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Overexpression of Copper Zinc Superoxide Dismutase Impairs Human Trophoblast Cell Fusion and Differentiation

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The syncytiotrophoblast is the major component of the human placenta, involved in feto-maternal exchanges and secretion of pregnancy-specific hormones. Multinucleated syncytiotrophoblast arises from fusion of mononuclear cytotrophoblast cells. In trisomy 21-affected placentas, we recently have shown that there is a defect in syncytiotrophoblast formation and a decrease in the production of pregnancy-specific hormones. Due to the role of oxygen free radicals in trophoblast cell differentiation, we investigated the role of the key antioxidant enzyme, copper/zinc superoxide dismutase, encoded by chromosome 21 in *in vitro* trophoblast differentiation. We first observed that overexpression of superoxide dismutase in normal cytotrophoblasts impaired syncytiotrophoblast formation. This was associated with a significant decrease in mRNA transcript levels and secretion of hCG and other hormonal markers of syncytiotrophoblast. We confirmed abnormal cell fusion by overexpression of green fluorescence protein-tagged superoxide dismutase in cytotrophoblasts. In addition, a significant decrease in syncytin transcript levels was observed in superoxide dismutase-transfected cells. We then examined superoxide dismutase expression and activity in isolated trophoblast cells from trisomy 21-affected placentas. Superoxide dismutase mRNA expression ($P < 0.05$), protein levels ($P < 0.01$), and activity ($P < 0.05$) were significantly higher in trophoblast cells isolated from trisomy 21-affected placentas than in those from normal placentas. These results suggest that superoxide dismutase overexpression may directly impair trophoblast cell differentiation and fusion, and superoxide dismutase overexpression in Down's syndrome may be responsible at least in part for the failure of syncytiotrophoblast formation observed in trisomy 21-affected placentas. (*Endocrinology* 142: 3638–3648, 2001)

IN HUMANS, FETAL cytotrophoblasts play a key role in the embryo implantation process and placental development. In early pregnancy mononuclear cytotrophoblasts proliferate and invade the maternal endometrium to form the anchoring villi (1, 2). Cytotrophoblasts also fuse and differentiate into a continuous layer of multinucleated syncytiotrophoblast. This cell layer, which covers the chorionic villi, is bathed with maternal blood in the intervillous spaces from early gestation (3, 4). The syncytiotrophoblast layer plays a major role throughout pregnancy, as it is the site of numerous placental functions, including ion and nutrient exchange and the synthesis of steroid and peptide hormones required for fetal growth and development (5, 6). Some of these hormones, such as hCG, human placental lactogen (hPL) and placental GH (PGH; also called GH variant) are specific to pregnancy and can be used as markers of syncytium formation (7–9).

It has been established both *in vivo* and *in vitro* that the syncytiotrophoblast layer arises from the differentiation and fusion of mononuclear cytotrophoblasts. Isolated mononuclear cytotrophoblasts have been shown to aggregate and fuse to form a nonproliferative multinucleated syncytiotrophoblast that synthesizes and secretes specific hormones required for fetal development (10, 11). This cytotrophoblast differentiation is stimulated *in vitro* by a number of factors, including epidermal growth factor (EGF) (12, 13), granulocyte-stimulating factor (14), hCG (15, 16), glucocorticoids (17), and estradiol (18). Several studies also have shown that hypoxia inhibits cytotrophoblast differentiation and fusion (19–21). These results raise the interesting possibility that a change in cellular oxidative status may play a regulatory role in cytotrophoblast fusion and differentiation into syncytiotrophoblast.

Reactive oxygen species, including hydrogen peroxide (H_2O_2), superoxide ion ($O_2^{\cdot -}$), and hydroxyl radical ($^{\cdot}OH^-$) are generated in cells in response to stimulation by various hormones, growth factors, and cytokines (22, 23). The oxygen radicals generated appear to act as second messengers in transmembrane signaling pathways to modulate cellular functions such as cell proliferation and differentiation (24,

Abbreviations: EGF, Epidermal growth factor; GFP, green fluorescence protein; hPL, human placental lactogen; INT, 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride; PGH, placental GH; PPIA, cyclophilin A; SOD-1, superoxide dismutase; T21, trisomy-21.

25). Cellular oxidative status is determined by the balance between reactive oxygen species production and their destruction by a variety of antioxidant enzymes. The primary antioxidant activity in the cell that regulates the level of $O_2^{\cdot -}$ and its reactive progeny is that of the superoxide dismutases (SODs). Mammalian cells have a mitochondrial Mn-SOD; a cytoplasmic Cu Zn-SOD, which also is found in peroxysomes; and an extracellular SOD, which is a Cu, Zn-SOD that is immunologically distinct from the classical Cu, Zn-SOD (26). These metalloenzymes act to dismute generated superoxide radicals to oxygen and H_2O_2 (23). In turn, catalase along with peroxidases such as glutathione peroxidase catalyze the decomposition of H_2O_2 to water and oxygen (27).

Both cytosolic Cu, Zn-SOD (SOD-1) and mitochondrial Mn-SOD are expressed in human cytotrophoblasts (28, 29). Extracellular Cu, Zn-SOD appears to localize within the villous extracellular matrix around the arterioles of the placenta (30). A role for SOD-1 in placental development has been suggested by results showing reduced fertility in transgenic female mice lacking SOD-1. In SOD-$1^{-/-}$ female mice a postimplantation embryonic loss was observed with no placental development (31). Moreover, male SOD-1-deficient *Drosophila* are sterile, whereas SOD-1-deficient females exhibit markedly reduced fertility (32). However, little is known concerning a possible role for SOD-1 in human placental development. Recently, we demonstrated a modulation of SOD-1 expression and activity with *in vitro* differentiation of human villous cytotrophoblasts (33). Interestingly, we also have shown a failure of cytotrophoblast differentiation into syncytiotrophoblast in trisomy 21 (T21)-affected placentas (34, 35). It has been known for some time that SOD-1 is located on human chromosome 21, and that it is overexpressed in different T21-affected cell types (36). To better understand the role of SOD-1 in trophoblast differentiation, we employed an *in vitro* model of differentiation of human villous cytotrophoblast into syncytiotrophoblast to study the effect of overexpression of SOD-1 on the differentiation of these cells and to determine the levels and activities of this enzyme in cytotrophoblast cells isolated from T21-affected placentas that are unable to undergo normal differentiation and fusion to multinuclear syncytiotrophoblast.

Materials and Methods

Placental tissue collection

Term placentas were obtained after elective cesarean section from healthy mothers near term with uncomplicated pregnancies. French law allows termination of pregnancy with no gestational age limit when severe fetal abnormalities are observed. Samples of placental tissues were collected at the time of termination of pregnancy at 12–24 wk gestation (expressed in weeks of amenorrhea) in T21-affected pregnancies and gestational age-matched control cases. Gestational age was confirmed by ultrasound measurement of crown-rump length at 8–12 wk gestation. Fetal Down syndrome was diagnosed by karyotyping of amniotic fluid cells, chorionic villi, or fetal blood cells. We checked that placental tissue was affected by T21 by determination of DNA polymorphism markers (37). In no case was T21 due to translocation, and no mosaicism was observed. Termination of pregnancy was performed in control cases affected by severe bilateral or low obstructive uropathy or major cardiac abnormalities. Fetal karyotype was normal in all controls. Placental samples were used for cytotrophoblast cell isolation or were immediately frozen in liquid nitrogen.

RNA isolation and analysis

Total RNA was extracted from frozen placental samples by means of the single step guanidinium-phenol-chloroform method described by Chomczynski and Sacchi (38) and from cultured cells following the procedure developed by QIAGEN (Valencia, CA). The total RNA concentration was determined at 260 nm, and its integrity was monitored by 1% agarose gel electrophoresis. Relative mRNA levels of the different genes were measured with the TaqMan5′ nuclease fluorogenic quantitative PCR assay essentially as previously described (39). The nucleotide sequences of the primers and probes are listed in Table 1. Each sample was analyzed in duplicate, and a calibration curve was run in parallel for each analysis. The level of transcripts of the constitutive housekeeping gene product cyclophilin A was quantitatively measured in each sample to control for sample to sample differences in RNA concentration and quality. The PCR data are thus reported as the number of transcripts per number of cyclophilin A molecules.

Cell culture

Villous tissue was dissected free of membranes, rinsed, and minced in Ca^{2+}-, Mg^{2+}-free HBSS. Cytotrophoblast cells were isolated after trypsin-deoxyribonuclease digestion and discontinuous Percoll gradient fractionation, using a slight modification of the method of Kliman and Alsat (10, 11). The villous sample was submitted to sequential enzymatic digestions in a solution that contained 0.125% powdered trypsin (wt/vol; Difco, Detroit, MI), 5 IU/ml deoxyribonuclease I, 25 mM HEPES, 4.2 mM $MgSO_4$, and 1% (wt/vol) penicillin/streptomycin (Biochemical Industries, Kibbutz Beit Haemek, Israel) in HBSS and monitored under light microscopy. The first and/or second digestions were discarded after light microscopy analysis to eliminate syncytiotrophoblast fragments, and the following four or five sequential digestions were kept. The cells collected during these last digestions were purified on a discontinuous gradient of Percoll (5–70% in 5% steps). The cells that migrated in the middle layer (density, 1.048–1.062 g/ml) were plated on culture dishes (10^6 cells/cm^2), attached to the dishes, and 3 h after plating they were carefully washed by three efficient washes with culture medium. Following this procedure, we determined that at 3 h of culture 95% of the cells isolated from normal or T21 placentas were cytokeratin 7 positive using a specific monoclonal antibody (dilution, 1:200; DAKO Corp.), less than 0.5% were vimentin positive (dilution, 1:200; Amersham International, Arlington Heights, IL), and the other cells were mononucleated cells and identified as macrophages. None of these cells was hPL positive using a polyclonal specific antibody (dilution, 1:500; DAKO Corp.). Cells were plated in triplicate either on glass slides for immunocytochemistry studies or onto 60-mm culture dishes (10^6 cells/cm^2). They were cultured for 3 d as previously described (11).

Cell staining

To detect desmoplakin or E-cadherin, cultured cells were rinsed with PBS, fixed, and permeabilized in methanol at −20 C for 25 min. A monoclonal antidesmoplakin or E-cadherin antibody (1:400; Sigma, St. Louis, MO) was then applied, followed by fluorescein isothiocyanate-labeled goat antimouse IgG (Sigma), as previously described (19).

Immunoblotting

To detect hPL, cell extracts were prepared as previously described (19), solubilized protein (5 μg) was immunoblotted using a rabbit polyclonal antibody against hPL (1:250; DAKO Corp.), and the specific band was revealed by chemiluminescence (Pierce Chemical Co., Rockford, IL; supersignal, Interchim) after incubation with an antirabbit peroxidase-coupled antibody (19). To detect SOD-1, cell extracts were prepared as previously described (19), and solubilized protein (5 μg) was immunoblotted using a sheep polyclonal antibody against human SOD-1 (The Binding Site Ltd., Birmingham, UK). The specific band was revealed by chemiluminescence (Pierce Chemical Co., Supersignal) after incubation with an antisheep peroxidase-coupled antibody.

Hormone assay

The hCG concentration was determined in culture medium using an enzyme-linked fluorescence assay (Vidas System, BioMerieux, Marcy

TABLE 1. Characteristics of the PCR assays used in this study

Genes	Primers and probes	bp	GenBank accession no.	Proteins
CGA	(+) TCCCACTCCACTAAGGTCCAA (−) CCCCATTACTGTGACCCTGTT FAM-CACAGCAAGTGGACTCTGAGGTGACG-TAMRA	106	V00518	hCGα
CGB	(+) GCTACTGCCCCACCATGACC (−) ATGGACTCGAAGCGCACATC FAM-CCTGCCTCAGGTGGTGTGCAACTACC-TAMRA	94	J00117	hCGβ
CSA	(+) GCATGACTCCCAGACCTCCTT (−) TGCGGAGCAGCTCTAGATTGG FAM-TTTCTGTTGCGTTTCCTCCATGTTGG-TAMRA	157	J03071	hPL
GHV	(+) AGAACCCCCAGACCTCCCT (−) TGCGGAGCAGCTCTAGGTAG FAM-TTTCTGCTGCGTTTTCACCCTGTTG-TAMRA	96	J03071	PGH
Leptin	(+) ACATTTCACACACGCAGTCAGT (−) CCATCTTGGATAAGGTCAGGAT FAM-TGGAGCCCAGGAATGAAGTCCAAA-TAMRA	96	U18915	Leptin
KRT7	(+) GGACATCGAGATCGCCACCT (−) ACCGCCACTGCTACTGCCA FAM-TCACGGCTCCCACTCCATCTC-TAMRA	171	X03212	Cytokeratin 7
PTN	(+) GAGATGTAAGATCCCCTGCAACT (−) CTCGCTTCAGACTTCCAGTTCT FAM-AGCAATTTGGCGCGGAGTGCAA-TAMRA	125	M57399	Pleiotropin
PPIA	(+) GTCAACCCCACCGTGTTCTT (−) CTGCTGTCTTTGGGACCTTGT FAM-AGCTCAAAGGAGACGCGGCCCA-TAMRA	97	Y00052	Cyclophilin A
HERV-W	(+) CGGACATCCAAAGTGATACATCCT (−) TGATGTATCCAAGACTCCACTCCA	100	/	Syncytin
SOD-1	(+) CTGAAGGCCTGCATGGATTC (−) CCAAGTCTCCAACATGCCTCTC FAM-TCATCCTTTGGCCCACCGTGTTT-TAMRA	138	X02317	CuZn superoxide dismutase

l'Etoile, France). Assay sensitivity was 2 mU/ml. The hPL concentration was assayed (Amerlex IRMA, Amersham Pharmacia Biotech) in 4-fold concentrated conditioned medium. The assay sensitivity was 0.5 μg/ml. All values are the mean ± SEM of triplicate determinations.

SOD-1 activity

Cultured trophoblast cells were washed three times with ice-cold PBS and harvested by scraping into ice-cold buffer [0.25 M sucrose, 20 mM Tris (pH 7.4), 1 mM $MgCl_2$] with a cell scraper. Cells were pelleted by centrifugation at 1000 × *g* for 5 min and then frozen at −80 C. Cell pellets were disrupted by sonication in 100 μl 10 mM sodium phosphate buffer, pH 7. SOD-1 activity was measured as previously described (40). Briefly, xanthine-xanthine oxidase was used to generate an $O_2^{\cdot -}$ flux, and the reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to red formazan by $O_2^{\cdot -}$ was monitored at 505 nm at 30 C. The rate of INT reduction in the absence of samples was used as the reference rate (0.02 ± 0.005 absorbance/min). Each assay tube contained 1 mM EDTA, 25 μM INT, 50 μM xanthine, 1 U/ml catalase, and enough xanthine oxidase to ensure 100% noninhibition, plus 50 mM 3-[cyclohexylamino]-1-propane-sulfonic acid buffer, pH 10.2. All data were expressed in units of SOD activity per mg protein.

DNA transfection

Cytotrophoblasts were isolated from placentas after elective cesarean section in healthy mothers with uncomplicated pregnancies at term. Transfection of cytotrophoblast primary cultures was performed by lipofection using the TransFast transfection reagent (Promega Corp., Madison, WI), according to a protocol adapted from Jacquemin's method (41). The transfection efficiency is 10 ± 3% as measured by β-galactosidase assay. Briefly, 2.5 μg pRSV-SOD-1 cloned from human syncytiotrophoblast mRNA (Pharos) or empty vector was mixed with TransFast reagent in a 1:1 lipid/DNA ratio. After 15-min incubation at room temperature, 2 ml of the TransFast reagent/DNA mixture were added to each plate (60 mm), and the cells were immediately placed in the incubator for 1 h. Cells were then overlaid with 4 ml complete medium and returned to the incubator for 24 h. The medium was sampled every day for 3 d to determine hCG levels. At 72 h the cells were harvested by scraping in the presence of 10 mM phosphate buffer, and the protein concentration was determined using BSA as a standard. SOD-1 mRNA and SOD-1 activity were assayed to assess transfection efficiency. Transfections were performed in triplicate with three different primary cell cultures and two different DNA preparations.

Construction of the SOD-1-green fluorescence protein (GFP) expression plasmid

An SOD-1-GFP gene fusion was created by amplifying a DNA product carrying the human SOD-1 gene from plasmid pRSVSOD-1 by PCR. The forward (5′-GCCGATCTCGAGATGGCGACGAAGGCCGTGTGC-3′) and reverse (5′-GACCGGCCGCGGGGCGATCCCAATTACACC-ACAAG-3′) primers used to amplify SOD-1 incorporated *Xho*I and *Sac*II restriction sites 5′ and 3′ to the gene, respectively. These sites were used to insert the gene into identical site within plasmids pEGFP-N1 and pEGFP-C3 (CLONTECH Laboratories, Inc., Palo Alto, CA). This created in-frame fusions with the GFP-coding region at the C-terminus (pEGFP-N1) or N-terminus (pEGFP-C3) of SOD-1.

Protein determination

Protein was determined according to Bradford's method (Bio-Rad Laboratories, Inc., Richmond, CA) using BSA as standard.

Statistical tests

Statistical analysis was performed using the StatView F-4.5 software package (Abacus Concepts, Inc., Berkeley, CA). Values are presented as the mean ± SEM. Significant differences were identified using Mann-Whitney analysis for hormonal secretions and ANOVA for transfections; $P < 0.05$ was considered significant.

Results

Effect of Cu/Zn SOD-1 overexpression in normal cytotrophoblasts

As shown previously, purified mononuclear cytotrophoblasts isolated from normal human term placenta aggregate, fuse, and form a large multinucleated syncytiotrophoblast 72 h after plating in culture (10, 11). This *in vitro* syncytiotrophoblast formation is associated with significant increases in hCGα mRNA, hCGβ mRNA, hPL mRNA, leptin, and PGH mRNA levels (34). Concomitantly, hCG, hPL, and leptin levels were shown to increase with time in conditioned medium of the differentiated syncytiotrophoblast (34).

To determine the effect of SOD-1 overexpression on cytotrophoblast differentiation and fusion into syncytiotrophoblast, we transiently transfected isolated cytotrophoblasts with SOD-1. The purity of the cytotrophoblast cell population was first checked by cytokeratin 7 immunostaining (Fig. 1A) before transfection. As shown in Fig. 1B, in SOD-1 transfected cells both SOD-1 mRNA and SOD-1 enzymatic activity were elevated. After transfection of these primary cultures, SOD-1 mRNA levels were increased about 5-fold, whereas SOD-1 activity was increased by approximately 30%. No increase in SOD-1 mRNA or SOD-1 activity was detected in control cells transfected with the empty vector. Western blot analysis showed that SOD-1 protein levels also were significantly higher (45%; $P \leq 0.001$) in transfected cells relative to control cells transfected with the empty vector (Fig. 1C).

With differentiation and fusion of cytotrophoblasts into syncytiotrophoblast, there is an increase in the mRNA transcript levels of the placental hormones, hCGα, hCGβ, hPL, and placental GH. The appearance of these hormones can be used as markers of syncytium formation. As shown in Fig. 2A, the mRNA levels of these marker hormones were significantly decreased in cells overexpressing SOD-1 compared with control cells transfected by the empty vector. Further,

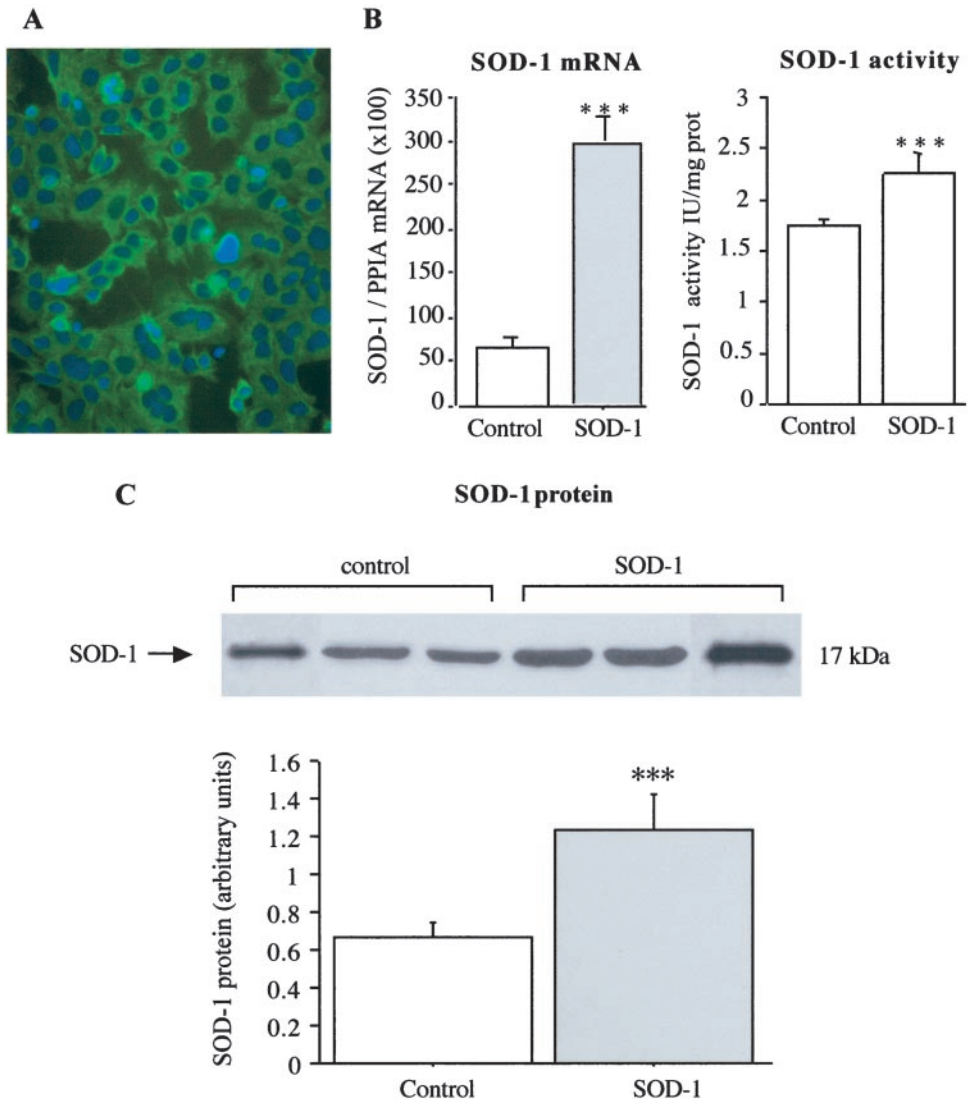

FIG. 1. Characterization of SOD-1 overexpression in normal cytotrophoblast cells. A, Cytokeratin 7 immunodetection after 24 h of culture of trophoblast cells isolated from normal placenta. Positive immunofluorescence staining is specific for cytotrophoblast cells. B, Transfection experiments. Cytotrophoblast cells isolated from normal placenta were transfected with an empty plasmid used as a control (Control) or with a SOD-1 expression vector (SOD-1). Data are expressed as levels of SOD-1 mRNA normalized by PPIA mRNA (peptidylprolyl isomerase A and cyclophilin A). SOD-1 activity in these cells is expressed as international units per mg protein. The results presented are expressed as the mean ± SEM of three culture dishes. C, SOD-1 protein levels after transfection were determined by Western blotting with a sheep polyclonal antibody to SOD-1. The autoradiogram (*upper panel*) shows a specific band of 17 kDa in normal cytotrophoblasts transfected either with an empty plasmid (Control; three separate dishes) or with a SOD-1 expression vector (SOD-1; three separate dishes). The *lower histogram* shows densitometry quantification of the autoradiograms (mean ± SEM of three culture dishes). The figure represents one of three experiments. ***, $P \leq 0.001$.

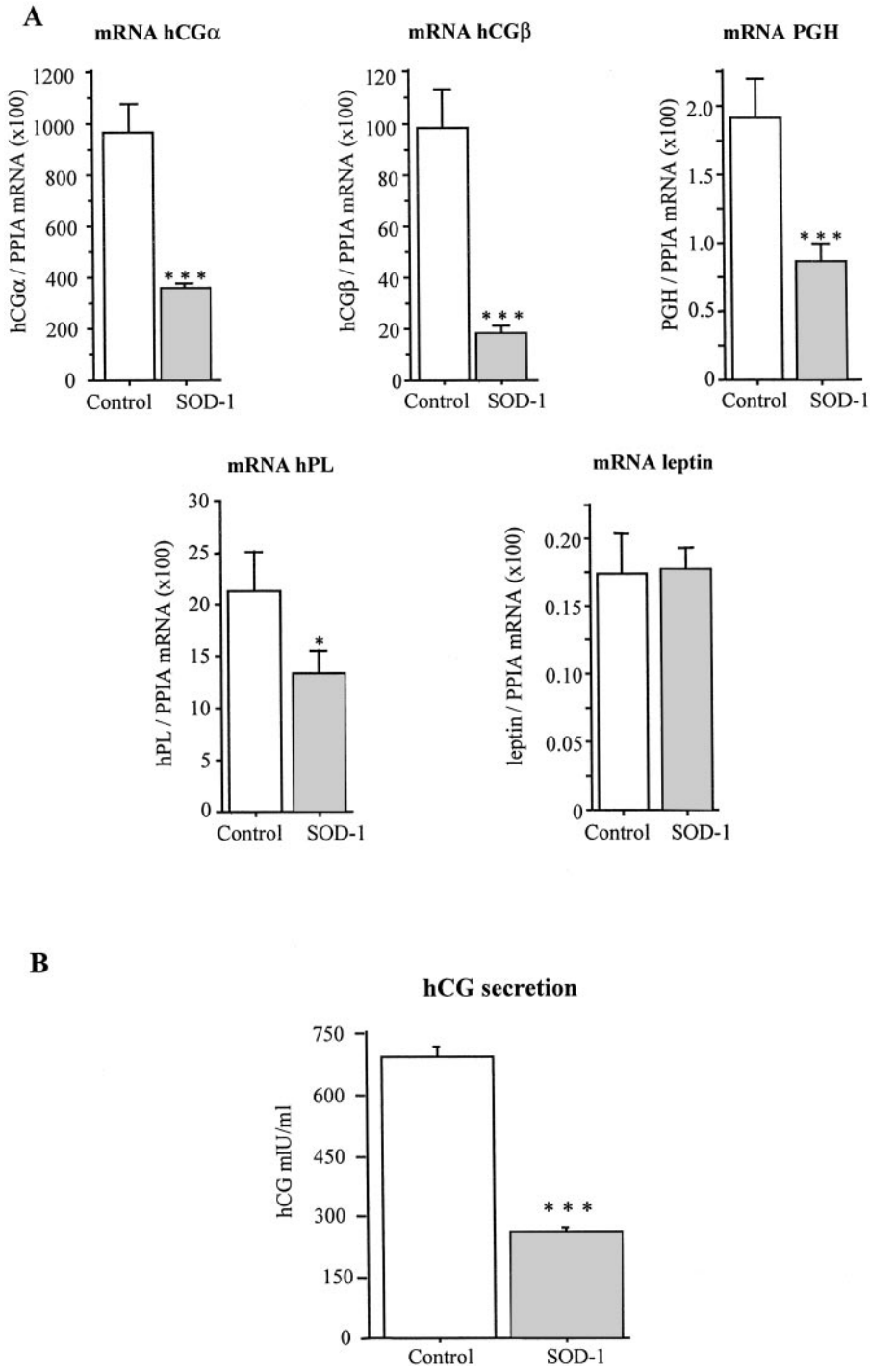

FIG. 2. SOD-1 overexpression in normal cytotrophoblasts inhibits the increase in placental hormone production noted with differentiation. A, hCGα, hCGβ, PGH, hPL, and leptin mRNA levels determined by quantitative real-time PCR in normal cytotrophoblasts transfected either with an empty plasmid used as a control (Control) or with a SOD-1 expression vector (SOD-1). These assays were carried out 72 h after plating. Values are the level of each hormonal mRNA normalized to the level of PPIA mRNA. B, Levels of hCG secreted into the culture medium 72 h after transfection determined by chemiluminescent immunoassay. Normal cytotrophoblasts were transfected either with an empty plasmid used as a control (Control) or with a SOD-1 expression vector (SOD-1). Data are the mean values for three separate dishes ± SEM. The figure illustrates one representative experiment of three performed. *, $P \leq 0.05$; ***, $P \leq 0.001$.

the overexpression of SOD-1 in normal cytotrophoblasts resulted in a significant ($P \leq 0.0001$) decrease in hCG secretion compared with control cells transfected by the empty vector (Fig. 2B). In other studies hPL production could be detected in the culture medium of SOD-1-transfected cells (data not shown).

A recent study has shown that a retroviral envelope glycoprotein, HERV-W, also called syncytin, is directly implicated in the trophoblastic fusion process (42). Indeed, the expression of recombinant syncytin in a variety of cell types was shown to induce the formation of giant syncytia. Further, the fusion of a human trophoblastic cell line expressing endogenous syncytin was found to be inhibited by an anti-syncytin antiserum. These data indicate that syncytin may mediate placental cytotrophoblast fusion. Thus, we determined the expression of syncytin mRNA in cytotrophoblasts by real-time quantitative RT-PCR (Fig. 3). First, we confirmed with our primary culture of human cytotrophoblast model that there is a significant increase ($P \leq 0.0002$) in syncytin mRNA levels during cytotrophoblast differentia-

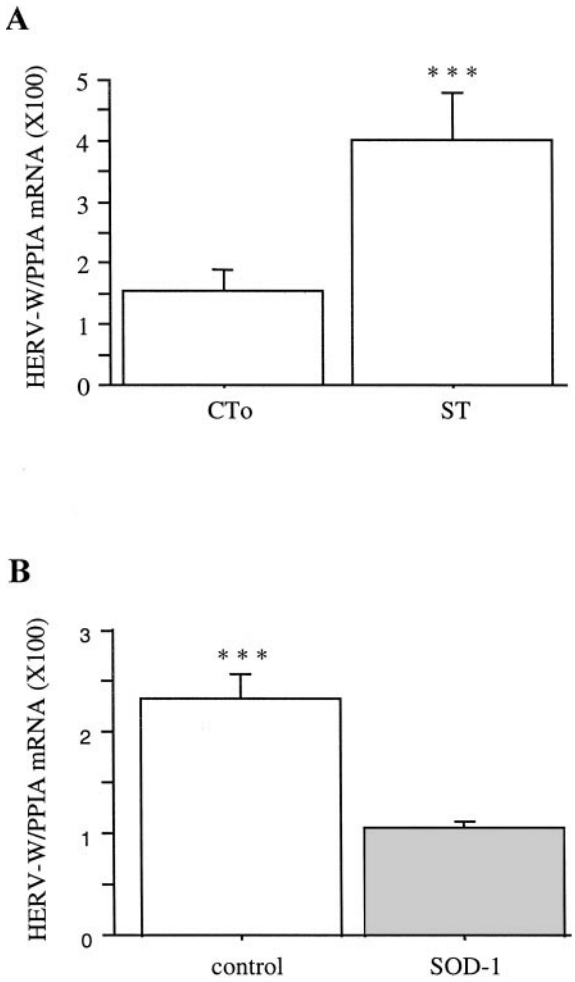

FIG. 3. SOD-1 overexpression in normal cytotrophoblasts blocks the increase in syncytin mRNA expression noted with differentiation. A, Syncytin mRNA levels were measured by real-time quantitative RT-PCR during normal cytotrophoblast differentiation and fusion. B, Syncytin mRNA levels were measured by real-time quantitative RT-PCR in normal cytotrophoblasts after transfection either with an empty plasmid (Control) or with a SOD-1 expression vector (SOD-1). Syncytin mRNA levels were normalized to PPIA mRNA levels (mean ± SEM). ***, $P \leq 0.001$. These assays were carried out 72 h after plating.

tion and fusion (Fig. 3A). In SOD-1-transfected cells, syncytin mRNA expression was decreased compared with that in control cells transfected with the empty vector (Fig. 3B).

Overexpression of GFP-tagged SOD-1 in normal cytotrophoblasts inhibits cell fusion

To further demonstrate that overexpression of SOD-1 may be implicated in the failure of cytotrophoblasts to fuse and differentiate, PCR products of SOD-1 were produced and then cloned into the pEGFP-N1 vector as described in *Materials and Methods*. This expression vector was used to transfect normal human cytotrophoblasts to overexpress GFP-tagged SOD-1. Cells overexpressing GFP-SOD-1 can be recognized within the cell population by the presence of green fluorescence due to the presence of the GFP tag. It was observed that cytotrophoblasts overexpressing GFP-tagged SOD-1 remained mononucleated and aggregated, as visualized by the detection of desmoplakin (Fig. 4). In contrast, cells on the same dish that were not overexpressing GFP-SOD-1 (absence of green fluorescence) fused to a multinucleated syncytium. Similar results were obtained using expression vectors containing GFP fused to either the C-terminus or N-terminus of SOD-1 (data not shown). Moreover, the GFP-tagged SOD-1 fusion protein was shown to retain catalytic activity, as determined by assay of increased SOD-1 activity (data not shown).

SOD-1 mRNA and protein levels in normal and T21-affected placentas

We recently observed that villous cytotrophoblasts isolated from T21-affected placentas either do not or poorly differentiate and fuse into multinucleated syncytiotrophoblast (34). Due to the known location of the human SOD-1 gene on chromosome 21 (43), we measured SOD-1 mRNA and protein levels in total tissue extracts prepared from five normal and seven T21-affected placentas matched for gestational age. Figure 5A shows SOD-1 mRNA levels in these total tissue extracts, normalized to pleiotropin, cyclophilin A (PPIA), and cytokeratin 7 mRNA expression as reference markers. No significant difference in SOD-1 mRNA transcript levels was found between T21-affected and normal total tissue extracts regardless of the reference gene used. Western blotting (Fig. 5B) showed no difference in SOD-1 protein levels between normal and abnormal tissues. These results were consistent with the similar SOD-1 mRNA levels in normal and T21-affected placentas.

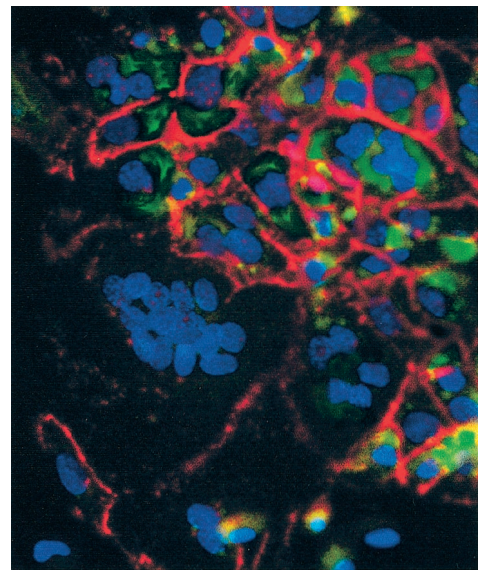

FIG. 4. Overexpression of GFP-SOD-1 protein in normal cytotrophoblasts inhibits cell fusion. Cells overexpressing GFP-SOD-1 can be detected by the appearance of *green* fluorescence. The cells expressing GFP-SOD-1 remain aggregated and do not fuse, as determined by the presence of desmoplakin (*red* fluorescence). In the same dish, cells that were not expressing the GFP-SOD-1 chimera protein were able to differentiate and fuse to a multinucleated syncytium, as determined by the absence of desmoplakin. Nuclei were labeled with DAPI (*blue* fluorescence).

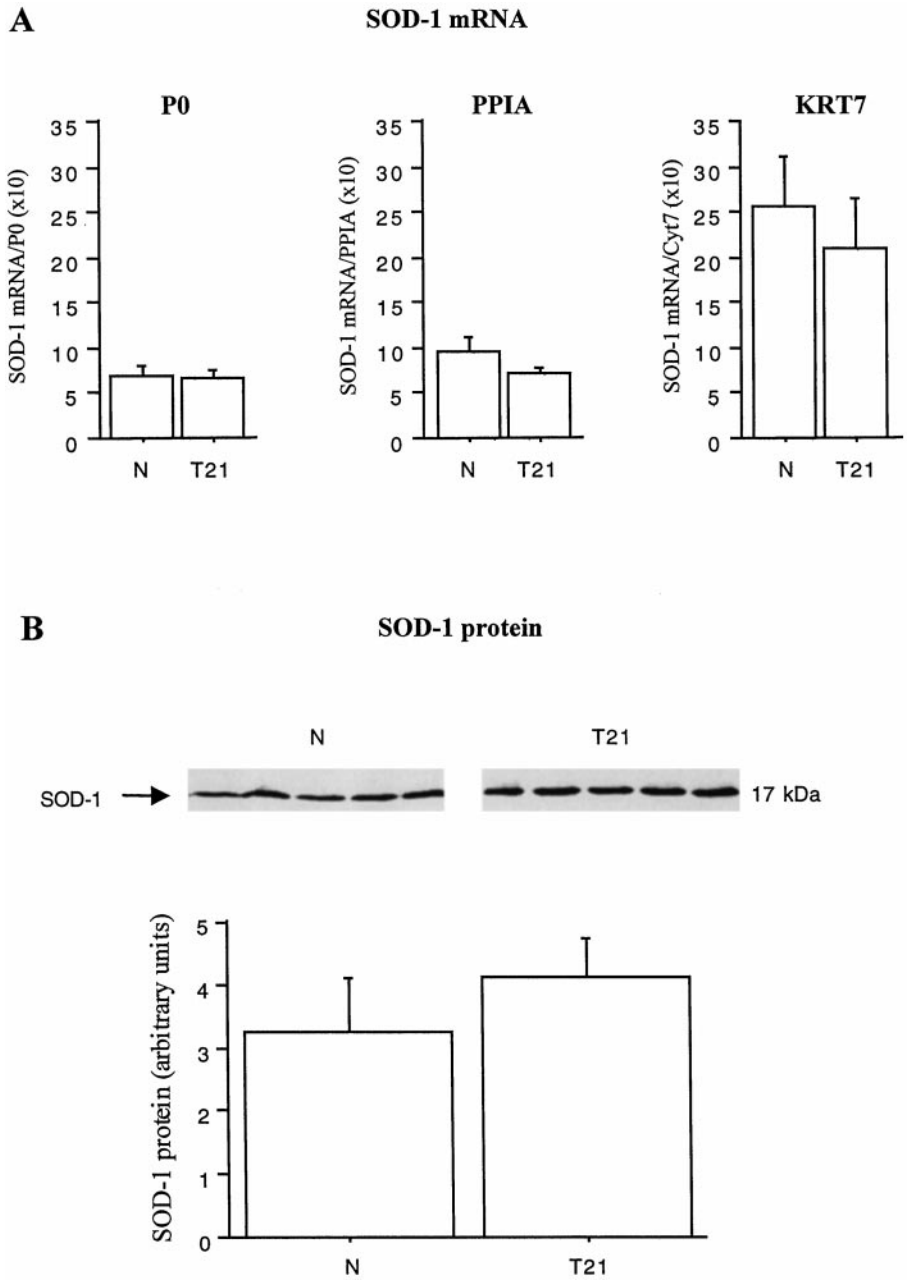

FIG. 5. SOD-1 mRNA and protein expression in total tissue samples obtained from normal (N) and T21-affected placentas (T21). A, SOD-1 mRNA levels determined by real-time quantitative PCR were normalized to pleiotropin, PPIA, and cytokeratin 7 mRNA levels (mean ± SEM). SOD-1 mRNA levels were determined in five normal placentas and seven T21-affected placentas. B, SOD-1 protein levels determined by Western blotting with a sheep polyclonal antibody to SOD-1. This autoradiogram shows a specific band of 17 kDa in normal (N) and T21-affected (T21) placentas. The *lower histogram* represents densitometry quantification of the autoradiograms (normal, n = 5; T21, n = 7).

SOD-1 mRNA and protein levels in trophoblast cells isolated from normal and T21-affected placentas

As cytotrophoblasts constitute a small percentage of the heterogeneous cell population found in placentas, we wanted to determine directly whether SOD-1 might be increased in an isolated population of T21 cytotrophoblasts. Thus, cytotrophoblast cells were purified and isolated from gestational age-matched normal and T21-affected placentas as described in *Materials and Methods*. As shown in Fig. 6A, SOD-1 mRNA was found in T21 trophoblast cells at a level about 1.5 times higher than that in normal trophoblast cells. This increase in SOD-1 mRNA expression was statistically significant ($P < 0.05$) and highly reproducible. SOD-1 protein levels and catalytic activity also were determined in both normal and T21-affected trophoblast cells (Fig. 6, B and C). Again, SOD-1 protein levels ($P < 0.01$) and enzymatic activity ($P < 0.05$) were significantly higher in T21 cells than in normal trophoblast cells, in keeping with the observed elevation of mRNA levels found in T21 cytotrophoblasts. These results establish and confirm that cells from individuals with this genetic disease, including cytotrophoblasts, have elevated levels of SOD-1 protein and catalytic activity. Further these cytotrophoblasts isolated from T21-affected placenta poorly differentiate into syncytiotrophoblasts.

Discussion

Few human cell types can fuse together and differentiate into multinucleated syncytia. This process is involved in the formation of myotubes (44), osteoclasts (45), and syncytiotrophoblast (3). The syncytiotrophoblast is the primary site of

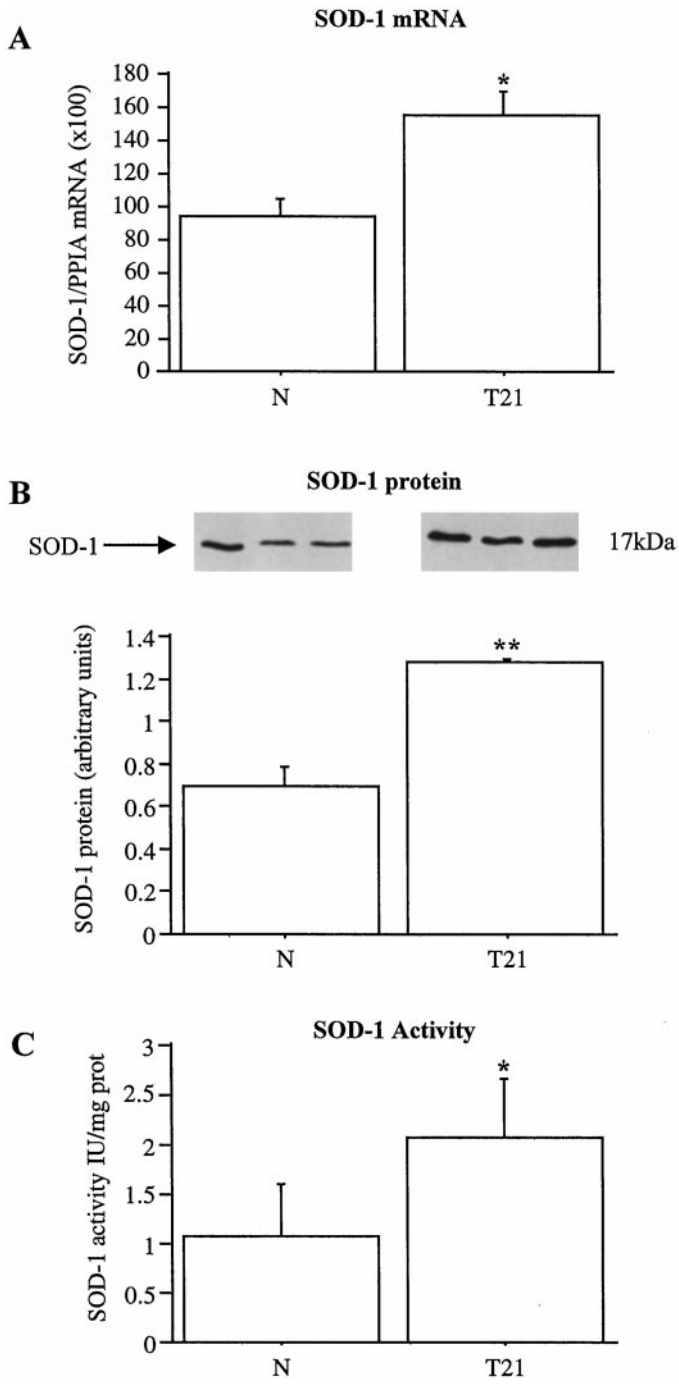

FIG. 6. SOD-1 mRNA and protein levels and catalytic activity in purified trophoblasts prepared from normal (N) and T21-affected (T21) placentas. A, SOD-1 mRNA levels determined by real-time quantitative PCR are normalized to PPIA mRNA levels (mean ± SEM). SOD-1 mRNA levels were determined in isolated trophoblasts prepared from five normal and seven T21-affected placentas. *, $P \leq 0.05$. B, Human SOD-1 protein levels determined by Western blotting with a sheep polyclonal antibody to SOD-1. The autoradiogram shows a specific band at 17 kDa in trophoblasts isolated from normal (N) and T21-affected (T21) placentas. The histogram represents densitometric quantification of the autoradiograms (normal, n = 5; T21, n = 7). C, Human SOD-1 activity is expressed in international units per mg protein. The results are expressed as the mean ± SEM. *, $P \leq 0.05$; **, $P \leq 0.01$.

several placental functions, including nutrient exchanges, metabolism, and steroid and peptide hormone synthesis, which are required for fetal growth and development (5, 6, 46). Despite a common morphological differentiation process, the three cell types that are able to differentiate into a syncytium differ notably. Syncytiotrophoblast *in situ* maintains a strong polarity, with microvilli on the apical membrane, whereas myotubes do not exhibit morphological polarity. The myoblast-myotube transition first requires the withdrawal of myoblasts from the cell cycle to G_0, whereas cytotrophoblasts that fuse to create the syncytiotrophoblast already are essentially in G_0 (10). In contrast to syncytiotrophoblast, osteoclasts have major locomotor activity.

The cell-cell fusion process involved in syncytiotrophoblast formation is poorly understood (47). *In vitro* studies have established that soluble factors such as EGF (12), PTH (11), and hCG (15) activate different intracellular signaling pathways to stimulate the differentiation of villous cytotrophoblasts into syncytiotrophoblast. The cellular processes leading to syncytial formation are associated with a concomitant increase in the intracellular level of cAMP (48, 49). This elevation in cAMP levels is required for the synthesis of numerous specific trophoblast proteins and hormones (for review, see Ref. 48). We also have reported a direct role for cAMP-dependent protein kinases in simulating cytotrophoblast fusion (51). On the other hand, we and others have observed that cytotrophoblast fusion and differentiation are inhibited by hypoxia (19–21). Similarly, the histological abnormalities of term placentas in pregnancy associated with underperfusion and hypoxia are characterized by cytotrophoblast prominence and abnormalities in syncytiotrophoblast differentiation (52, 53). This suggests that the oxidative state of the cytotrophoblast may be a key element in regulating differentiation into syncytium and points to a direct role for oxygen-derived free radicals in the modulation of cell fusion.

SOD-1 is a cytoplasmic enzyme that protects cells from oxygen-derived free radicals (26). SOD-1 transforms the superoxide anion $O_2^{\cdot -}$ into hydrogen peroxide, which is then converted to water by peroxisomal catalase and glutathione peroxidase. This two-step process eliminates H_2O_2 and other reactive oxygen species that could otherwise interact with macromolecules, such as DNA, proteins, and lipids, to alter their structure and function. However, any alteration of the balance between the first and second steps may induce an oxidative stress related to the misregulation of H_2O_2 production.

The SOD-1 gene is located on human chromosome 21 (43). The activity of this enzyme is increased by about 50% in the red blood cells (54), platelets (55), lymphocytes, polymorphonuclear granulocytes, and fibroblasts (56) of individuals with Down's syndrome (T21). In this study we first established that SOD-1 mRNA, protein, and activity were present in isolated normal human trophoblast cells, confirming and extending previous reports based on RT-PCR, differential display, and immunostaining (12, 28, 57). It then was shown that SOD-1 expression and activity in purified trophoblast cells isolated from T21-affected placentas were about 50% higher than those in normal trophoblasts, in keeping with a gene dosage effect. This increase in SOD-1 activity in cy-

totrophoblasts isolated from T21-affected placenta is associated with a defect of fusion and syncytiotrophoblast formation. These *in vitro* data are in agreement with recent data reporting histomorphological features of chorionic villi in T21-affected pregnancies, showing increased double layer proliferative trophoblasts (58). This suggests that overexpression of SOD-1 leads to increased oxidative stress and trophoblast injury, tending to stimulate proliferation and decrease differentiation, as observed in other pathological conditions.

However, when SOD-1 expression was compared in total tissue extracts from T21-affected placentas and normal controls matched for gestational age, no significant differences was found in SOD-1 transcript or protein levels. This finding appears to be due to the heterogeneous composition of the whole placenta. Total placenta extracts contain material of fetal origin, including fibroblasts, endothelial cells, and trophoblast cells. It also contains material of maternal origin, including red blood cells, along with endothelial and decidual cells. Therefore, SOD-1 levels in total placental extracts reflect SOD-1 expression in various cells of both fetal and maternal origins. SOD-1 is known to be highly expressed in maternal red blood cells and decidual cells (59). As cytotrophoblasts make up only a small percentage of the heterogeneous cell population found in placentas, we were unable to detect an elevation in SOD-1 levels in total tissue extract from the T21-affected placentas.

In this study experimental evidence suggests that an alteration in the oxidative state of human trophoblast cells related to overexpression of SOD-1 appears to be associated with a failure of differentiation and fusion into syncytiotrophoblast. This was illustrated by the inability of cells overexpressing SOD-1 to undergo cell-cell fusion. Indeed, we observed that primary normal human trophoblasts overexpressing SOD-1 tagged with GFP remained mononucleated and aggregated, as visualized by the detection of desmoplakin. In contrast, cells on the same dish that did not express SOD-1-GFP were able to fuse to a multinucleated syncytium. The inability of cells overexpressing SOD-1 to fuse and differentiate into syncytiotrophoblast was associated with a significant decrease in the transcript levels of genes encoding for pregnancy specific hormones such as hCG, hPL, and placental GH. These hormones are specifically expressed only by the differentiated syncytiotrophoblast. It should be noticed that this significant decrease in syncytiotrophoblast hormonal markers expression (*i.e.* an 80% decrease in hCG transcript levels) is in contrast to the relatively low transfection efficiency (10%) of these primary trophoblast cultures with SOD-1. Oxygen radicals have been reported to regulate cell signaling pathways and even to participate as second messengers in cellular signal transduction (25, 60). Thus, it is likely that a change in the oxidative state of a cell as a result of increased SOD-1 activity would have a significant effect on cell signaling and on the biological properties of cells. Thus, one possible explanation is that a single transfected cell may influence the regulatory properties of a number of its nontransfected neighboring cells through cell-cell contact (direct cell-cell communication) to regulate gene expression. A second possibility to explain this apparent discrepancy is that the altered generation and secretion of paracrine factors such as hormones, cytokines, cyclic nucleotides, or oxygen radicals in cells expressing SOD-1 in turn may affect neighboring cells. In this regard, cytokine secretion (14, 61), cAMP (51), and oxygen radicals (19) all have been implicated in trophoblast differentiation. These potential changes in cellular regulatory pathways can have significant effects on gene expression and cellular differentiation.

Other evidence has suggested that endogenous retroviral gene expression may be involved in mediating cell fusion. Indeed, high expression of retrovirus is one of the characteristics of human syncytiotrophoblast (62, 63). This observation of retroviral particles in placenta along with the presence of fused placental cells morphologically reminiscent of virally induced syncytia led to the proposal that an ancient retroviral infection may have been a pivotal event in mammalian evolution (63). Recently, syncytin gene expression, which codes for a retroviral envelop protein, was shown to be increased and required for trophoblastic cell fusion (42). In this communication, we confirmed by real-time PCR that the transcript levels of syncytin increased with the differentiation and fusion of cytotrophoblasts into syncytiotrophoblast. Further, it was shown that impaired cell fusion related to overexpression of SOD-1 was associated with a decrease in the transcript level of syncytin.

Individuals with Down's syndrome appear to exhibit increased oxidative stress (64, 65). They develop Alzheimer-like neuronal changes by the third or fourth decade of life, the incidence of autoimmune diseases and cataracts is significantly increased (66), and the overall aging process is accelerated (67). In addition, evidence that SOD-1 may be involved in the pathophysiology of Down's syndrome includes the demonstration that SOD-1 gene overexpression can impair neurotransmitter transport and alter neuromuscular junctions (68, 69). Here evidence is presented indicating that elevated SOD-1 levels may play a critical role in modulating the cell differentiation, especially the cell-cell fusion, process involved in human syncytiotrophoblast formation. Results presented in this communication also clearly demonstrate a relationship between elevated SOD-1 levels in human cytotrophoblasts and the decreased production of pregnancy-specific hormones found with differentiation to syncytiotrophoblast. Taken together, the results presented here suggest that the elevated level of SOD-1 noted in T21 may be responsible at least in part for the failure of cytotrophoblasts to fuse and form multinucleated syncytiotrophoblast as noted in Down syndrome (34, 35).

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