Changes in the levels of lipoperoxide and antioxidant factors in human placenta during gestation.

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Abstract

The concentration of lipoperoxides in maternal blood increases as gestation progresses. The concentration in pregnant women at 40 weeks gestation is 1.6 times higher than in nonpregnant women. The concentration in the cord blood, however, is 70% lower than that in maternal blood. To study the role of placental tissue in the difference in the lipoperoxide concentration between the cord blood and maternal blood, we investigated the lipoperoxide concentration, antioxidant activities and in vitro lipoperoxide formation in placental tissue during pregnancy. The lipoperoxide concentration was 50% lower in placental tissue of 40 weeks gestation than in tissue of 5-11 weeks gestation. Catalase and superoxide dismutase activities in placental tissues increased as gestation progressed, while glutathione peroxidase activity and alpha-tocopherol concentration did not change significantly during the gestational period. The in vitro formation of lipoperoxides in placental tissue decreased as gestation progressed. These results show that placental tissue suppresses lipoperoxide formation in the late gestational age, lowers the concentration of lipoperoxides in the blood and protects the fetus against oxygen toxicity.

KEYWORDS: lipoperoxides, antioxidant factors, placenta, human, gestation

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Changes in the Levels of Lipoperoxide and Antioxidant Factors in Human Placenta During Gestation

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The concentration of lipoperoxides in maternal blood increases as gestation progresses. The concentration in pregnant women at 40 weeks gestation is 1.6 times higher than in nonpregnant women. The concentration in the cord blood, however, is 70% lower than that in maternal blood. To study the role of placental tissue in the difference in the lipoperoxide concentration between the cord blood and maternal blood, we investigated the lipoperoxide concentration, antioxidant activities and in vitro lipoperoxide formation in placental tissue during pregnancy. The lipoperoxide concentration was 50% lower in placental tissue of 40 weeks gestation than in tissue of 5–11 weeks gestation. Catalase and superoxide dismutase activities in placental tissues increased as gestation progressed, while glutathione peroxidase activity and α-tocopherol concentration did not change significantly during the gestational period. The in vitro formation of lipoperoxides in placental tissue decreased as gestation progressed. These results show that placental tissue suppresses lipoperoxide formation in the late gestational age, lowers the concentration of lipoperoxides in the blood and protects the fetus against oxygen toxicity.

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Recent studies show that there is a close relationship between radical molecules such as superoxide or lipoperoxides and many diseases (1). In human pregnancy, placental blood circulation is completed at 17–20 days of gestation, and the maternal blood flows into placental villi spaces and supplies nutrients and oxygen to the fetus. The placental tissue actively transports a small quantity of lipid by pinocytosis (2) from the maternal blood to the cord blood, and at the same time, it is possible that superoxide and lipoperoxides also reach the fetal tissue and affect its development. A defense mechanism active in placental tissue has been postulated. Previously, Yoshioka et al. reported that, in rats, the lipoperoxide concentration in blood increased along with growth and peaked at about the 15th day of life (3), and maternal, fetal and placental levels of lipoperoxides and antioxidants changed during pregnancy (4). The present study examined the changes in the lipoperoxide content and antioxidant factors in maternal and fetal blood, and placental tissue during pregnancy. In vitro lipoperoxide formation in placental tissue was also

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studied to demonstrate the defense mechanism of placental tissues during pregnancy.

Materials and Methods

Placental samples were obtained from the Center for Adult Diseases, Kurashiki, Japan. Placenta was rinsed in cold saline to remove the blood, placed on filter paper to adsorb excess water, incised into small pieces and weighed (raw material).

Determination of lipoperoxides and antioxidant factors. The raw material was homogenized in 9 volumes of cold saline in a glass homogenizer and was filtrated through gauze. The lipoperoxides of the filtrate were determined by the method of Ohkawa et al. (5). In brief, to 0.2 ml of 10% (w/v) homogenate, 0.2 ml of 81% (w/v) sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution adjusted to pH3.5 with 20% (w/v) sodium acetate and 1.5 ml of 0.8% (w/v) thiobarbituric acid (TBA) solution were added. The mixture was made up to 4.0 ml with distilled water and then heated in a boiling water bath for 60 min. After cooling, 1.0 ml of distilled water and 5.0 ml of a mixture of n-butanol and pyridine (15:1 v/v) were added. The mixture was shaken vigorously and centrifuged. The absorbance of the organic layer was measured at 532 nm with a Shimadzu UV-3000 spectrophotometer. The same homogenate (10%) was used for the α-tocopherol determination by the method of Ishibashi et al. (6). In brief, the sample (0.5 ml) was added to 1.0 ml of 6% (w/v) pyrogallol in ethanol and mixed vigorously. One ml of tocol (2 μg/ml) in ethanol was added as an internal standard. The mixture was incubated at 70°C for 2 min, 0.2 ml of 60% (w/v) KOH was added, and the incubation was continued at 70°C for 30 min. After cooling in an ice bath, the mixture was added to 2.5 ml of distilled water and 5.0 ml of n-hexane, mixed vigorously for 5 min and centrifuged for 5 min at 3000 rpm. Four ml of the n-hexane layer was evaporated under N2 gas and dissolved in 200 μl of n-hexane. An appropriate aliquot was measured for α-tocopherol with a Shimadzu high performance liquid chromatography system. The column was Zorbax SIL (4.6 mm × 25 cm), and the column temperature was set at room temperature. The elute was n-hexane : ethanol : dioxane (97.6 : 0.4 : 20 v/v), and the flow rate was 1.5 ml/min. The α-tocopherol peak was detected with fluorescence spectromonitor RF-530 (Ex 298 nm, Em 325 nm) and identified with chromatopac C-RIB.

The starting material was homogenized in 9 volumes of 0.15 M NaCl-10 mM sodium phosphate buffer (pH 7.4). Catalase activity of the supernatant obtained by centrifugation at 700 × g for 8 min was determined by the method of Cohen et al. (7), and superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities of the supernatant obtained by centrifugation at 100,000 × g for 1 h were determined by the methods of Beauchamp et al. (8) and Demus-Oole et al. (9), respectively. For catalase, the supernatant was mixed with 1/100 volume of ethanol and incubated for 30 min in an ice water bath, followed by the addition of 1/10 volume of 10% (w/v) Triton X-100. The mixture was further diluted 100-1,000 fold to obtain an enzyme sample. The sample (0.1 ml) was mixed with 1 ml of 6 mM H2O2 in an ice bath, and after 3 min, 0.2 ml of 6 N H2SO4 was added. After letting the mixture stand at room temperature, 1.4 ml of 0.01 N K2MnO4 was added and mixed vigorously. The absorbance of the mixture was determined at 480 nm within 1 min. For GPx, the supernatant (0.05 ml) was added to 2.63 ml of 0.05 M sodium phosphate buffer (pH 7.0), followed by addition of 0.1 ml of 4.2 mM reduced nicotinamide adenine dinucleotide phosphate, 10 μl of one enzyme unit glutathione reductase (GSSG-Red), 0.1 ml of 0.15 M reduced glutathione (GSH) and 10 μl of 1.125 M NaN3. After the preincubation at 25°C for 5 min, the reaction was started by adding 0.1 ml of 2.2 mM H2O2 to the mixture, and the activity was determined by the decrease in absorbance at 340 nm. For SOD, 50 mM carbonate buffer (pH 10.2), 0.5 mM EDTA, 0.1 mM xanthine and 25 μM NBT were added to the supernate. After preincubation at 37°C for 3 min, a suitable amount of xanthine oxidase was added. Then, after 10 min, absorbance at 560 nm was determined. The final volume was 3.0 ml.

In vitro lipoperoxide formation. Placental tissues of 7-, 10-, 18-, 22- or 40-week-old fetuses were homogenized in 4 volumes of 0.15 M NaCl-10 mM sodium phosphate buffer (pH 7.4), and filtrated through gauze. The protein concentration of the filtrate was determined by the method of Markwell et al. (10). The filtrate was diluted with 0.15 M KCl-10 mM Tris-HCl buffer (pH 7.4) so as to give a final protein concentration of 1 mg/ml. Lipid peroxidation was determined by the method of Buege et al. (11). The sample was preincubated at 37°C for 5 min, and FeCl3 (0.5 mM) or sodium ascorbate (0.1 mM) was added. To 1-ml aliquots of the solution, 100 μl of 0.01% (w/v) butylated hydroxytoluene was added to stop the reaction. Two ml of a mixture of 15% (w/v) trichloroacetic acid, 0.375% (w/v) TBA and 0.25 N HCl was added to the sample, which was then boiled at 100°C for
15 min and cooled. The mixture was centrifuged at 2,500 rpm for 10 min, and the absorbance of the supernatant was determined at 515 nm.

**Determination of blood lipoperoxides.** Blood lipoperoxides were determined by the fluorometric assay of Yagi [12] using "lipoperoxides-test" (Wako Pure Chem. Ind., Osaka, Japan). In brief, blood (0.05 ml) was added to 1.0 ml of physiological saline in a centrifuge tube, shaken gently and centrifuged at 3,000 rpm for 10 min. To the supernatant (0.5 ml), 4.0 ml of 1/12 N H_2SO_4 was added, mixed well, and 0.5 ml of 10% (w/v) phosphotungstic acid was added. After standing at room temperature for 5 min, the mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was discarded, and the precipitate was mixed with 2.0 ml of 1/12 N H_2SO_4 and 0.3 ml of 10% phosphotungstic acid and centrifuged at 3,000 rpm for 10 min. The precipitate was suspended in 4.0 ml of distilled water, and 1.0 ml of TBA reagent was added. The mixture was heated in a boiling water bath for 30 min. After cooling, 5.0 ml of n-butanol was added, and the mixture was shaken vigorously. After centrifugation at 3,000 rpm for 10 min, the organic layer was collected for fluorometric determination, made at an excitation wavelength of 515 nm and emission wavelength of 535 nm.

**Determination of total glutathione.** Total glutathione was determined by the method of Griffith et al. [13]. Placental sample was mixed with 0.5 volume of 10% (w/v) 5-sulfosalicylic acid. The sample was centrifuged, and an aliquot of the supernatant was placed in a test tube. Triethanolamine (6 μl) was added to 100 μl of supernatant and the two were vigorously mixed. This solution was used for the determination of glutathione. The sample was mixed with 2.1 ml of 0.125 mM sodium phosphate (pH 7.5), 6.3 mM EDTA, 3 mM NADPH and 0.3 ml of 6 mM 5′, 5′ dithiobis (2-nitrobenzoic acid). Distilled water was added to make the final volume 3.0 ml. After 5 min of incubation at 30°C, 30 μl of 50 units/ml of glutathione reductase was mixed into the sample, and the absorbance at 412 nm was determined at 0, 20 and 40 min. The glutathione concentration was calculated as the concentration of GSH. The data were statistically analyzed by the Scheffé method [14].

**Results**

**Lipoperoxide concentration in the blood.** The lipoperoxide concentration in maternal blood increased as pregnancy progressed (Table 1). The concentration in non-pregnant females was 3.81 ± 1.43 nmoles/ml (mean ± SD) (n = 11), while the concentration in pregnant females at delivery was 1.6 times higher than in non-pregnant females. However, the concentration in the cord blood was only 30% of that in the at-term maternal blood.

**Lipoperoxide concentration in human placenta.** Fig. 1 shows the change in the lipoperoxide concentration in human placenta at various gestational ages. Although the concentration varied during the early period of gestation, the average value was higher than in the late period of gestation. The mean values of the lipoperoxide concentration were 228.9 nmoles/g wet weight (w. w.) at 5–11 weeks of gestation, 253.7 nmoles/g w.w. at 12–15 weeks of gestation, and 114.9 nmoles/g w.w. at birth after 37–42 weeks of gestation. The first value was statistically higher than the last two values (p < 0.01). There was no difference in the lipoperoxide concentration between the placentas from normal deliveries (113.6 ± 31 nmoles/g w.w. (mean ± SD) (n = 35)) and those from Caesarean sections (125 ± 34.6 nmoles/g w. w. (mean ± SD) (n = 7)).

**Antioxidant factors in human placenta.** Fig. 2 shows the change in antioxidant factors in human placenta at various gestational ages. SOD

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**Table 1** Lipoperoxide concentration in human blood specimens [a]

<table>
<thead>
<tr>
<th></th>
<th>Lipoperoxides (nmoles/ml blood)</th>
<th>Mean ± SD</th>
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<tbody>
<tr>
<td><strong>Non-pregnant</strong></td>
<td></td>
<td></td>
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<tr>
<td>5–9 weeks</td>
<td>3.81 ± 1.43 (11)</td>
<td></td>
</tr>
<tr>
<td>10–19 weeks</td>
<td>3.21 ± 0.96 (21)</td>
<td></td>
</tr>
<tr>
<td>20–29 weeks</td>
<td>4.05 ± 1.22 (8)</td>
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<tr>
<td><strong>Delivery</strong></td>
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<tr>
<td>41 weeks</td>
<td>6.17 ± 2.38 (26)</td>
<td></td>
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<tr>
<td><strong>Umbilical cord</strong></td>
<td>1.86 ± 0.24 (6)</td>
<td></td>
</tr>
</tbody>
</table>

*a:* Numbers of specimens are shown in parentheses.

*b:* Statistically different from that of the non-pregnant (p < 0.01).

*c:* Statistically different from that of the delivery (p < 0.025).
and catalase activities in placenta increased as gestation progressed, and these activities at early gestation were only 30% and 12% of that at delivery, respectively. No significant changes in GPx activity and the \( \alpha \)-tocopherol concentration were found during gestation.

*In vitro formation of lipoperoxide formation by placental tissue.* When the placental tissue of 40 weeks of gestation was incubated at 37°C, there was little increase in lipoperoxide formation (Fig. 3). However, the addition of ferrous chloride or ascorbic acid to the incubation mixture caused a marked increase in lipoperoxide formation. As shown in Fig. 3, with ferrous chloride, lipoperoxides increased in the first 60 min; thereafter, the rate of increase slowed. With ascorbate, lipoperoxides gradually increased as time passed. Dose dependency of these reagents to lipoperoxide formation was observed. With ferrous chloride, lipoperoxides increased as the ferrous chloride concentration increased. However, with ascorbate, lipoperoxides increased dose dependently to a concentration of 0.1 mM ascorbate, but over this concentration, there was no increase. Accordingly, we used 0.5 mM as the ferrous ion concentration, and 0.1 mM as the ascorbate concentration in the following experiments.

*The change in antioxidant factors during induced lipoperoxide formation.* The activities of placental antioxidant factors decreased when placenta was incubated at 37°C. As shown in Fig. 4, all of the antioxidants decreased after 2 h of incubation. Among the antioxidants, SOD, catalase and GPx activities decreased 14%, 28% and 48% of the zero-time level, respectively. The \( \alpha \)-tocopherol and total glutathione concentration decreased 48% and 40%, respectively. The decrease in antioxidants was large when lipoperoxide formation was induced by ferrous ion or ascorbate. When lipoperoxides were raised five times above the control value by the addition of ferrous chloride, \( \alpha \)-tocopherol decreased to only 9.7% of zero time.

*In vitro lipoperoxide formation by placental tissue at various gestational ages.* In vitro lipoperoxide formation by placental tissue at
various gestational ages (7 weeks, 10 weeks, 18 weeks, 22 weeks, 40 weeks) was induced in the presence or absence of FeCl$_2$ (0.5 mM) or ascorbate (0.1 mM). Lipoperoxide production at various gestational ages in the absence of FeCl$_2$ or ascorbic acid is shown by a solid line in Fig. 5. The highest lipoperoxide production (3.3 nmol/h) was observed in the placental tissue at 7 weeks of gestational age. The lipoperoxide production decreased when placental tissues of later gestational age were used. The value at 40 weeks of gestation was less than 1 nmole after 3 h of incubation (Figs. 3, 5). Lipoperoxide formation in placental tissues was accelerated by the addition of FeCl$_2$ or ascorbate regardless of the gestational age. The peak of lipoperoxide production decreased as gestation progressed. Although lipoperoxide production in early-gestation tissue after 3 h of incubation with FeCl$_2$ was lower than that with ascorbate, the initial velocity was high in all placental tissues incubated with FeCl$_2$. The FeCl$_2$-induced reaction reached a submaximum at 30 min of incubation. Lipoperoxide production was found to be more rapid in the placenta at an early gestational age than at a late gestational age.
Discussion

During the evolutionary process in which the living organisms were able to utilize oxygen, they had to acquire a defense mechanism against oxygen toxicity. Active oxygen species act with unsaturated fatty acids present in phospholipid to form lipid peroxide, which is a major factor influencing the breakdown and turnover of biomembranes (15). The relationship of diseases and lipoperoxides has been widely documented (1). Human females tend to develop hyperlipidemia in the late period of pregnancy and to have an increase in serum lipoperoxides (16). Previously, the authors reported changes in maternal, fetal, newborn and placental levels of lipoperoxides and antioxidants during pregnancy and development in rats (3, 4, 17). In the present study, we confirmed that the lipoperoxide concentration in the blood of pregnant women increased as gestation progressed, but it was kept lower in the cord blood than in the maternal blood. We also confirmed that the lipoperoxide concentration in placental tissue was higher in the early gestational period than in the late gestational period. As the lipoperoxide concentration was much higher in the placental tissue than in the blood vessels, the level of lipoperoxides in placental tissue may not be affected by the concentration in the blood. Furthermore, antioxidant activity in cord serum is lower than that in the serum of adults (18). Therefore, it was postulated that the placental tissue itself plays a role in the defense mechanism which lowers the fetal blood level of lipoperoxides.

The role of placental tissue was studied by determination of antioxidant factors and in vitro lipoperoxide formation. SOD and catalase activities in placental tissue increase as gestation proceeds. Maseki et al. (19) reported that SOD activity did not change at any time in the gestational period. They presented the activity per
Fig. 4  *In vitro* formation of lipoperoxides and the decrease in antioxidant factors induced by 0.5 mM FeCl$_3$ or 0.1 mM ascorbate in human placental tissue at 40 weeks of gestation. Each placental sample was treated as described in Fig. 3 except that the protein concentration was 5 mg/ml and the reaction was stopped at 2 h. Measured were: (a), lipoperoxide; (b), superoxide dismutase; (c), catalase; (d), glutathione peroxidase; (e), total glutathione; (f), α-tocopherol. Each point presents the mean value of 3 individual experiments. ●, Control; ×, 0.5 mM FeCl$_3$; ○, 0.1 mM ascorbate.

Fig. 5  *In vitro* formation of lipoperoxides induced by 0.5 mM FeCl$_3$ or 0.1 mM ascorbate in human placental tissue at various gestational ages. Each placental sample was treated as described in Fig. 3. Lipoperoxide formed was determined at every 30 min for 3 h. ●—●, Control; ×—×, 0.5 mM Fe$^{3+}$; ○—○, 0.1 mM ascorbate.
mg protein, but we think that the activity should be expressed per gram wet weight, since the concentration of proteins in tissue changed as gestation proceeded. Hien (20) and Sekiba et al. (21) reported a similar change in SOD. The change in catalase activity was marked. Both activities have been reported to be induced by exogenous radicals (22), so in the late placental tissue, enzymes may be induced by the radical species passing through the tissue. GPx activity was constant throughout the gestational period. Some GPx activity of glutathione S-transferase was reported, but GPx activity of the placental glutathione S-transferase was low (23). Awathi (24) stated that the role of GPx in the defense against radical-induced lipid peroxidation may not be so important. The \( \alpha \)-tocopherol concentration was also constantly low throughout the gestational period. \( \alpha \)-Tocopherol was only supplied by passive transport from the maternal blood (25). It was not clear whether the concentration of \( \alpha \)-tocopherol observed in the present study was sufficient to protect against oxidant attack in vivo.

Ascorbate induced lipid peroxidation at a low concentration in vitro, but inhibited it at a high concentration. This concentration-dependent phenomenon was well documented in various tissues and liposome experiments. Agarwal et al. (26) reported ascorbate induced lipoperoxide formation in liposomes, and concluded that the inhibitory action of ascorbate at a high concentration was due to the limited availability of oxygen. At a low ascorbate concentration, oxidation of ascorbate to the dehydro form proceeds to the formation of hydrogen peroxide, which yields hydroxy radicals known to promote lipid peroxidation. Abe et al. (27) concluded that ascorbate reduces the ferric ion to the ferrous ion, which in turn leads to lipid peroxidation. In the case of ferrous chloride-induced lipoperoxide formation, ferrous ion is converted to the stable ferric form by lipoperoxide formation, so the reaction progresses rapidly (28).

Lipid peroxidation is a damaging biological process. In vitro lipid peroxidation by tissue homogenate has been studied both in the enzymatically NADPH-induced and nonenzymatically induced (several metals such as Fe\(^{2+}\), perferryl ion and low concentration of ascorbate) systems. Diamant (29) reported that enzymatic lipid peroxidation of mitochondria or microsomes of the late placental tissue occurred more intensely than that of the early placenta and concluded that early placenta was functionally immature. These results strongly suggest that the placental tissue suppresses lipoperoxide formation and protects the fetus from many kinds of radicals.

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