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—Original Article—

Oxidative Stress Produced by Xanthine Oxidase Induces Apoptosis in Human Extravillous Trophoblast Cells

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Abstract. Oxidative stress has been recognized as an important factor in the pathophysiology of preeclampsia. It has been reported that the expression of xanthine oxidase (XO) in the cytotrophoblast and plasma hydrogen peroxide (H_2O_2) level are significantly higher in preeclamptics than in control women. The aim of this study was to clarify the biological influence of reactive oxygen species (ROS) produced by XO on extravillous trophoblast (EVT) cells. TCL1 cells, a human immortalized EVT cell line, were incubated with xanthine and XO (X/XO). We then measured the cell number, urate level of the culture media and the apoptotic cell ratio. Similar experiments were performed with additional administration of allopurinol, catalase, L-NAME or D-NAME, and with administration of H_2O_2 in substitution for X/XO. We assessed the effects of H_2O_2 on invasion ability, tube-like formation and protein expression of HIF1A and ITGAV of TCL1. Finally, the apoptotic cell ratio using primary cultured trophoblasts was measured following exposure to H_2O_2. X/XO decreased the relative cell number and increased the urate level and apoptotic cell ratio significantly. Elevation of the urate level and apoptotic cell ratio was attenuated by allopurinol and catalase, respectively. L-NAME and D-NAME had no influence on these effects. H_2O_2 also decreased the relative cell number. Pretreatment with H_2O_2 significantly inhibited the invasion ability, tube-like formation and HIF1A and ITGAV of TCL1. H_2O_2 also induced apoptosis in primary cultured trophoblasts. In conclusion, ROS produced by XO induced apoptosis and affected EVT function including invasion and differentiation.

Key words: Apoptosis, Extravillous trophoblast, Oxidative stress, Xanthine oxidase

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Many investigators have demonstrated that insufficient invasion of the extravillous trophoblast (EVT) into maternal tissue and impaired differentiation lead to the placental dysfunction and poor perfusion associated with preeclampsia and intrauterine growth restriction (IUGR) [1]. These events are described as "poor placentation," which is characterized by insufficiency of interstitial invasion and endovascular replacement by the EVT [2]. Poor placentation is likely related to multiple factors including immunological aberration and oxidative stress [3, 4].

Xanthine oxidase (XO), NADPH oxidase (Nox) and the mitochondrial electron transport system are major intracellular sources of reactive oxygen species (ROS). Of these, XO catalyzes hypoxanthine and xanthine into superoxide and uric acid by coupled reactions [5, 6].

Preeclampsia is often accompanied by hyperuricemia, and Many *et al.* actually demonstrated increased expression of XO and alterations due to oxidative stress in placentae from preeclamptic patients [7]. Furthermore, women with hyperuricemia at delivery show higher levels of serum uric acid in comparison to normal women early in pregnancy [8].

We have previously reported that the serum urate levels in women with preeclampsia correlated closely with plasma hydrogen peroxide (H_2O_2) levels and that both were significantly higher in women with preeclampsia than those of normal pregnant women [9]. Recently, it has been reported that preeclamptic patients exhibit higher H_2O_2 and lower nitric oxide (NO) in the maternal circulation from early gestation [10].

It is therefore plausible that XO activity and ROS production may significantly influence EVT function during placental development. However, there is little evidence showing that H_2O_2 affects biological behavior of trophoblast cells [11, 12]; furthermore, it has not yet been demonstrated that XO activity is involved in oxidative stress on trophoblast cell via uric acid and H_2O_2 production.

The aim of this study was to examine the effects of ROS produced by XO on the EVT *in vitro*. For this purpose, we administered xanthine and XO (X/XO) or H_2O_2 to TCL1 cells, a cell line derived from human EVT, and then assessed cell growth, invasion and the protein expression of HIF1A and ITGAV, which has been shown to be associated with EVT differentiation [13, 14].

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Materials and Methods

Reagents

For the ECM (extracellular matrix), growth factor-reduced Matrigel was purchased from BD Bioscience (Bedford, MA, USA).

Xanthine and xanthine oxidase were purchased from Sigma Chemical (St Louis, MO, USA). Allopurinol, an inhibitor of xanthine oxidase, and hydrogen peroxide (H_2O_2) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Catalase, a degrading enzyme of H_2O_2, was purchased from MP Biomedicals (Solon, OH, USA). N-omega-Nitro-L-arginine methyl ester hydrochloride (L-NAME), an inhibitor of NO synthase, was purchased from ICN Biomedicals (Aurora, OH, USA). NG-Nitro-D-arginine methyl ester hydrochloride (D-NAME), the control isomer of L-NAME, was purchased from Bachem (Bubendorf, Switzerland).

An HIF1A antibody was purchased from BD Transduction Laboratories (Bedford, MA, USA). The ITGAV antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The HLA-G antibody was purchased from Abcam (Tokyo, Japan). Alexa Fluor 546-labeled goat anti-mouse IgG (Invitrogen by Life Technologies) and FITC-labeled goat-anti mouse IgG (AnaSpec, Fremont, CA, USA) were used as secondary antibodies.

Cell lines and cell culture

TCL1 cells were established from mixed primary cultures of cells isolated from chorionic membranes obtained from elective, preterm caesarean sections [15]. Primary cultures contained 8–10% EVT. Isolated cells were immortalized by retroviral expression of the SV40 large antigen; single-cell cloning revealed that cells with an epithelial morphology were the only type present after six months of culture. Cells showed no tumorigenicity either *in vitro* or *in vivo*. The cloned population expressed human chorionic gonadotropin, alpha, beta, and colony stimulating factor 1, and lacked markers for decidualized endometrial cells, macrophages, or natural killer (NK) cells. TCL1 cells were positive for cytokeratin, and negative for vimentin. In addition, TCL1 cells constitutively expressed MMP-A, but MMP-B was expressed only when cells were cultured in the presence of an ECM, a characteristic restricted to the phenotype of the invading cytotrophoblast (CT) [15–17]. Cells were cultured in RPMI1640 (Nipro, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen) or conditioned serum in a humidified atmosphere containing 95% air and 5% CO_2 at 37 C for 24 h.

The medium was then replaced with complete medium containing 10% FBS and the indicated concentrations of X/XO, catalase, allopurinol and L-NAME. Control cells were incubated with complete medium alone. At the indicated times, cells were harvested and the total cell number was determined by counting with a Coulter counter. Furthermore, following the same culture conditions as in the previous experiments, TCL1 was incubated with the indicated concentrations of H_2O_2, and the cell number was determined at the indicated times. The relative numbers of cells were calculated by dividing the cell number after the indicated incubation time by the initial cell number.

Determination of uric acid concentration

Uric acid concentration was determined through a commercial laboratory service (SRL, Tokyo, Japan). After incubation of the TCL1 cells under various conditions, conditioned media were collected and centrifuged to remove cell components and debris. Uric acid was then quantified based on the uricase POD method with a JCA-BM 8000 series autoanalyzer (JEOL, Tokyo, Japan) and Pureauto S UA enzyme liquids (Sekisui Medical, Tokyo, Japan).

Detection of apoptosis

Apoptosis was confirmed by a terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin uridine triphosphate (dUTP) nick-end labeling (TUNEL) assay using a TUNEL Label Mix (Roche Diagnostics, Tokyo, Japan), according to the manufacturer's protocol. Cells were viewed (magnification ×40–400), and photographed using an Olympus IX71 microscope. Apoptotic cells were measured by counting the number of TUNEL-positive cells. At least three fields per well were observed; each experimental condition was tested in triplicate.

In vitro migration assay

A quantitative measure of the degree of *in vitro* invasion of TCL1 cells was obtained in a modified Boyden chamber assay (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. Briefly, after pretreatment with various agents, a 0.5 ml suspension of TCL1 in serum-free media was added to the upper compartment of the Boyden chamber at a density of 2×10^5 cells/well and incubated for 24 h at 37 C with 10% FBS-supplemented media in the lower compartment. Non-migrating cells were removed with a cotton swab, and the remaining cells were fixed and stained (Diff-Quik Stain Set, Dade Behring, Newark, DE, USA). Filters were removed from the chamber and mounted for visualization under an Olympus IX71 microscope. The number of cells migrating to the lower side of the filter was determined by counting all invaded cells in each membrane.

Tube-like formation assay

Growth factor-reduced Matrigel (BD Bioscience, Franklin Lakes, NJ, USA) was added (300 µl) to each well of a 24-well plate and allowed to polymerize for one hour at 37 C. After pretreatment with various agents for two hours, 2×10^5 TCL1 cells were seeded. Cells were incubated at 37 C in room air for twelve hours, viewed (magnification ×40–400), and photographed using an Olympus IX71 microscope. Tube-like formation was quantitated by counting the number of tube-like structures formed by the connected capillary bridge [37]. At least three fields per well were observed; each experimental condition was tested in triplicate.

Immunofluorescence

A total of 2×10^5 exponentially growing cells were seeded on coverslips. After incubation, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with phosphate buffered saline (PBS) containing 0.5% Triton-X. After blocking with 3% bovine serum albumin for 30 min, cells were incubated with primary antibodies overnight at 4 C followed by incubation with the secondary antibody for 45 min at room temperature; nuclei were stained with Hoechst 33852. After washing twice, cells were mounted onto slide glasses with VECTASHIELD Mounting Medium (Vector Laboratories,

Burlingame, CA, USA). Cells were observed using a confocal fluorescent microscope (Olympus BX50).

Western blotting

Cells were lysed with lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% (w/v) sodium dodecyl sulfate (SDS), and 10% glycerol. Cellular proteins were electrophoresed in an SDS gel together with a prestained molecular weight marker (Bio-Rad Laboratories, Hercules, CA, USA), transferred onto Immobilon-P (Millipore, Bedford, MA, USA), and analyzed for the expression of proteins by an immunoblotting system (GE Healthcare Japan, Tokyo, Japan). The amount of each protein was quantified using NIH image software.

Isolation of human primary cytotrophoblast cells

Human chorionic villi tissues were obtained from patients who underwent therapeutic termination of pregnancy at 6–7 weeks of gestation. Primary EVT cells were isolated from chorionic villi tissues as previously described by Loke and Burland [18]. Briefly, tissues were minced separately and digested with EDTA (ICN Biochemicals, Thame, Berks, UK) containing 0.25% trypsin (Sigma, St. Louis, MO, USA) and 50 kU/ml DNase I (Sigma) for 15 min at 37 C. The cell suspension was filtered through a nylon sieve to remove the gross villous core residues and centrifuged at 400 *g* for 20 min. The pellet was resuspended in bicarbonate buffered DMEM (Invitrogen, Eugene, OR, USA) containing 10% FBS, 1% penicillin and streptomycin, 2 mM glutamine, and 25 mM HEPES and layered onto preformed Percoll gradients, which were then centrifuged at 1200 *g* for 20 min. The cytotrophoblast cells were collected from the upper diffuse "band" by manual aspiration and seeded onto dishes. The purity of the trophoblastic cell fraction was assessed by positive staining for HLA-G. These tissue samples were obtained with the patients' informed consent, and this study was approved by the Ethics Review Board of Kyushu University.

Statistical analysis

Statistical analysis was performed using ANOVA, the Bonferroni test and the unpaired t-test in GraphPad Prism® (GraphPad Software, San Diego, CA, USA). A P value <0.05 was considered statistically significant.

Results

Administration of X/XO to the culture medium inhibited cellular growth of TCL1

To elucidate the influence of XO on EVT cellular growth, TCL1 cells were incubated with 2.3 mM xanthine and 15 mU/ml XO (X/XO). As shown in Fig. 1, the relative cell number was 1.31 ± 0.12 at 12 h and 1.47 ± 0.13 at 24 h. In the presence of X/XO, the growth of TCL1 cells was decreased to 0.68 ± 0.12 and 0.53 ± 0.14, respectively. Simultaneously, the uric acid level of the culture medium was elevated up to 4.1 mg/dl. This is consistent with production of superoxide as well as uric acid production by X/XO (Fig. 2). The decrease in cell number was completely inhibited by catalase, whereas the increase in urate level was significantly suppressed by allopurinol (P<0.05). Neither the cell numbers nor the urate level

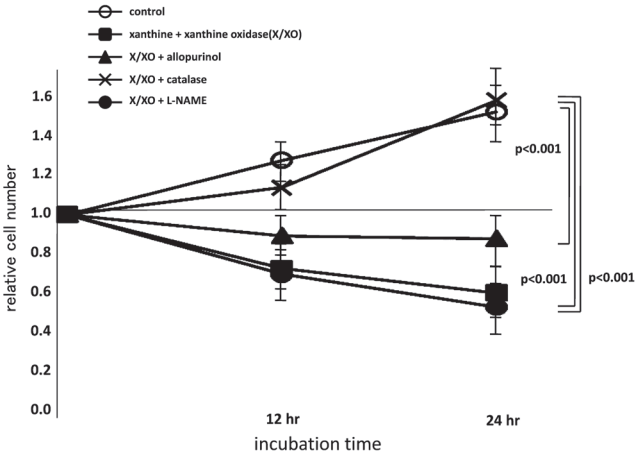

Fig. 1. Cellular growth of TCL1 following incubation with X/XO. TCL1 cells: open circle, control; filled circle, X/XO (2.3 mM xanthine + 1.5 mU/ml xanthine oxidase); open square, X/XO + allopurinol (20 μM); cross, X/XO + catalase (1 U/ml); filled square, X/XO+ L-NAME (10 μM); filled triangle, X/XO+ D-NAME (10 μM). Data are the mean ± standard deviation of three independent experiments. Statistical analysis was performed with ANOVA and the Bonferroni test.

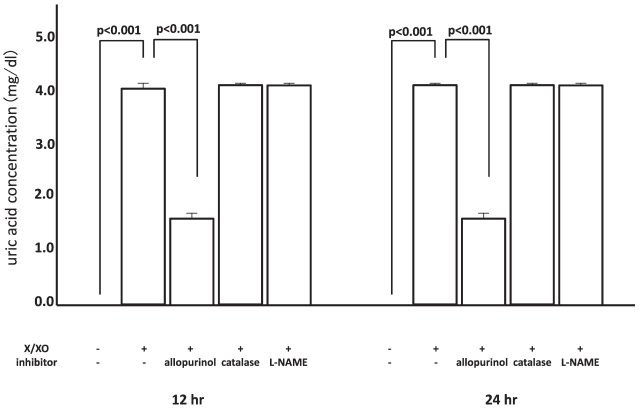

Fig. 2. Uric acid level of the culture medium for TCL1 following incubation with X/XO. Data are the mean ± standard deviation of three independent experiments. Statistical analysis was performed with ANOVA and the Bonferroni test.

was affected by L-NAME and D-NAME. These data suggest that superoxide produced by X/XO is mainly converted to H_2O_2, resulting in its cytotoxicity.

H_2O_2 inhibited cellular growth of TCL1

Because H_2O_2 production plays a role in the cytotoxic effect of X/XO, we examined the effect of H_2O_2 on the cellular proliferation of TCL1 cells. As shown in Fig. 3, the relative numbers of cells incubated with 0, 0.01, 0.1 and 1.0 mM of H_2O_2 for 6 h were 1.15 ± 0.09, 1.22 ± 0.10, 0.84 ± 0.90 and 0.65 ± 0.11, respectively. At 12 h of incubation, the relative numbers of cells were 1.30 ± 0.15, 1.21 ± 0.14, 0.93 ± 0.13 and 0.45 ± 0.11, respectively. At 24 h, the

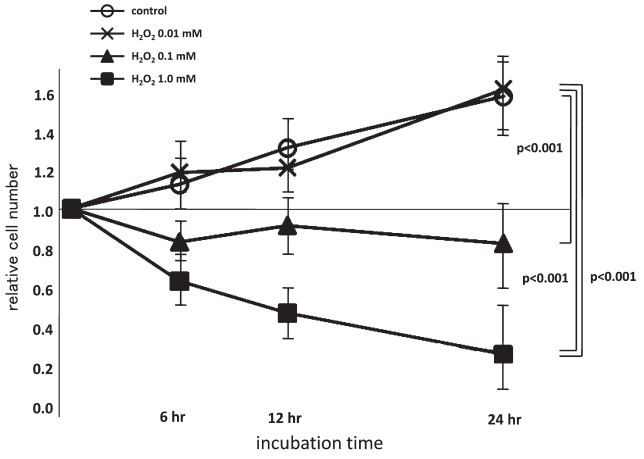

Fig. 3. Cellular growth of TCL1 incubated with H_2O_2. TCL1 cells. Open circle, control; cross, 0.01 mM H_2O_2; filled triangle, 0.1 mM H_2O_2; filled square, 1.0 mM. Data are the mean ± standard deviation of three independent experiments. Statistical analysis was performed with ANOVA and the Bonferroni test.

relative numbers of cells were 1.59 ± 0.19, 1.62 ± 0.18, 0.87 ± 0.22 and 0.23 ± 0.18, respectively. H_2O_2 exhibited its cytotoxic effect in a dose-dependent manner with no effect seen at or below 0.01 mM H_2O_2.

ROS produced by X/XO induced apoptosis in TCL1

To verify whether the decrease in cell numbers following the treatment with X/XO was the consequence of apoptosis induction, a TUNEL assay was performed. The TUNEL-positive ratio at 6 h of incubation with X/XO was 27.5 ± 9.0% compared with 4.0 ± 2.6% in the control (Fig. 4). This significant increase ($P<0.05$) in apoptotic cells was completely suppressed by catalase (2.3 ± 2.2%) and partially suppressed by allopurinol (11.0 ± 8.7%) and L-NAME (19.0 ± 8.2%). D-NAME did not affect the apoptotic cell ratio induced by X/XO (32.6 ± 10.7%). Administration of H_2O_2 in substitution for X/XO also increased the apoptotic cell ratio (28.4 ± 11.3%). These findings indicate that X/XO produces ROS such as superoxide and H_2O_2 and subsequently induces apoptosis in TCL1.

H_2O_2 decreased the number of migrating TCL1 cells

To examine the effect of H_2O_2 on the invasion ability of TCL1, we performed a pore membrane motility assay using the modified Boyden chamber method. The number of invading cells was 31.3±8.6 in the control (Fig. 5). Following pretreatment with 0.01 and 0.1 mM of H_2O_2 for 2 h, which did not affect cell survival at any dose in TUNEL assay (data not shown), the number of invading cells significantly decreased to 4.3 ± 3.2 ($P<0.05$) and 7.3 ± 4.7 ($P<0.05$), respectively. These results indicate that H_2O_2 substantially reduced the invasion ability of TCL1.

H_2O_2 modified "tube-like formation" and altered HIF1A and ITGAV protein expression in TCL1

Following 12 h of incubation on Matrigel, TCL1 cells exhibited a morphological change that mimicked endothelial cells, termed "tube-like formation" (Fig. 6). Pretreatment with 0.1 mM of H_2O_2 for 2 h abrogated this morphological change, but pretreatment with 0.01 mM of H_2O_2 did not. We then performed a Western blot analysis to examine the expression of HIF1A and ITGAV. Following incubation with 0, 0.01 and 0.1 mM of H_2O_2 for 12 h, the relative intensities of HIF1A protein expression were 0.47 ± 0.09, 0.11 ± 0.04 and 0.13 ± 0.02, respectively (Fig. 7). Similarly, ITGAV protein expression was 0.53 ± 0.05, 0.14 ± 0.02 and 0.19 ± 0.04, respectively. These data show that an excessively oxidative state blocks the normal differentiation of TCL1.

H_2O_2 increased the apoptotic cell ratio of primary cultured trophoblasts

To further explore the effects of ROS on EVT, we performed a TUNEL assay in a homogeneous population of primary cultured trophoblastic cells. Similar to TCL1 cells, more than 90% of cells were confirmed to be HLA-G positive in an immunostaining assay (Fig. 8A). The apoptotic cell ratio at 6 h of incubation with 0.1 mM H_2O_2 was 31.5 ± 8.1%, whereas that of the control was 5.3 ± 2.3% (Fig. 8B). These findings reinforce the conclusion that ROS influences the cell fate of EVT *in vivo*.

Discussion

Oxidative stress has recently been implicated in numerous pathological conditions including malignancy, cardiovascular disease, metabolic disease, neurological disorders, inflammatory reaction, and aging [19–23]. It is caused by the imbalance between ROS production and antioxidant activity. Major intracellular sources of ROS include Nox, XO and the mitochondrial electron transport system. Shyamali *et al.* demonstrated that endothelial damage in inflammatory brain disease may be mediated by elevated Nox4 activity [24], and Xue *et al.* showed that both Nox and XO contribute to endothelial dysfunction in ischemic reperfusion injury [25].

Oxidative stress also plays a significant role in pathologic conditions in pregnancy including preeclampsia and IUGR [26, 27]. Until recently, the hyperuricemia in preeclampsia was attributed solely to impaired renal function; however, XO and Nox have now been shown to play a role in elevated uric acid concentration [1, 31]. As described above, XO catalyzes xanthine into superoxide and uric acid by coupled reactions [5, 6]. Both compounds generate superoxide, which is converted into peroxynitrite (ONOO-) by NO, H_2O_2 by superoxide dismutase (SOD) [28] or O_2 by transition metals such as ferrum ion (Haber-Weiss reaction) [29]. There are few publications showing that H_2O_2 affects biological behavior of trophoblast cells; in addition, little is known about the source of H_2O_2 and the involvement of XO activity [14, 30].

In our study, suppression of trophoblast cell growth by administration of X/XO was inhibited by catalase and not by L-NAME and D-NAME, which suggested that generated superoxide was mainly converted into H_2O_2. In our results, it seems peculiar that the concentration of uric acid was virtually unchanged between 12 h and 24 h. This is supposedly because the reaction of X/XO progressed rapidly and completely, resulting in saturation of uric acid generation. Theoretically, the elevation of 4 mg/dl uric acid is nearly equivalent to the production of 0.24 mM H_2O_2 if all superoxide

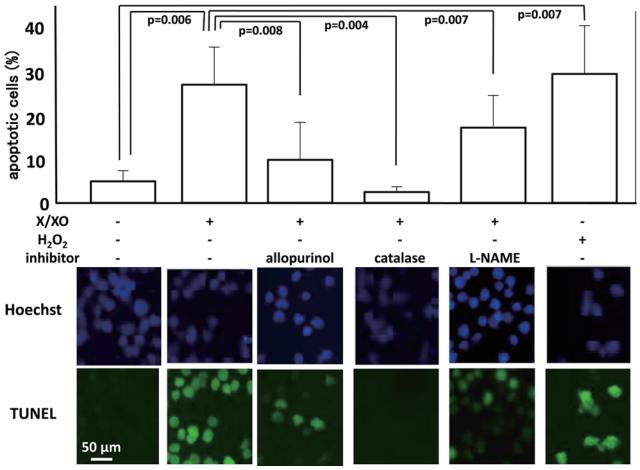

Fig. 4. Induction of apoptosis in TCL1 by X/XO or H_2O_2. TCL1 cells were incubated with X/XO or H_2O_2 (0.1 mM) with or without catalase (1 U/ml), allopurinol (20 μM), L-NAME (10 μM) or D-NAME (10 μM). Microscopy at ×400 magnification was performed after 6 h of incubation, and the ratio of TUNEL-positive cells to cells detected by Hoechst was calculated. Scale bar=50 μm. Data are the mean ± standard deviation of three independent trials. Statistical analysis was performed with ANOVA and the Bonferroni test.

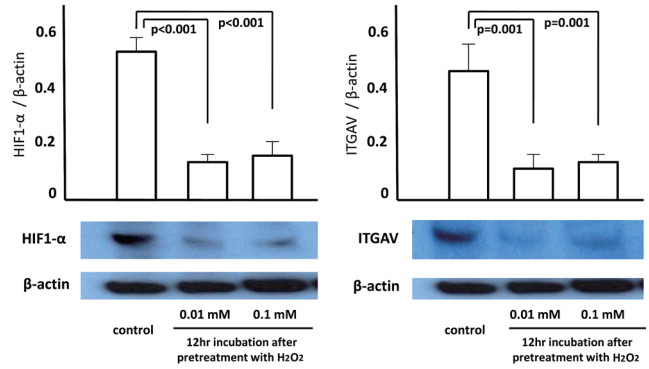

Fig. 7. Suppression of HIF1A and ITGAV expression in TCL1 cells by H_2O_2. Asynchronously growing cells incubated with 0, 0.01 or 0.1 mM H_2O_2 were seeded on poly-L-lysine and incubated in room air for 12 h. Cellular proteins were then extracted, electrophoresed and transferred onto Immobilon-P before analysis by immunoblotting using an HIF1A antibody (left panel) or an ITGAV antibody (right panel). Both of them were examined in relation to β-actin expression as a protein loading control. Data are presented as the mean ± standard deviation of three independent trials. Statistical analysis was performed with ANOVA and the Bonferroni test.

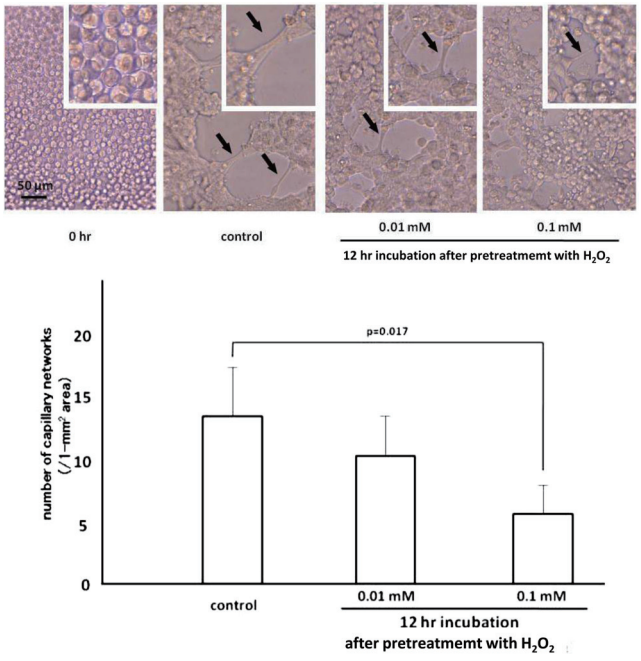

Fig. 6. Inhibition of tube-like formation in TCL1 cells by H_2O_2. TCL1 cells pretreated with 0, 0.01 or 0.1 mM H_2O_2, were seeded on Matrigel and viewed under a microscope at 0 and 12 h. Scale bar=50 μm. The number of capillary networks (arrow) per 1-mm^2 surface area was counted at a magnification of ×400. Data are presented as the mean ± standard deviation of three independent trials. Statistical analysis was performed with ANOVA and the Bonferroni test.

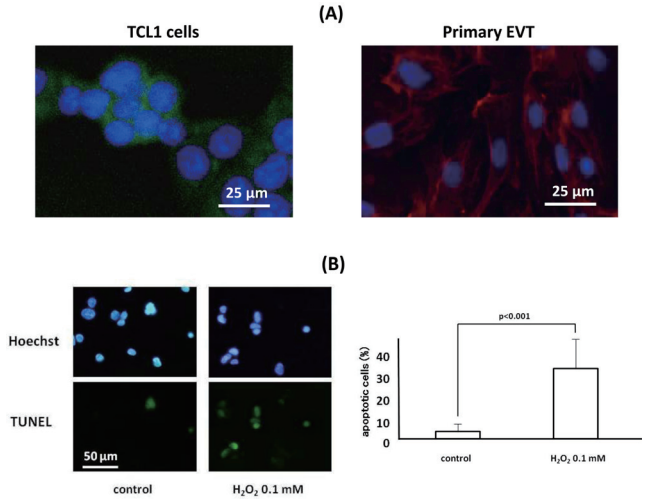

Fig. 8. Induction of apoptosis in primary cultured trophoblastic cells by H_2O_2. (A) TCL1 cells and a homogeneous population of trophoblastic cells were confirmed by immunofluorescence showing cells that were HLA-G positive (green portion in left panel and red portion in right panel). Scale bar=25 μm. (B) After primary cultured trophoblastic cells were incubated with 0.1 mM H_2O_2 for 6 h, microscopy at ×400 magnification was performed. The ratio of TUNEL-positive cells to cells detected by Hoechst was calculated. Data are the mean ± standard deviation of three independent trials**.** Scale bar=50 μm.

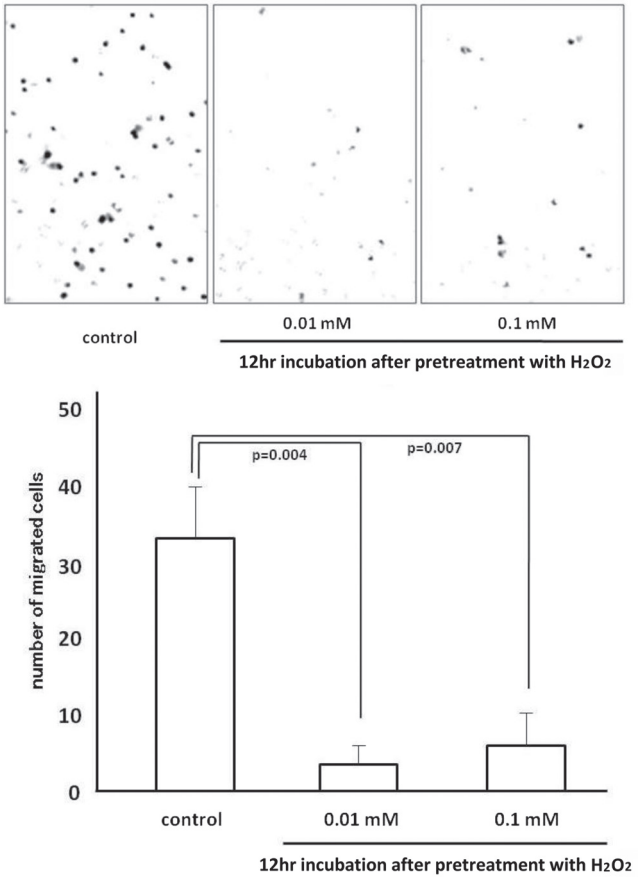

Fig. 5. Suppression of invasion ability in TCL1 cells by H_2O_2. TCL1 cells were pretreated with 0, 0.01 or 0.1 mM H_2O_2 for 2 h, and cells invasion assays were then performed. The number of migrated cells after 24 h in each Boyden chamber membrane was counted at magnification ×40. Data are the mean ± standard deviation of three independent trials. Statistical analysis was performed with ANOVA and the Bonferroni test.

generated by XO is converted into H_2O_2 [6, 32]. These features correspond to our result that cellular growth was suppressed by a minimum of 0.1 mM H_2O_2.

The regulation of trophoblastic cell apoptosis is closely associated with pregnancy outcome [33]. Increased apoptosis of the trophoblast is observed in the preeclamptic placenta [34]. Bainbridge *et al.* demonstrated that xanthine oxidase immunoreactivity in skin biopsies from preeclamptic women is higher than those from control women [35]. Our data support the concepts that increased trophoblast cell death and xanthine oxidase activity seem to be involved in the pathophysiology of preeclampsia and suggest that there is a possibility that oxidative stress in preeclampsia increases apoptosis of trophoblasts. It is noteworthy that H_2O_2 may suppress invasion and differentiation at even less than a fatal amount of exposure. Actually, pretreatment with 0.01 and 0.1 mM of H_2O_2 for 2 h did not affect cell survival at any dose in the TUNEL assay (data not shown). As our data does not clarify the detailed mechanism by which oxidative stress inhibits both proliferation and invasion, further examination will be needed.

The invasion and differentiation of the EVT in the early gestational period is essential to establishment of the normal fetal-maternal circulation [36]. Differentiation of the EVT occurs with both interstitial invasion and endovascular invasion [37]. We previously reported that the expression of HIF1A and ITGAV was closely related to differentiation of the EVT [12, 13]. In the present experiments, we demonstrated that oxidative stress attenuated trophoblast invasion, blocked normal morphological change, and altered protein expression. This suggests that excessive ROS production accompanied by hyperuricemia affects the biological behavior of the trophoblast. These observations, taken together, suggest that elevated uric acid in preeclamptic patients at least partly reflects the level of placental damage induced by XO-generated ROS in early pregnancy.

In conclusion, ROS derived from X/XO significantly affects trophoblastic cell function. This effect is mediated mainly by H_2O_2 and explains the relationship between oxidative stress in the placenta and certain pathologic conditions such as preeclampsia and IUGR.

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