statistics

For each chorionicamnion, we prepared several sections from three different sites, and took several electron micrographs from these sections. We observed ~30–40 chorion laeve trophoblast cells in each site. Sections were made from three different sites; 100 cells were observed per each fetal membrane. As shown in Results, precipitates indicative of NAD(P)H oxidase were mainly visible on the surface plasma membranes and microvillous membranes of the trophoblasts. We counted the number of trophoblasts, of which plasma or microvillus membranes were positively stained for NAD(P)H oxidase, among these 100 cells. This number represented the approximate percentage of trophoblasts positive for NAD(P)H oxidase. These data were statistically analysed using an unpaired Student t-test. Data are presented as mean ± SD. P < 0.05 was considered significant.

Results

Trophoblasts from fetal membranes without CAM were oval or polygonal-shaped. They contacted with each other by their
Figure 1. Transmission electron micrographs indicating NAD(P)H oxidase activity in chorion laeve trophoblasts from fetal membranes without chorioamnionitis (CAM). Specimens were taken from a term (37 weeks gestation) (a, b and e) or a preterm (32 weeks gestation) (c and d) pregnant woman; (e) and (d) were from an unstained specimen. Bar = 1 µm. (a) Four chorion laeve trophoblasts, numbered 1, 2, 3 and 4 were observed. Electron-dense precipitates indicative of NAD(P)H oxidase were visible on the surface plasma membranes of the No. 2, 3 and 4 trophoblasts (arrowheads), where these three cells had contact with each other. The area indicated by a large arrow is shown in (b) at a higher magnification. The No. 1 and No. 2 trophoblasts lay side by side, and their microvilli or surface plasma membranes were in contact with each other in the intercellular space (ics). These plasma membranes, however, lacked precipitates. Thus, we considered the No. 2, 3 and 4 cells to be NAD(P)H oxidase-positive trophoblasts, while the No. 1 cell was a negative trophoblast. Free surface plasma membranes of the No. 2 cell (small arrows) which had no contact with another trophoblast, also lacked precipitates. (b) Precipitates (arrowheads) were visible on the surface plasma membranes of two chorion laeve trophoblasts, which were in contact with each other. Arrows may indicate desmosomes. (c) Four trophoblasts were observed (Nos. 1–4). Nos. 2, 3 and 4 trophoblasts exhibited marked precipitates on the microvillus plasma membranes between their intercellular spaces (arrows), while No. 1 cell did not show precipitates. The area marked by an asterisk is shown in (d) at a higher magnification. (d) Precipitates were visible on the surface plasma membranes. (e) A cytochemical control experiment. Microvillus membranes of chorion laeve trophoblasts from term fetal membranes without CAM. Addition of catalase (1 mg/ml) to the reaction medium completely abolished the precipitates.
enzyme activity. Intracellular vesico-tubular structures positively stained for NAD(P)H oxidase, as observed in trophoblasts with mild CAM (described below) were not visible. Deposits were completely absent in cytochemical control experiments [substrate omission, replacement of NADPH with NADP, addition of catalase to the medium (Figure 1e), and section boiling]. In fetal membranes with mild CAM, some trophoblasts became irregular-shaped, while some remained oval. This morphological variation, however, did not affect the NAD(P)H oxidase staining pattern cited below. Precipitates were visible in the plasma or microvillous membranes of chorion laeve trophoblasts (Figure 2a, c). The numbers of trophoblasts with plasma membranes possessing NAD(P)H oxidase were 44, 48, 49, 54 and 60 (51.0 ± 6.2) per 100 trophoblasts counted, respectively, for each subject. Inter- section and inter-subject variations were also small. Thus, in the mild CAM group, the percentage of trophoblasts positive for NAD(P)H oxidase on their plasma membranes was 51.0 ± 6.2% (mean ± SD), significantly higher than that (25.6 ± 4.4%) of the without CAM group (P < 0.01). Occasionally, phagosome- or phago-lysosome-like structures were observed in laeve trophoblast cytoplasm (Figure 2b, b', b''), coinciding well with our previous observations (Matsubara et al., 2000). Precipitates indicating acid phosphatase activity were shown throughout the contents of these structures (Figure 2b'), and a TEM study demonstrated the presence of swollen mitochondria within them (Figure 2b''), indicating that the content of these structures was cell debris being digested by acidic lysosomal enzyme. Thus, we believe that the phagosome-like structure shown in Figure 2b was a phagosome or a phago-lysosome. NAD(P)H oxidase precipitates were visible on this phagosomal membrane (Figure 2b). In some trophoblasts (approximately one-tenth), precipitates were clearly observable in vesico-tubular structures in the cytoplasm (Figure 2c, d, e).

In severe CAM cases, destruction of the tissue was so severe that we could only distinguish the trophoblast layer by the presence of chorion laeve trophoblast basement membranes. Almost all trophoblasts exhibited severe cell destruction (data not shown). The relatively weak fixation procedure used here (0.25% glutaraldehyde) may have further prohibited the preservation of trophoblast morphology. We could not, therefore, perform morphological or cytochemical analysis. Precipitates were not visible in amniotic epithelial cells or fibroblasts in fetal membranes under the present experimental conditions (data not shown).

As partly described, there were no significant differences in staining pattern among fetal membranes with different gestational ages within the same group, nor were there significant variations in findings among sections taken from three different sites within the same subjects. Thus the observations cited above were believed to be representative findings of various chorioamnion specimens.

Discussion
In the present study, we made two important observations. First, enzyme-histochemically detectable NAD(P)H oxidase was present in chorion laeve trophoblasts without CAM at 32–38 weeks of gestation. Since the introduction of cerium into the histochemical demonstration of NAD(P)H oxidase (Briggs et al., 1975), its subcellular localization has been demonstrated mainly in professional phagocytic cells (Robinson and Badwey, 1995; Kobayashi et al., 1998), and thus, this enzyme has long been considered to be predominantly associated with phagocytes. Ten years ago, we demonstrated this enzyme on the microvillus membranes of syncytiotrophoblasts from normal term pregnant women, indicating that the presence of NAD(P)H oxidase was not exclusively confined to phagocytic cells (Matsubara and Tamada, 1991). Later, cardiovascular NAD(P)H oxidase was biochemically demonstrated (Griendling et al., 1994), but its subcellular localization has not yet been cytochemically determined. The present cytochemical study with a full series of cytochemical control experiments confirmed the presence of NAD(P)H oxidase in surface plasma membranes and microvillous membranes of chorion laeve trophoblasts. It has now become clear that chorion laeve trophoblasts possess NAD(P)H oxidase, and therefore ROS-generating capacity.

The second finding worthy of note was that changes in the distribution pattern of NAD(P)H oxidase occurred in chorion laeve trophoblasts with CAM. Three observations were made. First, the percentage of trophoblasts with surface plasma membranes possessing NAD(P)H oxidase increased [25.6 ± 4.4 versus 51.0 ± 6.2%; CAM(−) versus CAM(+)]. Second, precipitates indicative of NAD(P)H oxidase were visible on the phagosomal membranes. Third, this enzyme occasionally appeared in intracellular vesico-tubular structures. In PMN (professional phagocytes), when unstimulated or dormant, NAD(P)H oxidase is not detectable. However, when they are stimulated or activated, this enzyme appears in intracellular vesico-tubular structures and finally appears both on the surface plasma and phagosomal membranes (Briggs et al., 1975; Robinson and Badwey, 1995; Kobayashi et al., 1998). Thus, in PMN, the presence of NAD(P)H oxidase in the intracellular vesico-tubular structures, surface plasma membranes, and phagosomal membranes, is strong evidence that PMN are being activated or phagocytosing (Briggs et al., 1975; Robinson and Badwey, 1995; Kobayashi et al., 1998). These distribution patterns of NAD(P)H oxidase in phagocytosing PMN are identical to those observed in chorion laeve trophoblasts with mild CAM in the present study. Chorion laeve trophoblasts are not professional phagocytic cells, so it is not clear whether the appearance of NAD(P)H oxidase in the subcellular sites cited above also indicates trophoblast stimulation. However, since these trophoblasts undergo phagocytosis due to bacterial infection of fetal membranes similar to PMN (Matsubara et al., 2000), and since they are shown here to exhibit NAD(P)H oxidase distribution characteristic of stimulated PMN, it is quite reasonable to assume that the appearance of NAD(P)H oxidase on these sites also indicates that the trophoblasts were activated. We conclude that chorion laeve trophoblasts with CAM were activated and phagocytosing, releasing ROS around the fetal membranes.

What are the biological and pathophysiological roles of ROS production by chorion laeve trophoblasts in CAM? Their
Chorion laeve trophoblast NAD(P)H oxidase

Figure 2. Chorion laeve trophoblast NAD(P)H oxidase in fetal membranes with mild CAM at 39 (a, b, b' and b'') and 34 (c, d and e) weeks gestation. (c), (d) and (e) were from an unstained specimen. Bar = 1 µm (except for the right upper inset of b, and for d and e) (a) Three chorion laeve trophoblasts were observed, all of which exhibited electron-dense precipitates for NAD(P)H oxidase on their surface plasma membranes (arrowheads). (b) A phagosome or phagosome-like structure was visible at a lower (b, left lower inset) and a higher (b) magnification. Precipitates indicative of NAD(P)H oxidase were visible both on the membranes (arrowheads) lining this structure and the surface (arrows) of its content. The area indicated by a large arrow is shown at a higher magnification in the right upper inset. Membrane-bound round structures (double arrows in the right upper inset) were visible, which might be intracytoplasmic organelles (possibly swollen mitochondria), but their definite distinction as mitochondria was difficult, due to weak fixation used for detection for NAD(P)H oxidase. (b') Acid phosphatase enzyme histochemistry. Acid phosphatase staining was positive on lysosomes (arrowheads). The structure contained material (arrows) positively stained for acid phosphatase, strongly suggesting that this structure might be a phago-lysosome. (b') TEM observation. This trophoblast possessed a structure in which degenerated cell or cell debris (a large arrow) was clearly observed, indicating that this structure might be a phagosome. Small arrows indicated the swollen mitochondria in this cell debris (phagosomal content). (b') and (b'') clearly showed that material contained in the phagosome-like structure was degenerated cell or cell debris, being digested by lysosomal enzymes (acid phosphatase); (b) therefore indicated that NAD(P)H oxidase activity was present on the phagosomal membranes (arrowheads). Precipitates (arrows in b) were also visible on the surface of the phagosomal content, indicating that it may be a degenerated trophoblast still possessing enzyme activity. (c) Precipitates were observed not only on the surface plasma membranes (arrowheads), but also in the intracellular vesico-tubular structures (arrows). The area indicated by an asterisk is shown at a higher magnification in (d). (d) Four vesico-tubular structures with precipitates for NAD(P)H oxidase were clearly visible. (e) NAD(P)H oxidase-positive vesico-tubular structures observed in another section from the same specimen.
significance can be deduced by considering the roles played by phagocytosing professional phagocytes in the pathophysiology of CAM-related preterm delivery. Previously, we have demonstrated the activation of PMN from vaginal washings (Matsubara et al., 1998a; Yamada et al., 1998, 2000) and fetal membranes (Matsubara et al., 1999) in CAM. Activation of villous macrophages was also demonstrated in infection-related second trimester abortions (Matsubara et al., 1999b). These professional phagocytes, when activated by infectious stimuli, protect the host against infection. Activated phagocytes, on the other hand, may adversely affect the host. These cells, while undergoing phagocytosis and digestion, release various substances (peroxidase, proteinases, hydrolases, ROS, etc.) into the tissue environment (Weinberg and Athens, 1993). Of these substances, ROS have a particularly strong cytotoxic activity. These substances, including ROS, may attack the fetal membranes themselves (Sbarra et al., 1985), or the uterine cervical stroma (Kanayama and Terao, 1991), leading to preterm rupture of the membranes or ripening of the cervix, and finally to preterm delivery. Professional phagocytes, therefore, not only protect the mother against infection, but at the same time damage the host. Cell activation in CAM, however, is not confined to professional phagocytes. Recently, we found evidence that chorion laeve trophoblasts in CAM also exhibit morphological signs characteristic of stimulated professional phagocytes (Matsubara et al., 2000). Further, in the present study, it became clear that chorion laeve trophoblasts possessed NAD(P)H oxidase (ROS-generating system), and in CAM, they exhibit changes in the enzyme distribution, characteristic of stimulated PMN. Taking all these observations into account, we conclude that the NAD(P)H oxidase of chorion laeve trophoblasts may protect the mother against bacterial infection. This enzyme, however, may also damage the host in CAM, similar to professional phagocytes in fetal membranes. Chorion laeve trophoblast NAD(P)H oxidase may play some role in the pathophysiology of CAM.

In control subjects without CAM, though the percentage was smaller than that with mild CAM (25.6 versus 51.0%), some trophoblasts exhibited NAD(P)H oxidase on their plasma membranes. In mild CAM, while half (51.0%) of the trophoblasts showed positive NAD(P)H oxidase labelling, the remaining half were negative. Thus, chorion laeve trophoblasts exhibited this enzyme activity on a cell-to-cell basis and not in an all-or-none manner. The enzyme expression, which may represent the responsiveness of trophoblasts to stimuli, may differ at each cell level. In PMN NAD(P)H oxidase, the strength or species of stimulators can affect the expression of this oxidase at each cell level (Kobayashi et al., 1998; Jiang et al., 2000). The same mechanism may be working in laeve trophoblast NAD(P)H oxidase, and this may account for this variation and the lack of all-or-none phenomenon. Even without evidence of histologically confirmed CAM, some subtle substances may always be present in the chorioamnion and may act as stimuli for some trophoblasts. Thus, trophoblastic NAD(P)H oxidase may play a role in host defence even in fetal membranes without histologically confirmed CAM. As a bacterial infection becomes more severe, more laeve trophoblasts may express NAD(P)H oxidase on their plasma membranes, although their responsiveness appears to vary on a cell-to-cell basis. Furthermore, the responsiveness of trophoblasts may be influenced by the degree and nature of the stimulation, although there is no direct evidence of this at present. In conclusion, we demonstrated by enzyme-histochemistry that NAD(P)H oxidase was present in human fetal membrane chorion laeve trophoblasts. In CAM, this oxidase exhibited distribution patterns characteristic of stimulated PMN. Though laeve trophoblast NAD(P)H oxidase may play a significant role in the pathogenesis and pathophysiology of CAM, further studies on larger populations, using quantitative methods other than enzyme histochemistry (biochemistry, molecular biology etc.) are necessary to elucidate the biological roles this oxidase plays in physiological and pathological conditions.

Acknowledgements
We wish to thank Dr Hisanori Minakami (Department of Obstetrics and Gynecology, Hokkaido University, Hokkaido, Japan) and Dr Tetsuo Yamada (Department of Obstetrics and Gynecology, Jichi Medical School, Tochigi, Japan) for critically reading the manuscript and performing statistical analysis. We also wish to thank Dr Takashi Takayama and Dr Rie Usui (Department of Obstetrics and Gynecology, Jichi Medical School, Tochigi, Japan) for collecting and preparing the specimens.

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Received on January 24, 2001; accepted on June 8, 2001