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Increased resistance to apoptosis during differentiation and syncytialization of BeWo choriocarcinoma cells

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ABSTRACT

Transition from mononuclear villous cytotrophoblast into multinuclear syncytiotrophoblast in the human placenta is accompanied by changes in apoptosis-related proteins and an apparent increased resistance to induced apoptosis. We investigated the specific nature and timing of changes in Bcl-2, Bax, p53, and caspases 3 and 8 in forskolin-treated BeWo choriocarcinoma cells, a model for villous cytotrophoblast differentiation. BeWo cells were treated with forskolin or vehicle alone for up to 72 h and evaluated at 24 h intervals for syncytialization and quantitative expression specific apoptosis-related proteins and mRNAs. Syncytialization was quantified using fluorescent staining of intercellular membranes and enumeration of the percentage of nuclei in multinucleate cells, and differential localization of apoptosis-related proteins to multinuclear or mononuclear cells was determined by quantitative immunofluorescence. Forskolin treatment for up to 72 h resulted in 80% syncytialization, increased expression of Bcl-2 protein ($P < 0.01$) and mRNA ($P < 0.05$), and significantly decreased expression of protein and mRNA for Bax, p53, and caspases 3 and 8. Syncytialized cells expressed higher levels of Bcl-2 protein concurrent with increased resistance to cisplatin-induced apoptosis. Thus, syncytialization of BeWo cells was accompanied by altered transcription of apoptotic-related proteins characteristic of increased apoptosis resistance secondary to increased expression of the anti-apoptotic protein Bcl-2 and diminish expression of pro-apoptotic proteins.

Keywords: BeWo; Trophoblast; Placenta; Caspase 8; Caspase 3; Bcl-2; Intercellular Fusion

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1. INTRODUCTION

The surface of the human placenta is composed of multinucleate syncytiotrophoblast that expands throughout pregnancy by intercellular fusion from an underlying feeder layer of mononuclear cells (villous cytotrophoblast). Transition from mononuclear villous cytotrophoblast into multinuclear syncytiotrophoblast was accompanied by a variety of changes in the level and activity of apoptosis-related proteins [1,2]. Normal placental syncytiotrophoblast expressed antiapoptotic and pro-apoptotic proteins, as well as traditional indicators of apoptosis [3-8]. Some nuclei were positive for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), suggesting DNA fragmentation. Areas of the syncytium contained both anti-apoptotic proteins (Bcl-2 and myeloid cell leukemia sequence 1 [Mcl-1]) and pro-apoptotic Bak [1,3-9]. Activated caspase 8 and Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein (c-FLIP), an inhibitor of caspase 8, appeared to be expressed in villous cytotrophoblast and syncytiotrophoblast [5,6,10], whereas activated caspases 3 and 8 were found only in the syncytiotrophoblast [3-5,10-12]. In spontaneously differentiating isolated term villous cytotrophoblast, however, p53, procaspase 3, and activated caspases 3 and 8 were reduced [13,14]. Efflux of the membrane phospholipid phosphatidylserine (PS) is a classical characteristic of apoptosis and also a necessary component of syncytiotrophoblast formation [15,16].

Alterations in levels of apoptotic-related proteins may indicate decreased sensitivity to exogenous inducers of apoptosis. Although the syncytiotrophoblast in the human placenta is exposed to circulating maternal immune effector cells, the antigenically foreign fetal-placental unit appears normally resistant to immune rejection. Increased resistance to apoptosis may be one of several

complementary survival mechanisms that protect the syncytiotrophoblast. Experiments to test whether the syncytiotrophoblast is relatively apoptosis-resistant have been equivocal. *In vitro* treatment of mononuclear villous cytotrophoblast and syncytiotrophoblast with staurosporine preferentially induced TUNEL-positive apoptotic nuclei in mononuclear cytotrophoblast, suggesting increased syncytiotrophoblast resistance to apoptosis [17]. In the same study, however, treatment with a combination of tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) induced near equal percentages of apoptotic nuclei in both cell types. Exposure of villous explant cultures to TNF-α or IFN-γ resulted in a greater apoptosis in the syncytium than in villous cytotrophoblast [18,19].

The concurrent change in apoptotic-related proteins and the process of differentiation and fusion of the villous cytotrophoblast into the syncytiotrophoblast suggests a role for apoptotic mechanisms in trophoblast differentiation. The potential contribution of caspase-8 to trophoblast differentiation has been discussed thoroughly in two recent reviews [20,21]. Activation of caspase-8 was proposed as an indicator of villous cytotrophoblast differentiation and intercellular fusion [22]. However, studies of placental villi and in vitro differentiation of villous cytotrophoblast have not confirmed activation of caspase-8 during differentiation [11,13,14].

To understand the role of apoptotic-related proteins in villous cytotrophoblast differentiation, the specific nature and timing of the changes must be defined. The current data are unclear because of controversial and frequently contradictory results. We hypothesized that increased resistance to apoptotic injury was an outcome of this process, and that syncytialization would be accompanied by a concurrent decrease in levels of pro-apoptotic pathway components and an increase in anti-apoptotic factors. We used BeWo cells, a controllable model of villous cytotrophoblast differentiation and syncytialization. In this study, progressive changes in expression of Bcl-2, Bax, p53, caspases 3 and 8 mRNA and protein were correlated with formation of syncytia and increased resistance to cisplatin-induced apoptosis. Reduction in the level of caspase-3 and -8 protein levels was not accompanied by a concomitant increase in activated caspase-8, but was the apparent effect of diminished transcription.

2. MATERIALS AND METHODS

2.1. Cell Culture and Fusion Induction

BeWo, a continuous human choriocarcinoma cell line (CCL 98; ATCC, Rockville, MD), was maintained in F12K medium (Cellgro, Herndon, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, cat # 10438-034) and a 1× mixture of penicillin G sodium, streptomycin sulfate, and L-glutamine (Invitrogen) [15]. For assays of intercellular fusion BeWo cells were transferred to MEM (Cellgro) containing the same supplements. The human choriocarcinoma lines JAR and JEG-3 were maintained in RPMI-1640 medium (Cellgro) and MEM medium, respectively, with 10% FBS, penicillin G sodium, streptomycin sulfate, and L-glutamine.

BeWo cells undergo in vitro differentiation and intercellular fusion during treatment with 10 μM of forskolin, an activator of adenylate cyclase [16]. A stock solution of 10 mM of forskolin (Sigma-Aldrich Corp, St. Louis, MO) was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, cat # P4393). Forskolin was added to each choriocarcinoma cell line to a final concentration of 10 μM for up to 72 h with daily replacement with fresh medium and forskolin. An equal volume of DMSO was used as the vehicle control. C2C12 (ATCC, cat # CRL-1772), a murine myoblast cell line, was cultured in Dulbecco's modified Eagle's minimal essential medium with the same supplements. To induce fusion, culture medium was switched to differentiation medium in which 2% normal horse serum (Invitrogen, cat # 16050-114) was substituted for FBS [23]. Cells were cultured in differentiation medium for three days followed by two days in culture medium. Hematoxylin staining was performed to evaluate the degree of intercellular fusion.

2.2. Quantifying Intercellular Fusion

Intercellular fusion of choriocarcinoma cell lines was quantified using anti-E-cadherin staining to visualize intercellular membranes, as previously described in detail [16]. Cultures were evaluated at 24, 48, or 72 h after addition of forskolin. Cells grown on cover slips (10^4 cells seeded per cover slip) in 24-well plates were fixed with 4% formaldehyde for 20 min followed by permeabilization with 0.5% Triton X-100 (Sigma-Aldrich, cat # T8787) for 5 min at 4˚C. Cells were blocked in 2% goat serum (Sigma-Aldrich, cat # G9023) and 2% bovine serum albumin (BSA; Sigma-Aldrich, cat # A7906) in phosphate-buffered saline (PBS) for 30 min at room temperature. Anti-E-cadherin (**Table 1**) was added for 1 h at room temperature, followed by washing and the addition of a FITC-conjugated secondary antibody for 1 h at room temperature. The cover slips were mounted onto slides using DAPI-containing mounting medium (Vector Laboratories, Burlingame, CA). The staining patterns were observed and recorded using a Nikon Eclipse80i microscope equipped with blue and green filters. For each cover slip, 10 fields were randomly chosen and photographed at the magnification of 200×. In each field, the total numbers of DAPI stained nuclei were counted and the percentage of nuclei in multinucleate cells was determined.

Table 1. Quantifying intercellular fusion.

Technique	Primary Antibodies				Secondary Antibodies			
	Antibody	Antibody Type	Source	Antibody Dilution	Antibody	Antibody Type	Source	Antibody Dilution
Fusion	E-cadherin	Mouse monoclonal	BD, cat # 610181	1:400	FITC-anti-mouse IgG	Goat polyclonal	JIR, cat # 115-095-003	1:200
Western blot	Bcl-2	Mouse monoclonal	BD, cat # 610538	0.2 µg/ml	HRP-anti-mouse IgG	Goat polyclonal	JIR, cat # 115-035-003	1:5,000
	caspase 3	Mouse monoclonal	BD, cat # 610323	0.2 µg/ml				
	caspase 8	Mouse monoclonal	BD, cat # 551242	0.2 µg/ml				
	p53	Mouse monoclonal	SC, cat # sc-126	1:200				
	Bcl-xL	Mouse monoclonal	SC, cat # sc-8392	1:200				
	Bax	Mouse monoclonal	SC, cat # sc-7480	1:200				
	β-actin	Mouse monoclonal	SA, either cat # A1978 or A2228	0.2 µg/ml				
	mouse Bcl-2	Mouse monoclonal	SC, cat # sc-23960	1:200				
	mouse caspase 3	Mouse monoclonal	BD, cat # 611048,	1:2000				
	mouse caspase 8	Mouse monoclonal	BD, cat # 551242	1:2000				
	cleaved caspase 3	Rabbit monoclonal	CST, cat # 9664	1:1000	HRP-anti-rabbit IgG	Goat polyclonal	JIR, cat #111-035-144	1:5,000
	cleaved caspase 8	Rabbit monoclonal	CST, cat # 9496	1:1000				
	mouse p53	Goat polyclonal	SC, cat # sc-1312	1:200	HRP-anti-goat IgG,	Rabbit polyclonal	JIR, cat #305-035-003	??
Immuno-fluorescence	Bcl-2	Rabbit polyclonal	SC, cat # sc-492	1:400	Texas Red-anti-rabbit IgG	Donkey polyclonal	SC, cat # sc-2784	1:200
	caspase 3	Goat polyclonal	SC, cat # sc-1224	1:400	Texas Red-anti-goat IgG	Rabbit polyclonal	SC, cat # sc-3919	1:200
	caspase 8	Rabbit polyclonal	SC, cat # sc-7890	1:400	Texas Red-anti-rabbit IgG	Goat polyclonal	SC, cat # sc-2780	1:200
	p53	Rabbit polyclonal	SC, cat # sc-6243	1:400	rhodamine-anti-rabbit IgG	Goat polyclonal	SC, cat # sc-2091	1:200

BD, BD Bioscience, San Jose, CA; *CST*, Cell Signaling Technology, Danvers, MA; *FITC*, Fluorescein isothiocyanate; *HRP*, horseradish peroxidase; *JIR*, Jackson ImmunoResearch Laboratories Inc, West Grove, PA; *MC*, monoclonal antibody; *PC*, polyclonal antibody; *SA*, Sigma-Aldrich Corp, St. Louis, MO; *SC*, Santa Cruz Biotechnology, Santa Cruz, CA.

2.3. Western Blot Analysis

Cells were lysed in buffer (20 mM Tris, pH 7.4, 125 mM NaCl, 20 mM NaF, 0.1% SDS, 10% glycerol, 0.5% sodium deoxylate, 1% Triton X-100, 1 mM PMSF, 2 µg/ml aprotinin, leupeptin, and pepstatin) and centrifuged [16]. The lysates were separated in a 4% - 20% gradient gel (Invitrogen) and transferred onto Immobolin PVDF membranes (Millipore, Billerica, MA). The membranes were blocked in 3% BSA and incubated with the appropriate primary antibodies and horseradish peroxidase-conjugated antibodies (see **Table 1**). Additional antibodies that reacted more strongly against murine Bcl-2, caspase 3, caspase 8, and p53 were used in Western blots of lysates from C2C12 cells. Membranes were stripped with Restore Western blot stripping buffer (Pierce, Rockford, IL) and reblotted with additional antibodies. Protein bands were quantified by densitometry of autoradiograms using a Scion Image Program (Scion Corporation, Frederick, MD). The density of each band was normalized against the paired β-actin band, and the ratio of the expression level of each target protein in forskolin-treated cells to vehicle-treated cells was calculated.

2.4. Real-Time PCR

Total RNA was extracted from the cells using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's manual. Total cDNA was reverse transcribed using the SuperScript II First-Strand Synthesis system for RT-PCR (Invitrogen). Briefly, 5 µg of total RNA was mixed with 50 ng of random hexamers and dNTP and denatured at 65˚C for 5 min. A cDNA mixture containing RT buffer, 5 mM MgCl2, 10 mM DTT, 2 U RNaseOUT, and 10 U SuperScript reverse transcriptase was added to the denatured RNA. Reverse transcription was performed at 25˚C for 10 min followed by 50˚C for 50 min. RNA was digested from cDNA by adding RNaseH at 37˚C for 20 min. Real time PCR was carried out in a 25 µl mixture of 1 µl cDNA, 2 µl 20mM primer pair, 12.5 µl CYBR green PCR master mix (Applied Biosystems, Foster City, CA), and 9.5 µl of water. Each reaction was performed in triplicate. Primers used in this study include: p53 (forward 5'-CCC AGC CAA AGA AGA AAC CA-3"; reverse 5'-GTT CCA AGG CCT CAT TCA GCT-3'), Bax-1 (forward 5'-CAA ACT GGT GCT CAA GGC CC-3'; reverse 5'-GCA CTC CCG CAC AAA GAT G-3'), Bcl-2 (forward 5'-CAG ATG CAC CTG ACG CCC TT-3'; reverse 5'-CCC AGC CTC CGT TAT CCT GGA-3'), Bcl-xL (forward 5'-GGG GTA AAC TGG GGT CGC ATT-3'; reverse 5'-CTT GCG AAG TTG GCG TCC A-3'), caspase 3 (forward 5'-AGA ACT GGA CTG TGG CAT TGA-3'; reverse 5'-GCT TGT CGG CAT ACT GTT TCA G-3'), caspase 8 (forward 5'AGG AGG AGA TGG AAA GGG AAC TT-3'; reverse 5'-ACC TCA ATT CTG ATC TGC TCA CTT CT-3'), and 18S control (forward 5'-CGG CTA CCA CAT CCA AGG AA-3'; reverse 5'-GCT GGA ATT ACC GCG GCT-3'). RT-PCR was performed using a 7500 Real Time PCR System (Applied Biosystems) with a program of 95˚C for 5 min followed by 40 cycles of 95˚C for 15 s and 60˚C for 1 min. The 7500 System SDS software (Applied Biosystems) was used to analyze the data related to the 18S RNA control. The ratio of the mRNA level in forskolin to vehicle treated cells was calculated as $2^{(CtDMSO-Ctforskolin)}$.

2.5. Quantitative Immunofluorescence

BeWo cells were grown and stained with anti-E-cadherin, as described above. After washing, the cells were treated for 1 h at room temperature with a primary antibody against an apoptosis-related protein, followed by washing and the addition of an appropriate fluorescent secondary antibody (see **Table 1**).

All digital images were taken using a Nikon Eclipse 80i microscope equipped with blue, green, and red filters. Fluorescent staining was quantified using MetaVue, Meta Imaging Series® 6.1(Universal Imaging Corporation, Downingtown, PA) software. Fused and non-fused cells were identified and delineated in mask images based on nuclear staining with DAPI and intercellular membrane staining with anti-E-cadherin. These delineated areas were transferred to a companion red fluorochrome image for quantitative image analysis. Each companion area was required to be in exact register with the mask image. Thresholds were set for analysis and the fluorescent density of each region quantified. Quantitative data were expressed as the ratios of fluorescent intensity between mononuclear and syncytial cells in the same culture and represent the analysis of three independent experiments. The total number (n) of measurements in those experiments ranged from 20 to 39 (mean = 28.9, median = 29).

2.6. Apoptosis Induction

BeWo cells were grown on cover slips in 24 well plates and treated with forskolin for 48 h as described above. The culture medium was removed and replaced with medium containing cisplatin (Sigma-Aldrich, cat# P4394) at a concentration of 10 µM for 16 h. Medium was removed and cells were washed once with PBS and stained with blue-fluorescent Hoechst 33342 and green-fluorescent YO-PRO (Invitrogen). Hoechst 33342 brightly stains condensed chromatin in apoptotic cells and dimly stains the chromatin in live cells. YO-PRO can enter apoptotic cells but not the live cells. Diluted Hoechst 33342 (5 µg/ml) and YO-PRO (0.1 µM) were added to the cells and incubated for 30 min on ice. Cells were

washed and cover slips were mounted onto slides using VECTASHIELD mounting medium (Vector Labs). The staining patterns were recorded immediately using a Nikon Eclipse80i microscope equipped with blue and green filters and a Cool SNAP Photometrics camera.

2.7. Statistical Analysis

All quantitative data were expressed as mean ± standard deviation and analyzed using one way analysis of variance/least significant difference (Tukey).

3. RESULTS

The BeWo model is a highly reproducible model of villous cytotrophoblast differentiation and syncytialization. Forskolin induced progressive intercellular fusion; the number of nuclei in multinucleate cells were 20.8% ± 15.9% at 24 h (10.7% ± 2.6% in the vehicle-treated control; P = NS), 65.2% ± 5.2% at 48 h (10.9% ± 2.6% in control, $P < 0.01$; $P < 0.01$ compared with 24 h forskolin-treated cells), and 80.2% ± 4.1% at 72 h (8.9% ± 1.3% in control, $P < 0.01$; $P < 0.01$ compared with 48 h forskolin treated cells). These data are in very close agreement with our previous observations [16] and those of other investigators [24-27]. As we have cautioned in the past, maximum rates of intercellular fusion are not observed using media F-12, but require the use of media F-12K or MEM [28].

Bcl-2 expression was increased during forskolin-driven differentiation of BeWo compared with the vehicle-treated controls (**Figure 1(a)**). Increased transcription preceded elevations in protein levels (**Figure 1(b)**); mRNA levels were increased over controls by 48 h (7.7 fold increase, $P < 0.01$) and remained at a steady level through 72 h. A resultant significant increase in protein levels occurred by 72 h (6.9 fold, $P < 0.01$). Expression of Bcl-xL (B-cell lymphoma-extra large), an antiapoptotic protein in the Bcl-2 protein family, was not affected by forskolin treatment of BeWo cells (data not shown).

Expression of Bax, a pro-apoptotic protein, was suppressed during differentiation of BeWo cells (**Figure 1(a)**). Transcription of Bax was significantly decreased by 24 h ($P < 0.01$) and remained suppressed throughout all time points (**Figure 1(c)**). The level of protein was significantly decreased ($P < 0.01$) by 72 h of forskolin-induced differentiation. Thus the estimated ratio of Bcl-2 to Bax was progressively increasing throughout the differentiation process; 2.2 at 24 h, 5.0 at 48 h, and 10.0 at 72 h.

Treatment with forskolin also suppressed transcription of p53 mRNA (**Figure 1(d)**) at all time points: 39% at 24 h ($P < 0.01$), 56% at 48 h ($P < 0.01$), and 75% at 72 h ($P < 0.01$) (**Figure 1(c)**). Levels of p53 protein were also reduced significantly (**Figure 1(d)**) by 39% at 24 h ($P < 0.01$), 52% at 48 h ($P < 0.01$), and 62% at 72 h ($P < 0.01$).

Expression of the pro-forms of the effector caspase 3 (procaspase 3) and initiator caspase 8 (procaspase 8) were diminished during differentiation of BeWo cells (**Figure 1(a)**). The level of procaspase 3 mRNA was significantly decreased by 24 h of treatment (52% decrease, $P < 0.01$), without any significant further decrease thereafter (**Figure 1(e)**). Protein was also significantly decreased by 24 h (29% decrease, $P < 0.05$) and diminished further by 72 h (64% decrease, $P < 0.05$ compared to 24 h). Altered expression of procaspase 8 was delayed in comparison to procaspase 3; mRNA was reduced by 42% at 48 h ($P < 0.01$ compared to control) with no significant further reduction thereafter, and protein was progressively reduced over the 72 h time span; 21% reduction at 48 h ($P < 0.01$ versus control, $P < 0.05$ versus 24 h) and 46% reduction at 72 h ($P < 0.01$ versus control, $P < 0.01$ versus 24 h) (**Figure 1(f)**). We also measured levels of active caspases 3 and 8 to determine whether loss of procaspase proteins resulted from activetion (**Figure 1(g)**). No active caspase 3 bands were observed in forskolin-treated BeWo cells. Although a very small amount of activated caspase 8 was observed in BeWo treated with DMSO, the level did not significantly fluctuate during treatment with forskolin. The active fragments of both were readily observed after treating BeWo cells for 3 h with staurosporine, a known inducer of apoptosis (**Figure 1(g)**) [16].

To analyze the relationship between changes in apoptosis-related protein expression and intercellular fusion, we determined the distribution of protein between mononuclear and multinuclear cells by quantitative immunofluorescence (**Figures 2(a)** and **(b)**). Although we performed multiple time points with and without forskolin treatment (0, 24, 48, and 72 h), only the 72 h samples are presented.

Bcl-2 was the only protein in our study that increased during differentiation. Microscopically, the fluorescent signal for Bcl-2 appeared more intense in syncytial cells than in mononuclear cells, which was confirmed by quantification (**Figure 2(c)**). Increased expression of Bcl-2 was related to syncytialization, whether spontaneous (DMSO control) or forskolin-induced; fluorescence in DMSO treated cells was more intense ($P < 0.01$) in fused cells (90.4 ± 12.3) than mononuclear cells (77.6 ± 12.0). The same relationship was observed in cells treated with forskolin: mononuclear cells, 75.2 ± 9.6; fused cells, 97.5 ± 16.0 ($P < 0.01$). Treatment with forskolin appeared to augment the fluorescent intensity in populations of fused cells ($P = 0.05$), but not mononuclear cells (P = NS). Increased expression of Bcl-2 protein in syncytial cells was confirmed by immunoperoxidase labeling of human placental villi, in which intense

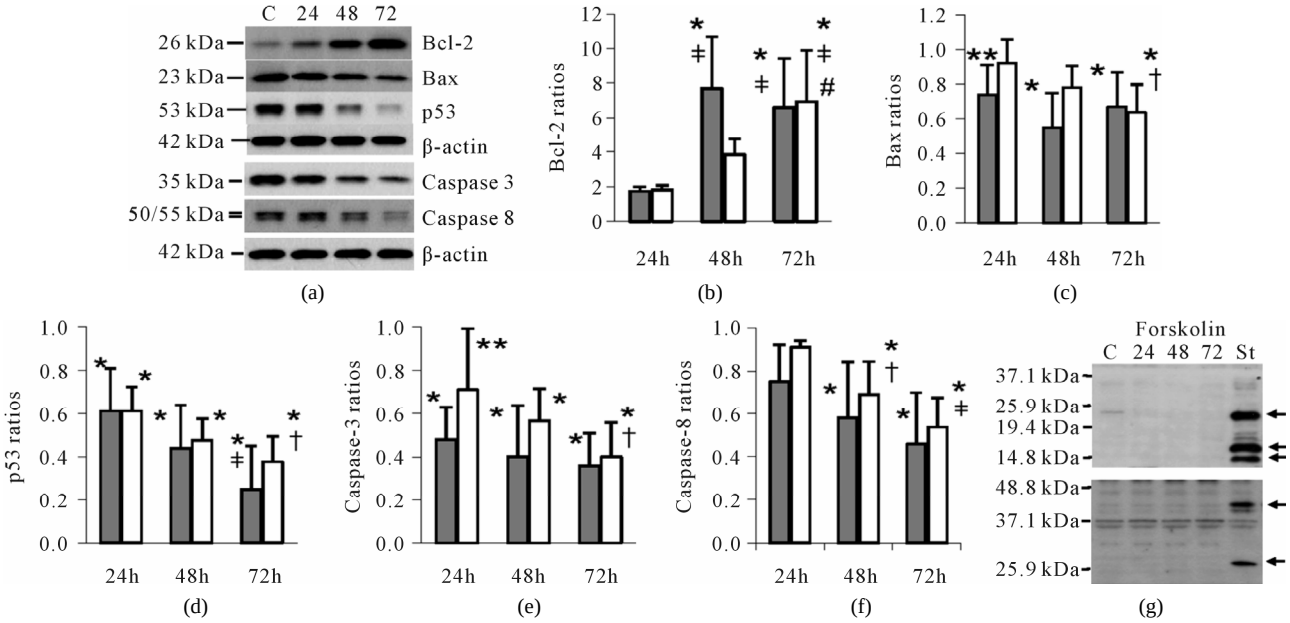

Figure 1. Expression of apoptosis-related proteins and mRNAs in BeWo cells undergoing forskolin-induced intercellular fusion. (a) Western blot analysis of extracts of BeWo cells treated with the DMSO vehicle control (Cont) for 72 h or forskolin for 24, 48, or 72 h; (b)-(f) Quantitative expression of Bcl-2 (b); Bax (c); p53 (d); caspase 3 (e); and caspase 8 (f) mRNA (filled bars) and protein (empty bars). Data are expressed as means and standard deviations of the ratios of forskolin-treated BeWo cells compared to the corresponding vehicle-treated controls at 24, 48, and 72 h of a minimum of five experiments at each time point. $^{**}P < 0.01$ or $^{****}P < 0.05$ relative to the matched negative control; $^{‡}P < 0.01$ or $^{†}P < 0.05$ relative to the 24 h time point; $^{\#}P < 0.01$ relative to the 48 h time point; (g) Analysis of active caspase 3 (upper blot) and active caspase 8 (lower blot) fragments (indicated by arrows) by Western blot in BeWo cells treated with vehicle control (c) or forskolin for 24, 48, or 72 h. A positive control for caspase activation was produced by inducing apoptosis in BeWo cells using 1 μM staurosporine for 3 h (St) [16].

staining was confined to syncytiotrophoblast (data not shown). Because of lower fluorescent intensity, relative levels of procaspase 3, procaspase 8, and p53 could not be accurately determined between mononuclear and syncytial cells (data not shown).

Changes in expression of apoptotic-related proteins may be unique to BeWo or secondary to intercellular fusion processes or forskolin-induced up-regulation of intracellular cAMP. We assessed the effects of forskolin on JAR and JEG-3 choriocarcinoma cells. BeWo cells readily form syncytia, whereas JAR and particularly JEG-3 cells do not routinely undergo extensive syncytialization [29,30]. We also evaluated a non-trophoblast model of intercellular fusion, C2C12 cells. C2C12 is a murine myoblast cell line that undergoes intercellular fusion when cultured in 2% normal horse serum instead of 10% fetal bovine serum [23]. Morphologic changes consistent with syncytialization were visible by hematoxylin staining in C2C12 cells cultured in differentiation medium compared to cells in growth medium (data not shown). Neither forskolin treatment of JAR nor JEG-3 nor intercellular fusion of C2C12 cells resulted in changes in apoptotic-related proteins at 24, 48, or 72 h (data not shown).

Cisplatin, a drug that induces apoptotic cell death in many cell types, was added to cultures of BeWo cells treated for 48 h with forskolin or controls treated with vehicle alone. Cells were evaluated for apoptosis by fluorescent staining using Hoechst 33342 and YO-PRO dyes as well as Western blot analysis for activation of caspases 3 and 8. Treatment of control cells with cisplatin resulted in apoptotic cells that stained with blue Hoechst 33342 and green YO-PRO dye (**Figure 3(a)**). Cells treated with forskolin displayed sparse staining (**Figure 3(b)**). Cisplatin induced activation of caspases 3 and 8 in control cells, but had no effect in forskolin-treated cultures (**Figure 3(c)**). Thus, cultures of predominantly syncytialzed BeWo cells appear to be relatively resistant to the induction of apoptosis by cisplatin.

4. DISCUSSION

To our knowledge this is the first study to define changes in expression of apoptosis-related proteins in a controllable model of villous cytotrophoblast differentiation and intercellular fusion, BeWo cells. Syncytialization was associated with increased levels of the anti-apoptotic protein Bcl-2 and decreased expression of several pro-apoptotic molecules; Bax, p53, caspase 3, and caspase 8. Levels of Bcl-xL remained unchanged.

Our data are similar to previous studies of primary

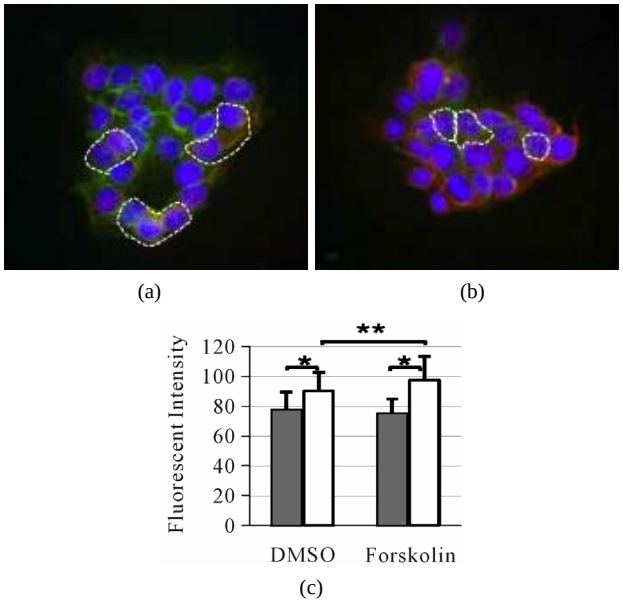

Figure 2. Quantitative immunofluorescence of Bcl-2 protein. Immunofluorescence was used to determine the distribution of Bcl-2, labeled with Texas Red-conjugated antibody, in BeWo cells treated with the DMSO vehicle alone (a) or forskolin (b) for 72 h. Staining of cell junctions with FITC-anti-E-cadherin (green stain) was used to distinguish mononuclear from fused cells and nuclei were stained with DAPI (blue stain). In the upper photograph fused cells are circles, whereas in the lower photograph mononuclear cells are circled. (c) Fluorescent staining of Bcl-2 was quantified in mononuclear cells (filled bars) or fused cells (empty bars) and expressed as fluorescent intensity from three independent experiments. $^{**}P < 0.01$ relative to indicated matched negative control, $^{****}P = 0.05$ relative to fused cells in DMSO treated cells.

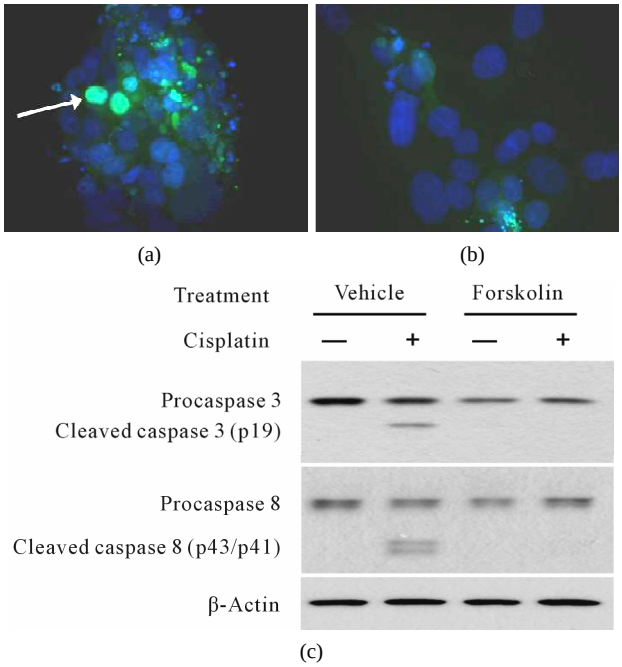

Figure 3. Cisplatin treatment of BeWo cells. BeWo cells were treated with either vehicle alone (a) or forskolin (b) for 48 h followed by 10 μM cisplatin for 16 h. YO-PRO and Hoechst 33342 were used to assess apoptosis (green apoptotic cells are indicated by the arrow in (a)). (c) Western blot analysis of cisplatin-induced (lanes with +) activation of caspase 8 in BeWo cell cultures pretreated with vehicle alone or forskolin.

villous cytotrophoblast cultures in which levels of procaspase 3 and p53 proteins diminished between 24 and 72 h of culture [13,14]. No differences were observed in levels of procaspase 8, Bcl-2, Bax, and Bcl-xL. Although the culture conditions normally support spontaneous syncytialization of villous cytotrophoblast, markers of differentiation were not assayed. Our data confirm decreased expression of procaspase-3 and p53 proteins extend those results; mRNA levels of each protein were also significantly reduces, suggesting an effect of differentiation at the transcriptional level. Several significant differences between studies should be noted. We observed differentiation-related increased expression of Bcl-2 protein and mRNA, with localization of the protein primarily in multinucleate cell. Our data are in agreement with reports of selectively increased expression of Bcl-2 in the syncytial layer of placenta [1,3-9]. It should be noted, however, that all *in vitro* models of villous cytotrophoblast differentiation, including the choriocarcinoma BeWo, primary cultures of villous cytotrophoblast, and villous explants, have inherent flaws and caveats that must be considered when interpreting data. Although BeWo is a choriocarcinoma, the characteristics of forskolin-induced syncytialization appear to closely replicate those seen *in situ*.

Decreased procaspase levels may result from diminished expression or increased consumption, one method of which is caspase activation. Using isolated placental villi, activation of caspase 8 was observed in mononuclear villous cytotrophoblast preceded syncytialization, whereas activation of caspase 3 was only observed in syncytiotrophoblast [4]. However in primary villous cytotrophoblast cultures, both activated caspases 3 and 8 were observed at 24 h of culture, and their levels decreased over the next 48 h, during the period in which syncytialization would be occurring [13]. We observed no evidence of caspase 3 or 8 activation and diminished caspase expression appeared to reflect down-regulation of transcription preceding syncytialization.

Diminished expression of pro-apoptotic proteins and increased Bcl-2 expression during transition of the villous cytotrophoblast to the syncytial phenotype may indicate a decreased sensitivity to induction of apoptosis. The syncytiotrophoblast has developed multiple active and passive mechanisms for preventing recognition and attack by the maternal immune and inflammatory systems [31]. The trophoblast may actively thwart potentially damaging maternal effector cells through expres-

sion of Fas ligand (FasL) on the cell surface and secretion of soluble apoptosis-inducing FasL into the maternal circulation [32]. The syncytiotrophoblast also expresses high levels of four different receptors for TNF-related apoptosis-inducing ligand (TRAIL) [33]. Macrophages are susceptible to apoptosis induced by TRAIL, thus over-expression of TRAIL may be an active mechanism that is important for maintaining the immune privilege status of the placenta.

Members of the Bcl-2 family are divided into two sub groups according to their roles in apoptosis; anti-apoptotic proteins (e.g. Bcl-2 and Bcl-xL) and pro-apoptotic proteins (e.g., Bax, Bad [Bclantagonist of cell death], Bid). The anti-apoptotic activity of Bcl-2 is often dependent on its relative concentration to Bax, so that an increasing Bcl-2/Bax ratio is indicative of resistance to apoptosis [34,35]. In other cell models over- expression of Bcl-2 inhibited Bax-induced activation of caspase 3 and rescued cells from apoptosis [36]. Syncytialized BeWo cells expressed increased levels of Bcl-2 and reduced protein level of Bax resulting in an increased Bcl-2/Bax ratio. Indeed, syncytialized BeWo cells were more resistant to cisplatin-induced apoptosis than were mononuclear BeWo cells.

Changing levels of Bcl-2 and Bax protein appeared to reflect altered transcription. Transcription of Bcl-2 and Bax are regulated by p53, which generally suppresses the expression of anti-apoptotic proteins and stimulates expression of pro-apoptotic molecules, such as Bax [37]. Decreased expression of p53 during BeWo cell syncytialization precedes significant changes in Bcl-2 expression, which may indicate a causal interrelationship.

Increased resistance to apoptosis appears to specifically relate to trophoblast syncytialization, rather than be a general phenomenon of intercellular fusion. Under normal physiologic conditions very few cells undergo intercellular fusion; one of which is the skeletal myoblast that fuses to form multinuclear myotubes. Fusion of a myoblast model, C2C12 cells, was not accompanied by alterations in expression of apoptosis-related proteins. Induced resistance to apoptosis is more critical for the syncytiotrophoblast, which is derived from immunologically foreign fetal tissue and a more likely target for maternal immune cells.

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