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Expression of Nitric Oxide Synthase Isoforms in the Human Placenta Is Not Altered by Labor

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Abstract. Nitric oxide has various biological activities including smooth muscle relaxation, anti-inflammatory activity, anti-coagulatory activity. As the human placenta is known to express nitric oxide synthases, this study investigated the possible effect of labor on the expression of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) in human placental tissues at term. Both eNOS and iNOS mRNA expression in placental tissues in labor were significantly higher than those in the amnion, chorion laeve, decidua vera and myometrium. The eNOS mRNA and protein expressions in placental tissues in labor (n = 12) were 1.6023 ± 0.1652 (eNOS/GAPDH, mean ± SEM) and 12.8 ± 1.3 arbitrary units (AU), respectively, which were similar to those not in labor (n = 10), 1.5806 ± 0.2042 (eNOS/GAPDH) and 11.4 ± 1.8 AU. The iNOS mRNA and protein expressions in the placental tissues in labor were 1.2831 ± 0.2436 (iNOS/GAPDH) and 10.7 ± 2.1 AU respectively, similar to those not in labor, 1.9254 ± 0.8004 (iNOS/GAPDH) and 13.3 ± 1.8 AU. The guanosine 3',5'-cyclic monophosphate (cGMP) cocentration in the placental tissues in labor was 23.6 ± 1.4 fmol/g wet tissue, similar to that not in labor, 26.1 ± 2.0 fmol/g wet tissue. These findings suggest that nitric oxide production in the human placenta is maintained during labor.

Key words: Guanosine 3',5'-cyclic monophosphate (cGMP), Nitric oxide synthase, Parturition, Pregnancy, Trophoblast cells
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NITRIC oxide is a short-lived free radical gas involved in the regulation of vascular smooth muscle tone, inflammation, cell-mediated immunity and coagulation [1]. Nitric oxide is hypothesized to contribute to the maintenance of pregnancy, by increasing uterine blood flow [2, 3], maintaining myometrial quiescence [4–6], and others. The major effect of nitric oxide on cell function is mediated through the activation of soluble guanylyl cyclase, which generates guanosine 3',5'-cyclic monophosphate (cGMP) as an intracellular second messenger [7]. We demonstrated that the local nitric oxide/soluble guanylate cyclase system was involved in the increase in ovine uterine artery blood flow during pregnancy [8], and that soluble guanylate cyclase activity in the human pregnant myometrium was down-regulated prior to the onset of labor at term [9].

Nitric oxide is produced by the enzymatic reactions of nitric oxide synthase (NOS) [10]. Three isoforms of NOS have been identified to date, two of which are constitutively expressed as neuronal NOS (nNOS) and endothelial NOS (eNOS) requiring calcium/calmodulin for their activity, whereas inducible NOS (iNOS) is calcium/calmodulin independent [11, 12]. Biochemical experiments demonstrated the presence of eNOS in the feto-placental vasculature or dissected placental villous tissue [13–16]. However, some investigators have also provided evidence for calcium/calmodulin-independent iNOS activity in placental tissues [15, 17–19], although other researchers reported that iNOS mRNA expression was not detected in normal human placental tissues by RT-PCR analysis [20, 21]. NOS expression and activity were also demonstrated

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in placental tissues of preeclampsia [18, 22, 23] as well as cultured trophoblast cells [24, 25]. Thus, the placenta is a potential source of nitric oxide production during pregnancy. We previously reported that maternal plasma concentrations of nitric oxide metabolites as well as cGMP are elevated in pregnancy then decline during labor, while such decrease is not observed in patients undergoing term elective cesarean section before the onset of labor [26]. These results suggest a possible labor-associated down-regulation of nitric oxide and cGMP production in the maternal vasculature and/or organs, such as the pregnant uterus, including the placenta. However, to our knowledge, it remains to be elucidated whether or not changes occur in nitric oxide production in placental tissue during labor.

Therefore, this study investigated the possible effect of labor on nitric oxide production in human term placental tissues. We firstly measured eNOS and iNOS mRNA expression in the amnion, chorion laeve, decidua vera and pregnant corpus myometrium tissues, in addition to placental tissues, to compare the potency of nitric oxide production in placental tissues with those in other intrauterine tissues. Secondly, we measured eNOS and iNOS mRNA and protein expression as well as cGMP content in placental tissues obtained from women undergoing elective cesarean section at term before the onset of labor and from those undergoing normal spontaneous vaginal delivery at term.

Materials and Methods

Reagent

All reagents were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise indicated.

Materials

Chorionic villous tissues were collected from pregnant women at the time of artificial termination of pregnancy (6–9 weeks of gestation, n = 6). Amnion, chorion laeve and decidua vera tissues were obtained from pregnant women undergoing cesarean section at term (37–38 weeks of gestation, n = 10). Myometrial tissues were obtained from middle portion of the uterine body of pregnant women undergoing hysterectomy at term (37–38 weeks of gestation; n = 6) due to complication by uterine cervical cancer (n = 4) or uterine myoma (n = 2). Term placenta tissue was collected from 4 different portions of one placenta, mixed together after removing decidual layer, and used as one specimen. Specimens obtained at elective cesarean delivery before the onset of labor, were used as "not in labor" specimens. Term placental tissues were also obtained from normal pregnant women at vaginal delivery at term, which were 8 multiparous (mean duration of labor; 8.5 ± 2.7 [SEM] hours, mean number of parity; 2.4 ± 0.8) and 4 primiparous (mean duration of labor; 15.3 ± 4.4 hours) pregnant women. These specimens were used as "in labor" specimens (IL; 37–40 weeks of gestation, n = 12). Informed consent was obtained from each patient after a full explanation of the study. Tissues were immediately frozen by liquid nitrogen and/or embedded in OCT (Optimal Cutting Temperature) compound (Sakura Finetek Inc., Torrance, CA, USA) and stored at –80°C. The experimental protocol was approved by the ethics committee of Kyoto University Graduate School of Medicine.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of eNOS, and iNOS mRNA expression

Total RNA was extracted as previously described [27]. Quantitative RT-PCR of human eNOS, iNOS and GAPDH mRNAs was carried out using real time TaqMan™ technology and analyzed on a Model 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) [28, 29]. Forward and reverse primers, and (Fam or Joe/Tamra) probes used for targeting amplification of part of the human eNOS [30], iNOS [31] and GAPDH coding regions [32], were: eNOS forward; 5'-GCATCACCAGGAAGAAGACCTT-3', eNOS reverse; 5'-TTCACTCGCTTCGCCATCA-3', eNOS (Fam/Tamra) probes; 5'-Fam-AAGAAGTGGCCAAC GCCGTGAAGAT-Tamra-3', iNOS forward; 5'-GGC TGCCAAGCTGAAATTGAAT-3', iNOS reverse; 5'-CGTGATAGCGCTTCTGGCTCTT-3', iNOS (Fam/Tamra) probes; 5'-Fam-AGGAGCAGGTCGAGGAC TATTTCTTTCAGC-Tamra-3': GAPDH forward; 5'-GAAGGTGAAGGTCGGAGT-3', GAPDH reverse; 5'-CTTCTACCACTACCCTAAAG-3', GAPDH (Joe/Tamra) probes; 5'-Joe-CCGACTCTTGCCCTTCGAAC-Tamra-3'. Cycling parameters used were 2 min at

50°C, 30 sec at 60°C, and 5 min at 95°C, followed by 40 cycles of 20 sec at 94°C and 1 min at 60°C. Human eNOS and iNOS mRNA expression was estimated by dividing the eNOS and iNOS threshold cycle (C_T) values, by GAPDH C_T values, as previously described [28, 29].

Western blot analysis of eNOS and iNOS

Protein extraction, SDS-PAGE and immunoblotting were carried out as previously described [8]. Positive control tissues used were human umbilical vein endothelial cells (HUVEC) for eNOS positive control; cultured human promyelocytic leukemia cells (HL-60) after 24 hours stimulation of 10 nM phorbol ester 12-O-tetradecanoylphorbol 13-acetate and 10 ng/ml interleukin-1α for iNOS positive control. Monoclonal antibodies used were an antibody raised against human eNOS (1:250 dilution, Transduction Laboratories, Lexington, KY, USA) and an antibody raised against mouse iNOS, having cross reactivity for human iNOS (1:1000 dilution, Transduction Laboratories, KY, USA). The eNOS and iNOS protein expression was expressed as arbitrary units (AU) based on quantitative densitometric analysis of the blots.

Immunohistochemistry of eNOS and iNOS

After 1 hour incubation with primary antibodies at room temperature, staining was detected using the avidin-biotin-peroxidase (ABC) method kit for monoclonal antibody (Elite ABC, Vector Laboratories, Burlingame, CA, USA) with 3,3'-diaminobenzidine (DAB) following the manufacturer's recommendation. Monoclonal antibodies used were an antibody raised against human eNOS (1:100 dilution, Transduction Laboratories, Lexington, KY, USA) and an antibody raised against mouse iNOS, having cross reactivity for human iNOS (1:200 dilution, Transduction Laboratories). Adsorption study for iNOS immunostaining was carried out by mixing (1:1) monoclonal antibody against iNOS with the mouse macrophage extracts (Transduction Laboratories, Lexington, KY, USA).

Measurements of cGMP concentraiton in the placental tissues

The cGMP tissue concentration was measured as previously described [9]. Placental tissues were homogenized immediately after collection with ice cold 6% trichloroacetic acid (TCA). The homogenates were centrifugated at 12,000 ×g for 5 min. After extraction of TCA by water saturated diethylether, cGMP concentrations in the supernatants were measured by ELISA (Cayman Chemical Co., Ann Arbor, MI, USA). Inter- and intra-assay variations were both less than 10%.

Statistical analysis

Values are expressed as mean ± SEM. Significance was assessed by Mann-Whitney U test. *P*-values less than 0.05 were regarded as significant.

Results

eNOS and iNOS mRNA expression in intrauterine tissues

Expression of eNOS mRNA in placental tissues obtained before labor onset was 1.4805 ± 0.1102 eNOS/GAPDH (n = 6), which was significantly higher than those in amnion (0.0003 ± 0.0001 eNOS/GAPDH, n = 6), chorion laeve (0.0026 ± 0.0006 eNOS/GAPDH, n = 6), decidua vera tissues (0.0261 ± 0.0084 eNOS/GAPDH, n = 6) and myometrium (0.1700 ± 0.0400 eNOS/GAPDH, n = 6) (Fig. 1A, $P<0.001$ for all).

Expression of iNOS mRNA in placental tissues obtained before labor onset was 1.9204 ± 0.7300 eNOS/GAPDH (n = 6), which was significantly higher than those in amnion (0.1201 ± 0.0715 iNOS/GAPDH, n = 6), chorion laeve (0.3901 ± 0.1008 iNOS/GAPDH, n = 6), decidua vera (0.1608 ± 0.0426 iNOS/GAPDH, n = 6) and myometrial tissues (0.2600 ± 0.0500 iNOS/GAPDH, n = 6) (Fig. 1B, $P<0.05$ for all).

eNOS mRNA and protein expression in placental tissues obtained before labor onset

Expression of eNOS mRNA in placental tissues obtained after labor (IL) was 1.6023 ± 0.1652 eNOS/GAPDH (n = 12), which was similar to the level in the placenta obtained before labor onset (NIL), 1.5806 ± 0.2042 eNOS/GAPDH (n = 10) (Fig. 2A). The eNOS mRNA expression in the chorionic villous tissues in the first trimester was 1.9115 ± 0.3076 eNOS/GAPDH (n = 6), which was similar to those in

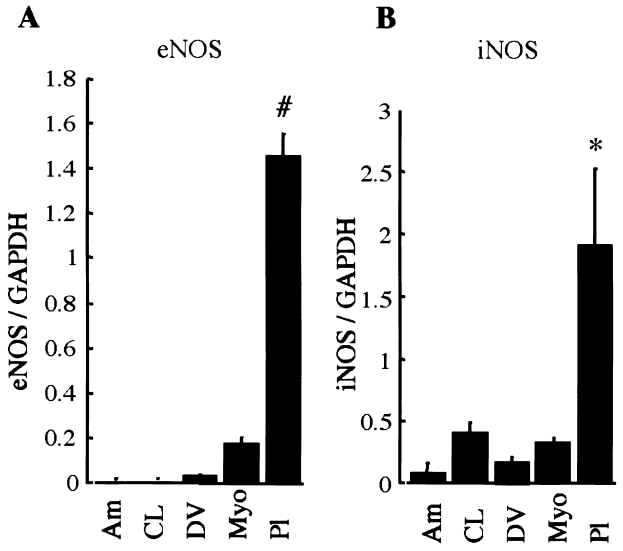

Fig. 1. Quantitative RT-PCR analysis of eNOS (A) and iNOS mRNA(B) expression, using real time Taqman™ technology in intrauterine tissues of amnion (Am), chorion laeve (CL), decidua vera (DV), myometrium (Myo) and placenta (Pl) obtained from third trimester pregnant women (n = 6). The eNOS or iNOS C_T values/G3PDH C_T values correspond to eNOS or iNOS mRNA expression, respectively, as described in Materials and Methods. Columns and error bars indicate the mean of six samples ± SEM, respectively. All assays were conducted in duplicate wells. #; $P<0.001$. *; $P<0.05$.

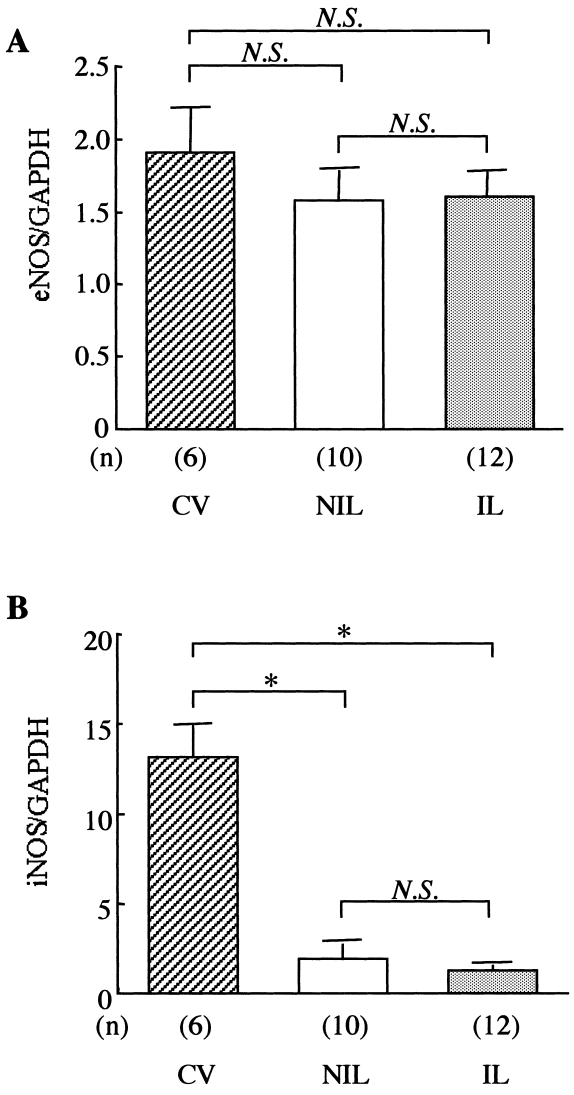

Fig. 2. Quantitative RT-PCR analysis of eNOS (A) and iNOS mRNA(B) expression, using real time Taqman™ technology in chorionic villous tissues in frist trimester (CV; hatched column) and in term placental tissues obtained from pregnant women not in labor (NIL; open column) and in labor (IL; closed column). The eNOS or iNOS C_T values/GAPDH C_T values correspond to eNOS or iNOS mRNA expression, respectively, as described in Materials and Methods. Columns and error bars indicate the mean ± SEM, respectively. *N.S.*; not significant. *; $P<0.05$.

term placenta both IL and NIL (Fig. 2A). The eNOS protein expression in placental tissues obtained after labor (IL) was 12.8 ± 1.3 arbitrary units (n = 5), which was similar to that in the placenta obtained before labor onset (NIL), 11.4 ± 1.8 arbitrary units (n = 5) (Fig. 3A). Immunostaining for eNOS was observed in syncytiotrophoblast and endothelial cells in the placental tissue both before (NIL) and after labor (IL) (Fig. 4A, B). Negative control using normal mouse IgG showed greatly reduced staining (Fig. 4C, D). There was no apparent difference between eNOS immunostaining in the placenta before labor (NIL) and that in the placenta after labor onset (IL).

iNOS mRNA and protein expression in the placental tissues

Expression of iNOS mRNA in placental tissues obtained after labor onset (IL) was 1.2831 ± 0.2436 iNOS/GAPDH (n = 12), which was slightly lower than the level in the placenta obtained before labor onset (NIL), 1.9254 ± 0.8004 iNOS/GAPDH (n = 10); however, the difference was not significant (Fig. 2B). The iNOS mRNA expression in the chorionic villous tissues in the first trimester was 13.1272 ± 1.7151 iNOS/GAPDH (n = 6), which was significantly higher than those in term placenta both IL and NIL ($P<0.05$ for both) (Fig. 2B). Western blot analysis showed a

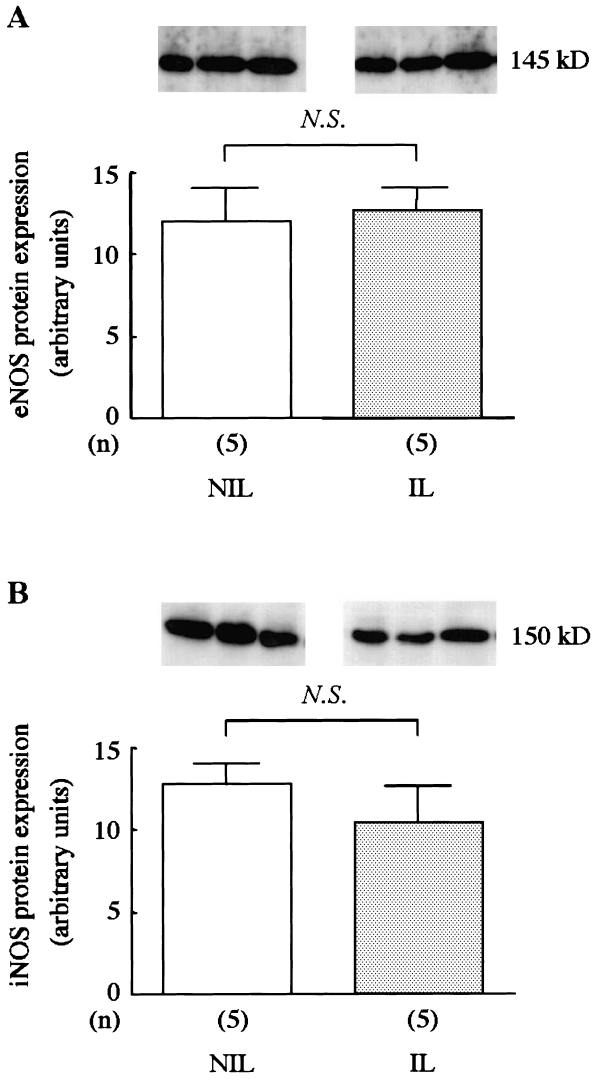

Fig. 3. Western blot analysis of eNOS (60 μg/lane; A) and iNOS (60 μg/lane; B) protein expression in placental tissues obtained from term pregnant women not in labor (NIL; open column) and in labor (IL; closed column). The 145-kD and 150-kD bands correspond to eNOS and iNOS protein expression, respectively, which were representatives of immunoblots of five specimens both before (NIL) and after (IL) labor onset. Columns and error bars indicate ± SEM, respectively. *N.S.*; not significant.

135kD band in stimulated HL-60 cells (data not shown) and a 150kD band in placental tissues (Fig. 3B), which was in agreement with previous reports [12]. Both the 135 kD and 150 kD bands were greatly reduced by iNOS antibody adsorption using mouse macrophage extracts (data not shown). iNOS protein expression in NIL placental tissues was 10.7 ± 2.1 arbitrary units (n = 5), which was slightly lower than that in the placenta obtained before labor onset (NIL), 13.3 ± 1.8 arbitrary units (n = 5) (Fig. 3B). There was no significant difference observed. Immunostaining for iNOS was observed in syncytiotrophoblast and endothelial cells in placental tissues both before (NIL) (Fig. 5A) and after (IL) (Fig. 5B) labor onset. Negative control using antibody adsorption by the mouse macrophage extracts (Fig. 5C, D) as well as normal mouse IgG (Fig. 5E, F) showed greatly reduced staining. There was no apparent difference between iNOS immunostaining in placenta obtained before labor onset (NIL) and that in placenta obtained after labor onset (IL).

cGMP concentration in placental tissues

The cGMP concentration in placental tissues obtained after labor onset (IL), 23.6 ± 1.8 pg/g wet tissues (n = 9) was similar to that in placenta obtained before labor onset (NIL), 26.1 ± 2.0 pg/g wet tissues (n = 9) (Fig. 6).

Discussion

The present study demonstrated that both eNOS and iNOS mRNA expression in the placental tissues are most prominent among intrauterine tissues of pregnant women at term (Fig. 1A, B), suggesting that a considerable amount of nitric oxide is produced in placental tissues at term. In this study, the eNOS (Fig. 4A, B) immunostaining was observed mainly in syncytiotrophoblast cells and endothelial cells. Myatt *et al.* [14, 16, 17], Conrad *et al.* [15] and Buttery *et al.* [13] reported that eNOS expression was observed in trophoblast cells in the human placenta. These findings are relevant to our present results. However, the reports on iNOS expression among investigators are not in agreement. Several studies have provided evidence of the presence of an iNOS activity in the human placenta, [17–19, 22], which is relevant to the present findings. By contrast, Garvey *et al.* [20] and Schonfelder *et al.* [21] did not find iNOS mRNA in normal placental tissue by RT-PCR. However, the present study identified both iNOS mRNA (Fig. 2B) and protein (Fig. 3B) expression in term placental tissues by quantitative RT-PCR and Western blot analysis, respectively. In the present study, we used

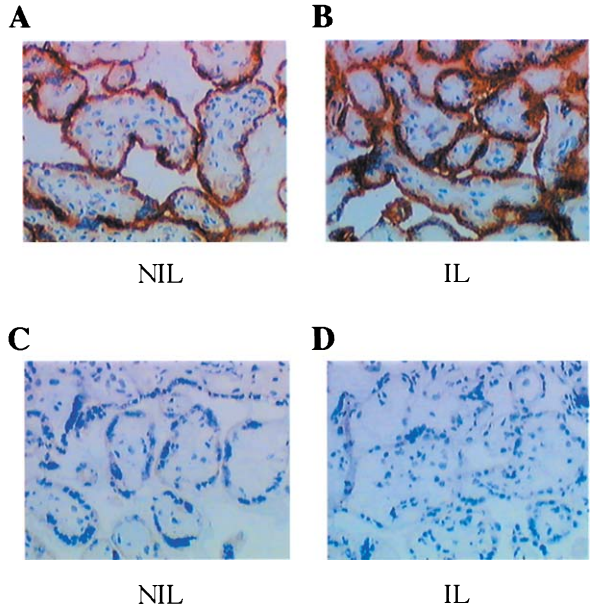

Fig. 4. Immunohistochemistry of eNOS protein in placental tissues obtained from pregnant women at 37 weeks of gestation before the onset of labor (NIL; A, C) and from women at 38 weeks of gestation in labor (IL; B, D). Brown indicates positive staining. Original magnification was × 200. Negative control using normal mouse IgG (C, D) showed greatly reduced staining. The staining is representative of three placental tissues obtained from pregnant women before the onset of labor and three specimens obtained after the onset of labor.

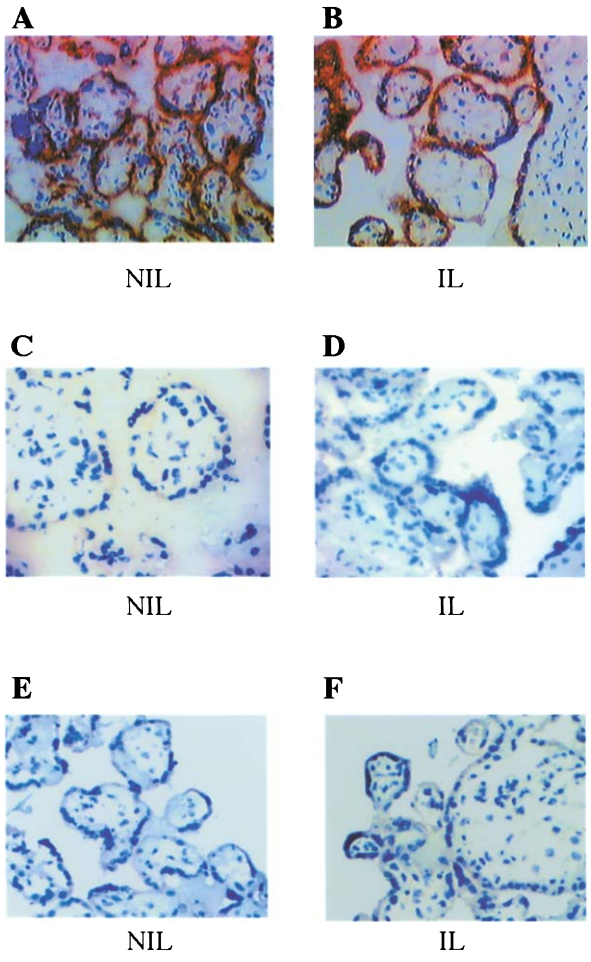

Fig. 5. Immunohistochemistry of iNOS in placental tissues obtained from pregnant women at 37 weeks of gestation not in labor (NIL; A, C, E) and from women at 38 weeks of gestation in labor (IL; B, D, F). Brown indicates positive staining. Original magnifications was × 200. Negative controls using antibody adsorption by mouse macrophage extract (C, D) and normal mouse IgG (E, F) showed greatly reduced staining. The staining is representative of three placental tissues obtained from pregnant women before labor (NIL) and three placental tissues from women after labor onset (IL).

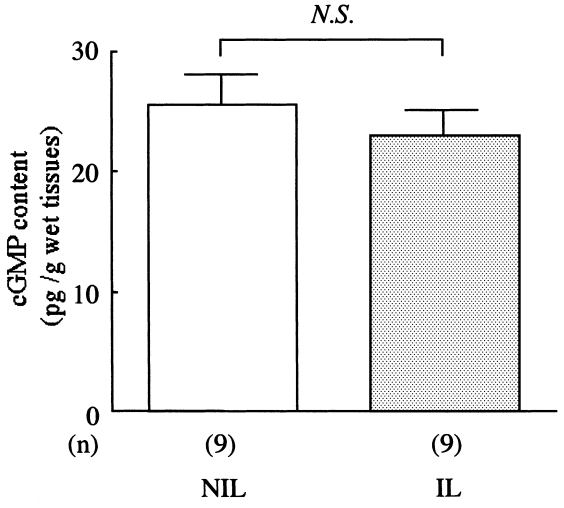

Fig. 6. cGMP content in placental tissues obtained from term pregnant women not in labor (NIL; open column) and from women in labor (IL; closed column). Columns and error bars indicate the means ± SEM, respectively. *N.S.*; not significant.

quantitative RT-PCR by real time TaqMan™ technology, which was reported to be superior in detection sensitivity to conventional Northern blot analysis [28]. As for tissue distribution of iNOS in the placenta, Myatt *et al*. reported that iNOS immunoreactivity was localized to stromal cells of the villous structure [18]. By contrast, Napolitano *et al.* [25] demonstrated iNOS mRNA expression in cultured placental trophoblastic cells. Thus, the tissue distribution of iNOS in human placenta remains controversial. In the present study,

iNOS immunostaining was observed mostly in syncytiotrophoblasts and endothelial cells (Fig. 5A, B). We carried out iNOS antibody adsorption study (Fig. 5C, D) to confirm our findings, in view of the conflicting reports on iNOS tissue distribution in the placenta. The results of our adsorption experiments with macrophage extract showed immunoreactivity to the antibody indicating iNOS.

In human feto-placental circulation, nitric oxide has been reported to attenuate the action of vasoconstrictive substances [33] and to contribute to the maintenance of reduced vascular resistance [34]. Infusion of nitric oxide donor into the maternal circulation reduces uterine artery resistance [35] and improves the waveforms of blood flow velocity in the umbilical artery [36]. Therefore, placental nitric oxide is hypothesized to play significant roles by contributing to the regulation of both utero-placental and feto-placental circulation. During active labor, maternal uterine arterial blood flow is intermittently but markedly interrupted by cyclic uterine contraction [37, 38]. Thus, uterine blood flow to the placenta is repeatedly reduced during uterine contraction followed by reperfusion during uterine relaxation. Shear stress [39] and/or changes in vascular tone [40] caused by such alterations in blood flow were reported to affect local nitric oxide production. However, the present study demonstrated that both eNOS and iNOS expression (Fig. 2, 3) as well as the tissue distribution pattern (Fig. 4, 5) in placental tissues obtained after the onset of labor were similar to those in the placenta obtained before the onset of labor (NIL). Moreover, cGMP concentration in placental tissues obtained after labor onset (IL) was also similar to that in the placenta obtained before labor onset (NIL) (Fig. 6), supporting the possibility that net nitric oxide production in placenta was maintained unchanged during labor. Taken together, it is plausible that both eNOS and iNOS expressed in the placental tissues are stable irrespective of labor, and may contribute to maintenance of placental vascular tone during labor. Longitudinal *in vivo* studies are necessary to confirm this possibility. Nitric oxide production is significantly affected by the stimulation of ischemia/reperfusion in several tissues, such as the heart [41], skeletal muscle [42] and kidney [43]. Thus, constant nitric oxide production in the human placenta during labor may be characteristic of the human placenta. Besides vasodilative nitric oxide, various kinds of vasoconstrictive substances were hypothesized to be involved in the regulation of placental blood flow, such as endothelin-1, prostaglandin, and angiotensin-II [44, 45]. Both endothelin-1 and angiotensin-II contract vascular smooth muscle, while nitric oxide and prostacyclin relax vascular smooth muscle and attenuate the contractive action of endothelin-1 and angiotensin-II [44, 45]. However, the entire scheme of changes of expression during labor has yet to be fully elucidated. Therefore, more investigation is necessary to confirm the physiological significance of the stable expression of both eNOS and iNOS in the regulation of placental blood flow.

The iNOS mRNA expression in the chorionic villous tissues in the first trimester was significantly higher than that in the term placenta (Fig. 2B). By contrast, no such difference was observed in eNOS expression (Fig. 2A). This discrepancy suggested the possibly important role of iNOS in the development of chorionic villi during early gestation. In rodents, Saxena *et al*. reported up-regulation of iNOS in implantation site [46] and Fuhrmann *et al.* demonstrated the importance of the combination of iNOS and progesterone in implantation [47]. By contrast, Ariel *et al.* identified eNOS immunoreactiviry in the human extravillous trophoblastic cells, suggesting the possible involvement of eNOS in human implantation [48]. Thus, the physiological role of iNOS as well as eNOS in the chorionic villous tissues in the first trimester is still obscure in humans [49]. The higher expression of iNOS in the first trimester chorionic villi than in the term placenta supports the relative importance of iNOS in implantation. However, more studies are necessary to confirm the possible role of augmented expression of iNOS mRNA in the chorionic villous tissues in early gestation. In contrast, eNOS expression did not differ among gestations, and was stable even during labor, supporting the relative important of eNOS in the maintenance of vascular tone throughout pregnancy.

The present study demonstrated that eNOS as well as iNOS immunostaining was observed in syncytiotrophoblast cells (Fig. 4, 5). Since syncytiotrophoblast cells are physiologically in direct contact with maternal blood flow within the intervillous space, it is plausible that nitric oxide produced by eNOS and/or iNOS in syncytiotrophoblast cells may affect blood cells in the maternal blood in the intervillous space. Indeed retro-placental blood obtained at placental delivery contained considerable amounts of nitric oxide me-

tabolites [26], although the exact origin of these substances remains unclear. The maternal blood cells in the intervillous space are hypothesized to be in a so-called hypocoagulative state by the action of various anti-coagulative substances [50, 51]. It is hypothesized that uterine contraction by labor decreases the blood flow in the placental intervillous space [52, 53]. Since nitric oxide also acts as a potent anti-coagulant [54], it is possible that such a constant nitric oxide production in syncytiotrophoblast cells may, at least partly, play a role in protecting intervillous maternal blood cells from over-coagulation by the influence of intermittent interruption of maternal blood flow during labor. Further *in vitro* and *in vivo* studies are necessary to clarify this possibility.

In summary, the present study demonstrated that eNOS as well as iNOS expression in the term placenta after the onset of labor were similar to those in the placenta obtained before the onset of labor, suggesting a constant nitric oxide production in placental tissues during labor.

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