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Cytotrophoblasts Up-Regulate Soluble Fms-Like Tyrosine Kinase-1 Expression under Reduced Oxygen: An Implication for the Placental Vascular Development and the Pathophysiology of Preeclampsia

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Sufficient cytotrophoblast (CT) invasion into the uterine wall and subsequent remodeling of maternal uterine vasculature is critical to establish uteroplacental circulation. The production of vascular endothelial growth factor (VEGF) family molecules is confirmed in placental cells including CTs, but it is not elucidated how the VEGF system in CTs is controlled by oxygen tension and how it is involved in the development of placental circulation. To address this, we explored the effect of oxygen tension on the expression of VEGF, placenta growth factor (PlGF), and their antagonist, soluble fms-like tyrosine kinase-1 (sFlt-1) using ELISA and real-time PCR in a primary CT cell culture. For comparison, the same was conducted in parallel using other cells comprising placenta, such as human umbilical vein endothelial cells (HUVECs) and villous fibroblasts (VFs). Reduced oxygen resulted in a pronounced increase in sFlt-1 mRNA amount and sFlt-1 release into the culture media in CTs, whereas this was not the case with HUVECs and VFs. Free (not bound to sFlt-1) VEGF was not detected in CT culture media regardless of oxygen concentration, even though VEGF expression was stimulated by reduced oxygen in CTs, which was similar to the stimulation in HUVECs and VFs. Free PlGF was also diminished in CT culture media by reduced oxygen. These results implicate that CTs possess a unique property to enhance sFlt-1 production under reduced oxygen, which could consequently antagonize angiogenic activity of VEGF and PlGF. The presented findings might provide a framework with which to understand the mechanism of uterine vascular remodeling and its perturbations as exemplified in preeclampsia. (*Endocrinology* 145: 4838–4845, 2004)

ADEQUATE DEVELOPMENT OF fetomaternal vasculature is a prerequisite for successful implantation of the embryo and its ensuing growth. On the other hand, aberrations of fetoplacental vasculature, as exemplified by shallow invasion of cytotrophoblasts (CTs) into the uterine spiral arteries and resultant immature remodeling of the uterine vessels, are thought to be central to the pathogenesis of preeclampsia and intrauterine fetal growth restriction (1–3).

CTs are highly specialized epithelial cells comprising the villous structure of the placenta. At early pregnancy, CTs located at the tips of the villi proliferate extensively and form trophoblastic cell columns, which are eventually anchored to the maternal side, the decidua. Then, they breach the superficial portions of the uterine walls and remodel the vessels by replacing the endothelial lining, resulting in the completion of the physical connections between the fetus and the mother (1, 4). As such, CTs seem to play a pivotal role in the development of fetoplacental vasculature.

It must be admitted that extended vascular pruning or remodeling taking place in the uterine wall represent fundamental mechanisms of the development of the fetoplacental circulation. Although vascular remodeling occurring at the fetomaternal interface is executed by coordinated interplay between the fetus and mother, several recent lines of evidence have established essential roles played by CTs in this process. Specifically, angiogenic factors, such as vascular endothelial growth factor (VEGF) (5–8) and placenta growth factor (PlGF) (9), are suggested to act locally via their receptors and thus control the vascular remodeling required for the establishment of the fetoplacental circulation.

In general, angiogenesis is driven by hypoxic conditions, as seen in a growing tumor (10), and diabetic retinopathy (11). Looking at placental development, which proceeds in an environment of relative low oxygen tension, we may speculate that low oxygen tension could serve to stimulate the production of angiogenic substances. Actually, the oxygen tension environment surrounding CTs drastically alters depending on the location in the placenta and the gestational age. As reported in previous work (12), local oxygen pressure in the placenta is lower in the villous side (18 mm Hg) compared with the maternal side (40 mm Hg) at 8–10 gestational weeks, but it increases up to 60 mm Hg after maternal blood is supplied into the intervillous space (later than 12 gestational weeks). Therefore, it can be hypothesized that

Abbreviations: CT, Cytotrophoblast; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; msFlt-1, membrane-spanning Flt-1 PlGF, placenta growth factor; sFlt-1, soluble fms-like tyrosine kinase-1; VEGF, vascular endothelial growth factor; VF, villous fibroblast.

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local oxygen tension of the placenta controls the activity of CTs required for the vascular remodeling and then works as a major regulator for the reconstitution of uterine vascular structure. The mechanism underlying uterine vascular remodeling is closely related in the pathophysiology of preeclampsia because incomplete uterine vascular remodeling has been suggested in preeclamptic placenta (1–3). At present, however, little is known regarding a link between oxygen tension and the production of angiogenic substances by CTs. Furthermore, it is unclear how these angiogenic substances are involved in the process of vascular remodeling.

With this background in mind, we attempted to explore how oxygen tension regulates the expression of VEGF and PlGF using primary cell culture of CTs. We also focused on the regulation of the expression of soluble fms-like tyrosine-kinase-1 (sFlt-1), which is a potent biological antagonist for both VEGF and PlGF (13–15), and thus how this could modify the effect of both angiogenic factors. As well as examining the relation of oxygen to the VEGF system regulation in CTs, it is important to observe any difference in response to oxygen tension between CTs and other cellular components comprising the placenta. To address this, we looked at the effects of oxygen in human umbilical vein endothelial cells (HUVECs) and villous fibroblasts (VFs) and showed that CTs in a reduced oxygen state enhance their capacity to produce sFlt-1.

Materials and Methods

All the sample collections and the experiments in this study were conducted with the approval of the Ethical Committee of Medical Faculty at the University of Tokyo.

Isolation of CTs and VFs

The procedures to isolate CTs and VFs were performed as previously described (16). Briefly, the first trimester placenta tissues (5–10 wk of gestation) were obtained from the cases of legal abortion with consent. Minced villi tissues were digested in PBS (Sigma, St. Louis, MO) supplemented with 0.125% trypsin (GibcoBRL, Grand Island, NY), 0.42 mM $MgSO_4$, and 20 U/ml DNase type 1 (Invitrogen, Carlsbad, CA) at 37 C for 20 min. After passing through a mesh (100-μm pore size), the collected cells were separated in three layered Percoll (Sigma) density gradients (4 ml of 40%, 4 ml of 25%, and 0% Percoll layers in a 15-ml conical tube). After the centrifugation at 800 $\times$ *g* for 20 min, the floating cells between 25 and 40% Percoll layer were collected. The cells were incubated with anti-CD9 antibody-bound (clone P1/33/2; Dako, Kyoto, Japan) magnetic beads (Cellection PanMouse IgG Kit; Dynal Biotech, Oslo, Norway) for 30 min at 4 C to exclude contaminating stromal cells.

VFs were removed due to their attaching to anti-CD9-bound magnetic beads in the previously mentioned process of CT isolation. The cell-magnetic bead complexes were incubated with DNase for 15 min at room temperature to detach the beads from these cells according to the manufacturer's instructions. After removing beads by magnetic collector, the detached cells were cultured in medium 199 (Sigma) supplemented with 10% fetal bovine serum (FBS; Sigma) on culture flasks. VFs proliferated rapidly by changing medium every 3 d and were obtained with high purity 14 d after the isolation.

Culture of HUVECs

HUVECs in the first passage were obtained from CAMBREX Bio Science (Walkersville, MD) and maintained in endothelial cell basic medium supplemented with a growth factor mixture containing hydrocortisone, heparin, β-fibroblast growth factor, VEGF, IGF-I, human epidermal growth factor, and FBS (0.2%) (EGM-2; CAMBREX Bio Science). All the experiments were conducted using the 3–5 times passed cells.

Cell lines

The choriocarcinoma-derived cell lines, BeWo, JAR, and JEG3, were obtained from American type culture collection. They were maintained in RPMI 1640 (Sigma) supplemented with 10% FBS.

Culture under different oxygen conditions

The freshly isolated CTs were resuspended in defined Keratinocyte SFM (GibcoBRL) at a concentration of 1×10^5 cells/ml and seeded on collagen type 4-coated 35-mm dishes (2 ml/dish). After 3 h of incubation in ambient oxygen culture condition (5% CO_2-20% O_2-75% N_2) at 37 C, the medium was refreshed to remove the debris, and then CTs were exposed to different oxygen conditions.

BeWo, JAR, JEG3, VFs, and HUVECs in 80% confluency were detached from the culture flasks with 0.05% trypsin and 0.53 mM EDTA (GibcoBRL). After being washed twice with PBS, they were diluted to 4×10^4 cells/ml in RPMI 1640 plus 10% FBS (BeWo, JAR, and JEG3), medium 199 plus 10% FBS (VFs), and endothelial basic medium plus 1% FBS (HUVECs) and were seeded on 35-mm dishes (2 ml/dish) with no extracellular matrix coating.

The cells were prepared in two sets of culture dishes, one for cell culture in ambient oxygen condition and the other for cell culture in reduced oxygen conditions (5% CO_2-2% O_2-93% N_2 or 5% CO_2-8% O_2-87% N_2) at 37 C, with the oxygen concentration being correctly regulated in a BioLabo Multigas Incubator (Jujifield, Tokyo, Japan) for different periods.

The assessment of cell viability and cell number in culture

The cultured cells were detached from culture dishes with 0.05% trypsin-EDTA (GibcoBRL) at 72 h of culture under different oxygen conditions. The cells were stained with trypan blue (GibcoBRL), and the viable cells without staining were counted on a hemocytometer.

Enzyme immunoassays

The culture media under ambient and reduced oxygen conditions were collected at different incubation periods. Free VEGF (not bound to sFlt-1), free PlGF, and sFlt-1 concentrations in each culture media were measured using sandwich ELISA (Quantikine ELISA kit; R&D Systems, Minneapolis, MN). All the procedures were performed according to the manufacturer's instructions. Total VEGF (the sum of free and sFlt-1-combined VEGF) concentrations were measured using competitive enzyme immunoassay (ChemiKine EIA kit; Chemicon International, Temecula, CA). The minimal detectable doses of assays were 5.0 pg/ml (free VEGF), 7.0 pg/ml (PlGF), 5.0 pg/ml (sFlt-1), and 195 pg/ml (total VEGF). When the concentrations were below the detectable dose, they were regarded as zero.

RNA isolation and reverse-transcriptase reaction

Total RNA of cells was isolated using RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amount of the isolated RNA was assessed spectrophotometrically. The total RNA was reverse transcribed into cDNA using the Rever Tra Ace kit (Toyobo, Osaka, Japan) in the volume of 40 μl, including 8 μl of 5$\times$ reverse-transcriptase buffer, 4 μl deoxynucleotide triphosphate, 2 μl RNA inhibitor, 2 μl Rever Tra transcriptase, 2 μl random primer, and 22 μl sample RNA in a thermal cycler (30 C for 10 min, 42 C for 20 min, and 99 C for 5 min).

Real-time PCR

Real-time PCR was carried out to semiquantify the amounts of mRNA expression of VEGF, PlGF, sFlt-1, and membrane-spanning Flt-1 (msFlt-1) in the cultured cells. The primer sequences, their location, and their annealing temperatures are summarized in Table 1. VEGF primers amplified the sequence between exon 1 and exon 3, which was shared by all known splicing variants of VEGF (17). The specific primers for

PlGF were designed to amplify the sequence common to all known PlGF splicing variants (PlGF, PlGF2, and PlGF3) (18, 19). To quantify sFlt-1 and msFlt-1 expression distinctively, the antisense primer sequences for them resided in unique regions to each mRNA, whereas the same sense primer was commonly used for both sFlt-1 and msFlt-1.

Real-time PCR was performed using the Light Cycler system (Roche Diagnostics, Lewes, UK). PCR reaction was performed in a total volume of 20 μl mixture including 5 ng cDNA, 2 μl LightCycler FastStart Reaction Mix SYBR Green 1 (Roche Diagnostics), 0.5 μM each primer, and 3 mM $MgCl_2$. After 10 min of denaturing at 95 C, 40 cycles of amplification were carried out (95 C denaturation for 15 sec, annealing for 10 sec, and 72 C extension). The annealing temperature for each primer pair is shown in Table 1, and the extension time depended on the PCR product size (1 sec per each 25 bp). β-actin mRNA was quantified in each sample as an internal control to normalize the level of mRNA among samples. After the amplification program, PCR products were analyzed by melting curve to confirm the amplification specificity. The PCR product inserted in pCRII-TOPO plasmid vector was subcloned using the TOPO TA cloning kit (Invitrogen), and the accordance of its nucleotide sequence with the ever-reported one was confirmed by sequencing analysis.

Statistical analysis

Statistical analysis was performed using Wilcoxon's test for paired data obtained from paired culture in which the cells isolated from the same placenta tissue were assigned to two different oxygen conditions (20 and 2% pair culture or 20 and 8% pair culture). All the values were presented as means ± SE, and $P < 0.05$ was considered significant. In the results of real-time PCR analysis, the mRNA amounts in samples obtained from culture groups in reduced oxygen conditions were shown as relative values to those under 20% O_2 conditions after compensation with β-actin mRNA amount in each sample. In each experiment, n equals the number of different individuals who supplied the placental tissues.

Results

Culture purity

The present culture method yielded 95% purity for CTs and 99% purity for VFs as confirmed by anticytokeratin and antivimentin staining (16).

The effect of reduced oxygen on viable cell number in culture

The cell viabilities of cultured CTs and VFs under ambient oxygen condition were maintained at least up to 144 h in culture as confirmed using WST-8 assay in our previous work (16). We compared viable cell numbers among culture groups of different oxygen conditions using trypan blue exclusion test. In all oxygen conditions, the percentage of stained cells at 72 h in culture was less than 0.5%, regardless of cultured cell types. The cell numbers of CTs were 2.11 ± 0.29 × 10^5 cells/dish (20% O_2, n = 12), 2.47 ± 0.38 × 10^5 cells/dish (8% O_2, n = 6), and 2.90 ± 0.54 × 10^5 cells/dish (2% O_2, n = 6; $P < 0.05$), indicating that the reduced oxygen condition promoted CT proliferation (Fig. 1A) in agreement with the previous report (20). VFs and HUVECs showed no significant change in the cell numbers under reduced oxygen condition (VFs: 2.23 ± 0.24 × 10^5 cells/dish in 20% O_2 and 2.18 ± 0.26 × 10^5 cells/dish in 2% O_2, n = 6; HUVECs: 2.29 ± 0.085 × 10^5 cells/dish in 20% O_2 and 2.31 ± 0.11 × 10^5 cells/dish in 2% O_2, n = 6; Fig. 1B).

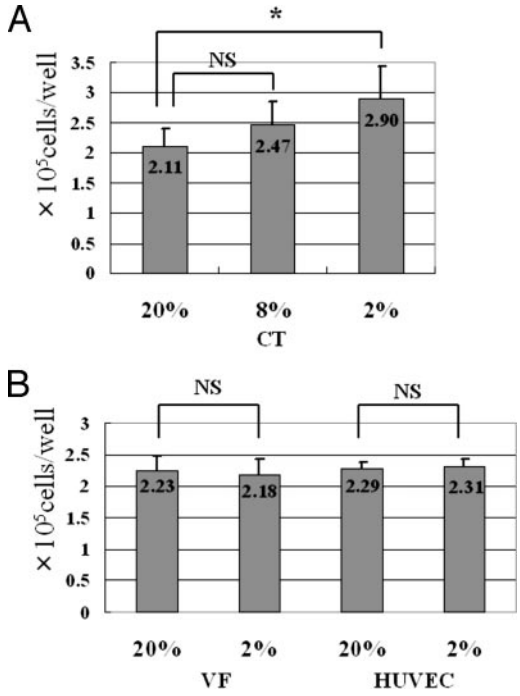

FIG. 1. The influence on the viable cell number in culture under reduced oxygen condition. The number of viable cells was assessed at 72 h of culture under different oxygen conditions by trypan blue exclusion test. CTs increased in cell number with the decrease of oxygen tension (A), whereas HUVECs and VFs showed no change (B). In A: n = 12, 20% O_2; n = 6, 8% O_2 and 2% O_2. In B: HUVECs, n = 6, 20% O_2 and 2% O_2; and VFs, n = 6, 20% O_2 and 2% O_2. *, $P < 0.05$. NS, Not significant.

TABLE 1. Primer sequences used in real-time PCR

	GenBank accession no.	Sense primer (location), antisense primer (location)	Annealing temp. (C)	Ref.
sFlt-1	U01134	5-GCACCTTGGTTGTGGCTGACT-3 (1808-1828) 5-GAGCCCGGGGGTCTCATTATT-3 (2450-2430)	63	30
msFlt-1	X51602	5-GCACCTTGGTTGTGGCTGACT-3 (1808-1828) 5-CCCTTCTGGTTGGTGGCTTTG-3 (2462-2442)	60	30
VEGF	NM003376.3	5-CGAAACCATGAACTTTCTGC-3 (1032-1051) 5-CCTCAGTGGGCACACACTCC-3 (1333-1314)	63	31
PlGF	NM002632	5-CCAGCCACAGCCTTACCTAC-3 (156-175) 5-AGACACAGGATGGGCTGAAC-3 (590-571)	60	[a]
β-Actin	X00351	5-CGACAACGGCTCCGGCATGTGC-3 (71-92) 5-CGTCACCGGAGTCCATCACGATGC-3 (513-490)	63	32

[a] Designed on "primer3" primer-making software (http://biowb.sdsc.edu/).

Reduced oxygen increased the secretion of sFlt-1 in CTs but not in HUVECs and VFs

Preliminarily, we examined whether coexisting VEGF and PlGF in culture media might hinder correct measurement of sFlt-1 by the ELISA kit used in this study. No interference was found with the standard curve for sFlt-1 by the presence of either recombinant VEGF or PlGF (Fig. 2, A and B).

sFlt-1 concentrations in CT culture media increased with time in culture up to 144 h (258 ± 95 pg/ml at 48 h, 1576 ± 622 pg/ml at 96 h, and 3796 ± 1156 pg/ml at 144 h; n = 6; Fig. 3A). When CTs were cultured for 72 h in different oxygen conditions, sFlt-1 concentrations in CT culture media were 920 ± 180 pg/ml (20% O_2, n = 18), 1223 ± 408 pg/ml (8% O_2, n = 8, $P < 0.05$), and 1865 ± 457 pg/ml (2% O_2, n = 10, $P < 0.01$; Fig. 3B). Thus, sFlt-1 concentrations increased with a decrease in O_2 concentration. sFlt-1 was also detected in the culture media of both HUVECs and VFs at 72 h. However, sFlt-1 concentrations in the culture media of HUVECs and VFs did not differ between 20 and 2% O_2 condition (HUVECs: 1120 ± 178 pg/ml, 20% O_2, n = 8 and 909 ± 186 pg/ml, 2% O_2, n = 8; VFs: 41 ± 16 pg/ml, 20% O_2, n = 10 and 40 ± 16 pg/ml, 2% O_2, n = 10; Fig. 3B).

Reduced oxygen increased sFlt-1 mRNA in CTs but not in HUVECs and VFs

To investigate whether oxygen conditions regulate sFlt-1 expression at the pretranslational level, mRNA of sFlt-1 was semiquantified using real-time PCR. The amounts of sFlt-1 mRNA expression in CTs at 72 h culture increased with a decrease in O_2 concentration (9.30 ± 3.24-fold, 2% O_2, n = 10, $P < 0.01$ and 2.12 ± 0.40-fold, 8% O_2, n = 8, $P < 0.05$) relative to 20% O_2 condition. sFlt-1 mRNA expression was not affected by O_2 concentration in HUVECs (0.83 ± 0.12-fold, 2% O_2, n = 8) relative to 20% O_2 and in VFs (0.90 ± 0.50-fold, 2% O_2, n = 10) relative to 20% O_2 (Fig. 3C).

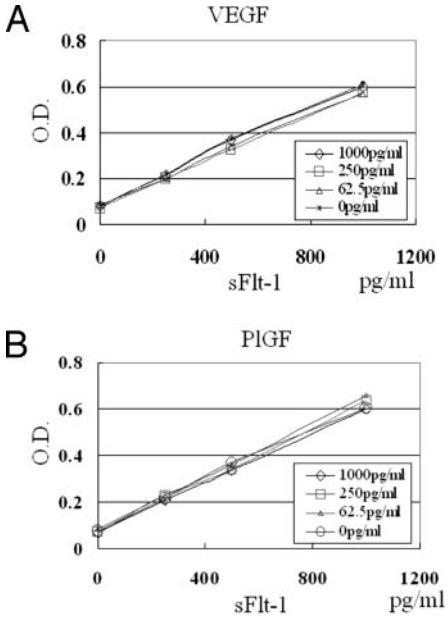

FIG. 2. The interference of coexisting VEGF (A) and PlGF (B) to measured sFlt-1 value. The dose OD standard curve of sFlt-1 was generated using sFlt-1 ELISA kit used in this study at the presence of different concentrations of VEGF (A) or PlGF (B; 0, 250, 500, and 1000 pg/ml). No major shift of standard curves was observed regardless of the concentration (up to 1000 pg/ml) of VEGF and PlGF added to the sFlt-1 standard solutions, indicating that measured sFLt-1 value was not interfered with by coexisting VEGF and PlGF.

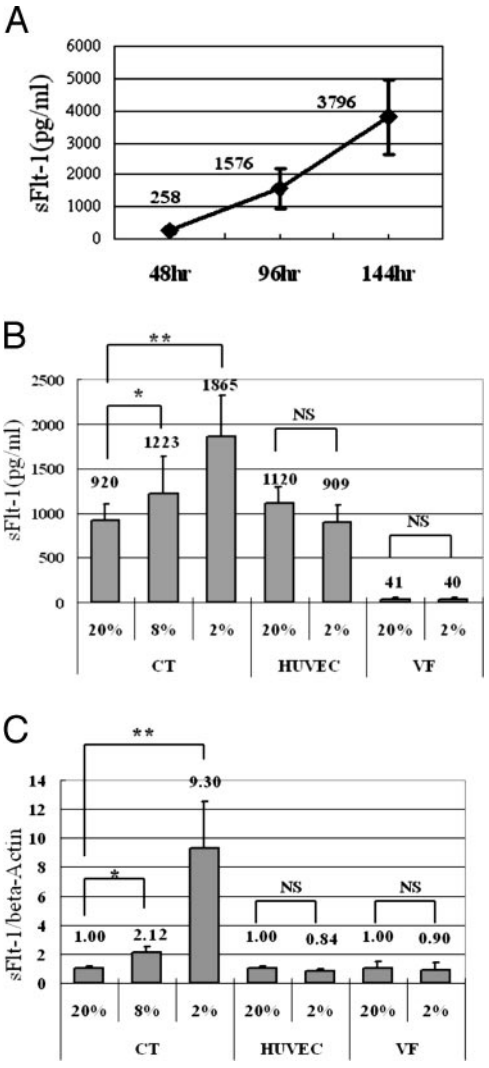

FIG. 3. The effect of reduced oxygen on the sFlt-1 expression in CTs, HUVECs, and VFs. A, The sFlt-1 concentrations in the culture media of CTs in 20% O_2 condition were measured using ELISA at 48, 96, and 144 h of culture. The concentrations increased with time in culture, revealing the continuous sFlt-1 release from cultured CTs. B, The sFlt-1 concentrations in the culture media were measured at 72 h of culture under different oxygen conditions (20, 8, and 2%). The sFlt-1 concentrations in CT culture media increased with a decrease of oxygen concentration, whereas those in HUVEC and VF culture media were unchanged. C, Real-time PCR analysis was performed to semiquantify the sFlt-1 mRNA at 72 h of culture under different oxygen conditions. Under reduced oxygen conditions (2 and 8%), sFlt-1 mRNA in CTs was strikingly increased compared with the 20% O_2 condition. Reduced oxygen (2% O_2) caused no significant difference in sFlt-1 mRNA in HUVECs and VFs. In A: n = 5. In B and C: CTs, n = 18, 20% O_2; n = 8, 8% O_2; and n = 10, 2% O_2; and HUVECs, n = 8, 20% O_2 and 2% O_2; and VFs, n = 10, 20% O_2 and 2% O_2. *, $P < 0.05$; **, $P < 0.01$. NS, Not significant.

Reduced oxygen increased msFlt-1 mRNA commonly in CTs and HUVECs

Real-time PCR analysis revealed that the amount of msFlt-1 mRNA in CTs slightly but significantly increased with a decrease in O_2 concentration (1.82 ± 0.48-fold, 2% O_2, n = 10, $P < 0.05$ and 1.20 ± 0.07-fold, 8% O_2, n = 8, P = not significant) relative to 20% O_2 condition (Fig. 4). The mRNA of msFlt-1 increased under reduced oxygen condition in HUVECs (1.54 ± 0.06-fold relative to 20% O_2, n = 6, $P < 0.05$) but not in VFs (Fig. 4). It should be noted that the degree of the increase in the msFlt-1 mRNA amount in CTs exposed to hypoxia was far smaller than that in sFlt-1.

VEGF expression was increased under hypoxia in CTs, HUVECs, and VFs

The sandwich ELISA kit used in this study detects VEGF that is not bound to coexisting sFlt-1 in culture media (21, 22). VEGF measurement by the competitive enzyme immunoassay is not hindered by the presence of sFlt-1 (22). In this study, the concentrations of VEGF were measured using these two enzyme immunoassays (*i.e.* sandwich ELISA for free VEGF and competitive enzyme immunoassay for total VEGF). Although free VEGF was observed in the culture media of the choriocarcinoma cell lines, JEG3 (1788 pg/ml at 72 h in 20% O_2) and JAR (1825 pg/ml at 72 h in 20% O_2), free VEGF was not detected in the culture media of CTs regardless of oxygen concentration (detectable limit, 5 pg/ml; Fig. 5A). Using the same ELISA kit, free VEGF in the culture media from VFs was detectable and significantly increased under lower O_2 concentration (27 ± 7.8 pg/ml, 20% O_2, n = 10 *vs.* 51 ± 11.5 pg/ml, 2% O_2, n = 10, $P < 0.01$; Fig. 5A). The concentrations of total VEGF in CT culture media increased significantly with a decrease in O_2 concentration (246 ± 41 pg/ml, 20% O_2, n = 18; 359 ± 70 pg/ml, 8% O_2, n = 8, $P < 0.05$; and 472 ± 58 pg/ml, 2% O_2, n = 10, $P < 0.01$; Fig. 5B).

Real-time PCR analysis revealed that the amounts of VEGF mRNA in CTs at 72 h of culture increased with a decrease in O_2 concentration (2.31 ± 0.43-fold, 2% O_2, n = 10, $P < 0.05$ and 1.53 ± 0.42-fold, 8% O_2, n = 8, $P < 0.05$) relative to 20% O_2 (Fig. 5C). Likewise, the amounts of VEGF mRNA in HUVEC and VF culture for 72 h in 2% O_2 significantly increased compared with the amounts of VEGF mRNA cultured in 20% O_2 (4.7 ± 1.45-fold for HUVECs, n = 8, $P < 0.05$; and 1.74 ± 0.41-fold for VFs, n = 10, $P < 0.01$; Fig. 5C).

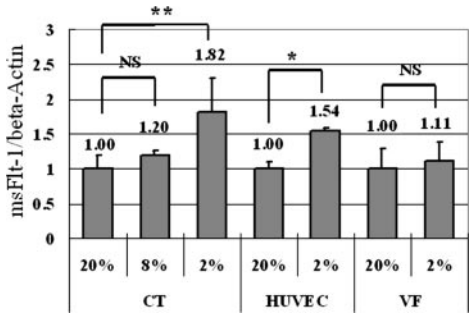

FIG. 4. The effect of reduced oxygen on msFlt-1 mRNA amount in CTs, HUVECs, and VFs. Real-time PCR analysis demonstrated that reduced oxygen slightly increased msFlt-1 mRNA amount in CTs and HUVECs at 72 h of culture but not in VFs. CTs: n = 18, 20% O_2; n = 8, 8% O_2; and n = 10, 2% O_2; HUVECs: n = 8, 20% O_2 and 2% O_2; and VFs: n = 10, 20% O_2 and 2% O_2. *, $P < 0.05$; **, $P < 0.01$. NS, Not significant.

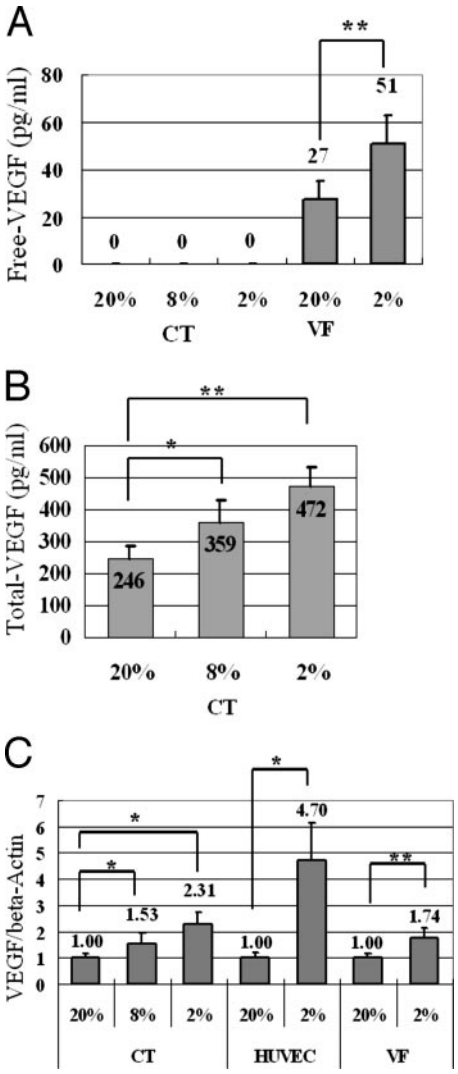

FIG. 5. The effect of oxygen tension on the VEGF expression in CTs, HUVECs, and VFs. Using two types of enzyme immunoassays, free (not bound to sFlt-1) VEGF (A) and total (the sum of free and sFlt-1-combined) VEGF (B) concentrations in their culture media were measured at 72 h of culture under different oxygen conditions. The free VEGF concentrations in culture media of CTs were under detectable limit regardless of the oxygen condition, whereas the free VEGF in VF culture media increased under 2% O_2 condition compared with 20% O_2 (A). The total VEGF concentrations in CT culture media increased with a decrease of oxygen concentration (B). C, Real-time PCR was conducted to investigate the VEGF mRNA amount at 72 h of culture under different oxygen conditions. Reduced oxygen significantly increased VEGF mRNA in all types of cultured cells. In A–C: CTs, n = 18, 20% O_2 and n = 10, 2% O_2; HUVECs, n = 8, 20% O_2 and 2% O_2; and VFs, n = 10, 20% O_2 and 2% O_2. *, $P < 0.05$; **, $P < 0.01$.

Reduced oxygen decreased free PlGF levels in the culture media of CTs

The ELISA kit for PlGF used in this study detects only free PlGF and not PlGF bound to sFlt-1 (21). Free PlGF concentrations in the culture media of CTs increased with time in

culture (1115 ± 166 pg/ml at 48 h, 1498 ± 140 pg/ml at 96 h, and 2146 ± 614 pg/ml at 144 h, n = 6; Fig. 6A). Free PlGF concentrations in the culture media of CTs cultured for 72 h reduced with a decrease in O_2 concentration (1293 ± 123 pg/ml, 20% O_2, n = 18; 1089 ± 124 pg/ml, 8% O_2, n = 8, $P < 0.01$; and 667 ± 70 pg/ml, 2% O_2, n = 10, $P < 0.01$; Fig. 6B). On the other hand, no significant difference in free PlGF concentrations was observed in the culture media of HUVECs cultured for 72 h in 20% O_2 (290 ± 35 pg/ml, n = 8) *vs.* 2% O_2 (295 ± 39 pg/ml, n = 8; Fig. 6B). PlGF was not detected in VF culture media under both 20 and 2% O_2 conditions (Fig. 6B). Real-time PCR analysis revealed that the amounts of PlGF mRNA in CTs were not altered among the 20% O_2, 8% O_2 (0.88 ± 0.06-fold relative to 20% O_2, n = 8), and 2% O_2 (1.31 ± 0.34-fold relative to 20% O_2, n = 10) conditions (Fig. 6C). Likewise, the amounts of PlGF mRNA in HUVECs were not altered between the 20 and 2% O_2 conditions (1.11 ± 0.31-fold relative to 20% O_2, n = 8; Fig. 6C).

In a previous report, reduced oxygen diminished PlGF mRNA amount in the choriocarcinoma-derived cell line, BeWo (23). To settle the controversial difference between our result on primary CTs and their finding, the PlGF mRNA amount in BeWo was compared between the 20 and 2% O_2 conditions at 72 h in culture. The PlGF mRNA level in 2% O_2 condition (0.35 ± 0.11-fold relative to 20% O_2, $P < 0.05$; the data were obtained from six independent experiments) was significantly lower than the level in 20% O_2 condition, which was in agreement with the reported finding (Fig. 6D).

Discussion

In this study, we investigated the effects of oxygen tension on the production of VEGF, PlGF, and their antagonist, sFlt-1, by CTs in comparison with HUVECs and VFs. It was revealed that CTs augmented the production of sFlt-1 in response to reduced oxygen, which is a unique property of CTs because this was not case with HUVECs and VFs. An increase in sFlt-1 production by CTs was brought about, at least in part, at the pretranslational level as judged by the concurrent pronounced increase in sFlt-1 mRNA. On the other hand, the amounts of msFlt-1 mRNA significantly increased in both CTs and HUVECs. Our data seem to be in agreement with the previous study demonstrating an increase in Flt-1 expression in HUVECs by reduced oxygen via binding of a hypoxia-inducible factor-1 to the Flt-1 promoter (24). However, the degree of increase in msFlt-1 mRNA caused by reduced oxygen was apparently smaller than that of sFlt-1. It is known that msFlt-1 and sFlt-1 are generated by alternative splicing of the common pre-mRNA through mechanisms that have not yet been identified (13). One possible explanation is that CTs may have unique splicing factors that might induce the splicing of Flt-1 pre-mRNA to an sFlt-1-dominative state. This question remains to be solved in future work.

It is conceivable that sFlt-1 might antagonize the activity of VEGF and PlGF by binding with them (13–15). In the present study, VEGF mRNA increased under a reduced oxygen condition in cells including CTs, HUVECs, and VFs, a finding in keeping with the observations in other tissues and neoplasms (10). In view of sFlt-1 being a VEGF antagonist,

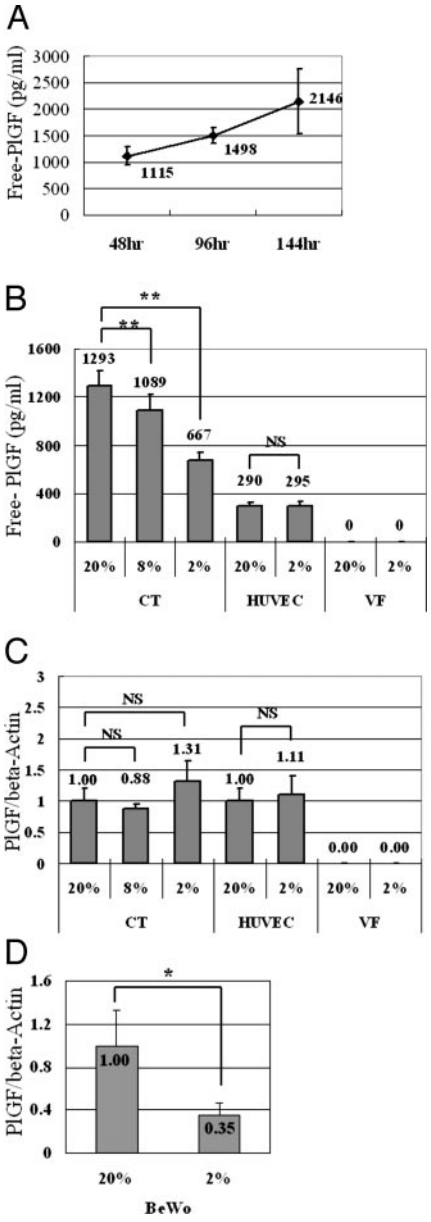

FIG. 6. The effect of oxygen tension on the PlGF expression in CTs, HUVECs, and VFs. A, The free (not bound to sFlt-1) PlGF concentrations in the culture media of CTs under 20% O_2 condition were measured using ELISA at 48, 96, and 144 h of culture. The concentrations increased with time in culture, revealing the continuous PlGF release from cultured CTs. B, The free PlGF concentrations in the culture media were measured at 72 h of culture under different oxygen conditions. The free PlGF concentration in CT culture media was reduced with a decrease of oxygen concentration, whereas the concentration in HUVEC culture media did not. Free PlGF was not detected in the VF culture media. C, Real-time PCR analysis was performed to semiquantify the PlGF mRNA in CTs and HUVECs at 72 h of culture under different oxygen conditions. The PlGF mRNA amounts were not altered regardless of oxygen conditions both in CTs and HUVECs. In A: n = 5. In B and C: CTs, n = 18, 20% O_2; n = 8, 8% O_2; and n = 10, 2% O_2; HUVECs, n = 8, 20% O_2 and 2% O_2; and VFs, n = 10, 20% O_2 and 2% O_2. *, $P < 0.05$; **, $P < 0.01$. NS, Not significant. D, Real-time PCR analysis was performed to semiquantify the PlGF mRNA in BeWo at 72 h of culture under different oxygen conditions. PlGF mRNA was reduced in BeWo in 2% O_2 compared with 20% O_2. The shown data were obtained from six independent experiments.

it is reasonable to speculate that the actual angiogenic potential of the VEGF system is determined by the balance between VEGF and sFlt-1. Notably, in this study, free VEGF was not detectable in the culture media of CTs regardless of oxygen level despite an apparent increase in the total VEGF concentrations in a reduced oxygen milieu, suggesting that sFlt-1 production, concurrently increased in a reduced oxygen milieu, may overrule the increase in VEGF production and, thereby, negate the biological effects of VEGF.

The secretion of sFlt-1-unbound (free) PlGF was diminished in response to reduced oxygen in CTs but not HUVECs, whereas PlGF mRNA amounts were not altered by reduced oxygen both in CTs and HUVECs. Due to an appropriate method to measure total PlGF being unavailable, it remains to be seen how hypoxia influences the production of PlGF in CTs. Although this needs to be addressed by future studies, given a marked increase in sFlt-1 production in CTs under a reduced oxygen milieu, the observed reduction in free PlGF could be a reflection of enhanced sFlt-1 production, as also could be the case with VEGF. We found that BeWo, a choriocarcinoma cell line, down-regulated PlGF mRNA amount under reduced oxygen, which was in accordance with the previous reported observation (23). BeWo demonstrates a different response to oxygen concentration milieu from CTs; *e.g.* BeWo keeps proliferating in ambient oxygen milieu, whereas primary CTs cease to proliferate. The observed discrepancy in the effect of reduced oxygen on PlGF mRNA between primary CTs and BeWo might be due to the difference in the cell characters.

In general, the VEGF system is activated under a hypoxic condition, a reasonable mechanism to hold oxygen tension constant (10). In this study, we observed that HUVECs and VFs stimulated VEGF expression that was unassociated with the up-regulation of sFlt-1 under a reduced oxygen condition, which is in support of the known concept that hypoxia augments angiogenesis by mechanisms involving the VEGF system. These cell properties of HUVECs and VFs seem to be appropriate to develop the vascular network in the villous stroma depending on oxygen demand from the growing fetus. When looking at CTs, however, the response of CTs to reduced oxygen is paradoxical because CTs increase sFlt-1 production in a reduced oxygen milieu. This doesn't seem to fit the hypothesis that VEGF-related angiogenesis is driven by hypoxia. Vascular remodeling in the uterine wall is a specific vascular event to placental development. CTs reconstitute the uterine vascular structure into low-resistance and high-conductance vessels. Considering that maternal endothelial cells are replaced by CTs, uterine vascular remodeling, despite progressing in the relatively hypoxic fetomaternal interface of early gestation, seems to put some restriction on angiogenesis by maternal endothelial cells. In this viewpoint, there might be some discrepancy between the well-accepted notion of angiogenesis and the uterine vascular remodeling. When based on our results, one possible interpretation of this paradox is that sFlt-1, produced by CTs in a reduced oxygen environment, might block angiogenesis by maternal endothelial cells and prepare an appropriate condition for CTs to progress uterine vascular remodeling. Although we just demonstrated that CTs had a property to up-regulate sFlt-1 expression by reduced oxygen and that unbound VEGF was undetectable in the media of CTs cultured under a reduced oxygen condition despite an apparent increase in total VEGF production, this line of reasoning might offer a basic paradigm for the establishment of a placental circulation.

Recently, concentrations of sFlt-1 in peripheral blood in patients with preeclampsia were reported to be higher than those in normal cases (21, 25, 26). Furthermore, pregnant rats given a large amount of sFlt-1 exhibited preeclampsia-like symptoms, and the same symptoms were observed even in case of nonpregnant rats when given sFlt-1 (21). Therefore, it seems logical to assume that sFlt-1 is closely interrelated to the pathology of preeclampsia. The predominant source of sFlt-1 elevation in preeclampsia is estimated to be placenta. CTs isolated from preeclamptic placenta secreted more abundant sFlt-1 than CTs from normal placenta (27). sFlt-1 mRNA level was significantly higher in placentas from preeclamptic patients (28). However, the mechanism of how sFlt-1 expression is elevated in preeclamptic placenta has not been elucidated. Preeclampsia is characterized by incomplete uterine vasculature and, thereby, reduced blood flow into the intervillous space, resulting in placental hypoxia (3, 4, 29). Based on present findings, it is conceivable that CTs in preeclampsia are exposed to abnormally low oxygen concentrations and, thus, produce excessive amounts of sFlt-1, leading to an elevation in sFlt-1 concentrations as is observed in clinical settings. In the same context, our findings regarding the response of VEGF and PlGF expression in CTs to reduced oxygen support the previous study in which VEGF mRNA was increased and PlGF mRNA was not altered in preeclamptic placenta (28).

In conclusion, we provided evidence that CTs possess a unique property in that they enhance their ability to produce sFlt-1, an antagonist for both VEGF and PlGF, in the face of reduced oxygen. Despite an increase in VEGF production in response to reduced oxygen, a concomitant even greater increase in sFlt-1 might override the effects of VEGF and, thereby, rather attenuate the ultimate capacity of CTs to stimulate angiogenesis. This unique response of VEGF system to oxygen tension in CTs might be closely involved in the mechanism of uterine vascular remodeling. Besides, the present findings might give some help for an understanding of the pathogenesis of preeclampsia.

Acknowledgments

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