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Thromboxane A_2 Limits Differentiation and Enhances Apoptosis of Cultured Human Trophoblasts

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ABSTRACT

Prostanoids influence differentiation in diverse cell types. Altered expression of cyclooxygenase and prostaglandins has been implicated in the pathophysiology of placental dysfunction, which results in preeclampsia and fetal growth restriction. We hypothesized that prostanoids modulate differentiation and apoptosis in cultured human trophoblasts. Villous cytotrophoblasts were isolated from term human placentas and cultured in serum-free medium. The level of human chorionic gonadotropin was used as a marker of biochemical differentiation of primary trophoblasts, and syncytia formation was used as a marker of morphologic differentiation. Of the prostanoids tested, we found exposure to thromboxane A_2 hindered both biochemical and morphologic differentiation of cultured trophoblasts. As expected, human chorionic gonadotropin levels in the media were elevated in a concentration-dependent manner in the presence of the thromboxane synthase inhibitor, sodium furegrelate, or the thromboxane A_2 receptor blocker SQ 29,548. Furthermore, thromboxane A_2 enhanced trophoblast apoptosis, determined using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining, cell morphology, and a concentration-dependent increase in p53 expression. We conclude that thromboxane A_2 hinders differentiation and enhances apoptosis in cultured trophoblasts from term human placenta. We speculate that thromboxane may contribute to placental dysfunction by restricting differentiation and enhancing apoptosis in human trophoblasts. **(*Pediatr Res* 50: 203–209, 2001)**

Abbreviations

hCG, human chorionic gonadotropin
FGR, fetal growth restriction
TX, thromboxane
PG, prostaglandin
TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

The function of the human placenta depends on multinucleated, terminally differentiated syncytiotrophoblasts. This epithelium is in direct contact with maternal blood and regulates maternal-fetal exchange of micronutrients and gases. The syncytiotrophoblast arises from fusion of relatively undifferentiated, mitotically active cytotrophoblasts. This process involves morphologic and biochemical differentiation. Morphologic differentiation is defined by fusion of mononucleated cytotrophoblasts with adjacent syncytium (1). Biochemical differentiation is characterized by production of hormones such as hCG and human placental lactogen (2–4). Preeclampsia and FGR are associated with trophoblast hypoxia and placental dysfunction (5). Villi from women with these conditions typically exhibit diminished villus formation, prominent cytotrophoblasts, enhanced syncytial knot formation, and apoptosis (6, 7). An increase in TX synthesis has been demonstrated in villi (8, 9) and trophoblasts (10, 11) from preeclamptic women compared with healthy control subjects. Importantly, FGR (12) and preeclampsia (13) are associated with elevated TXA_2 levels in the circulation of pregnant women. Although the effect of prostanoids on vascular reactivity is well known (14), recent studies indicate that prostanoids also regulate proliferation, differentiation, and apoptosis in many cell types (15–18). We tested the hypothesis that prostanoids influence the differentiation of cultured trophoblasts from term human placenta. We found that TXA_2 hinders both morphologic and biochemical differentiation and enhances apoptosis in primary human trophoblasts.

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METHODS

Cell isolation and culture. The study was approved by the Human Studies Committee of Washington University. Placen-

tas were obtained immediately after term singleton deliveries after uncomplicated pregnancies. Cytotrophoblasts were isolated by the trypsin-DNase, Percoll (Pharmacia, Piscataway, NJ, U.S.A.) gradient centrifugation method described by Kliman *et al.* (2), modified by inclusion of 10 mL of Dispase (Collaborative Research, Bedford, MA, U.S.A.) for each 50 g of tissue in the enzyme digestion mixture. Cell isolation and growth characteristics *in vitro* have been previously characterized (19–21). Isolated cytotrophoblasts were plated on polystyrene plates (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) in duplicates at 3×10^5 cells/cm^2 in medium 199 (Tissue Culture Facility, Washington University) with 10% fetal bovine serum (Hyclone, Logan, UT, U.S.A.), 20 mmol HEPES (Sigma Chemical Co., St. Louis, MO, U.S.A.), and 2 mmol L-glutamine (Sigma Chemical Co.) in a 5% carbon dioxide–air atmosphere at 37°C. After 4 h, the medium was replaced with serum-free medium 199. Media were changed every 24 h. All prostanoids were obtained from Cayman Chemicals (Ann Arbor, MI, U.S.A.) except 9α,11β-PGF$_2$, obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA, U.S.A.).

Hormone assays. All media samples were stored at −20°C until assayed for hCG or prostanoids. The concentration of hCG was determined by a microparticle enzyme immunoassay (Abbot Laboratories, Abbot Park, IL, U.S.A.) and expressed as milli-International Units per microgram cellular DNA. DNA was isolated from cells using DNAzol (Molecular Research Laboratories, Cincinnati, OH, U.S.A.) and quantified by spectrophotometry. TXA_2 levels were determined by measuring its stable metabolite, TXB_2, by a specific enzyme immunoassay (Oxford, Oxford, MI, U.S.A.), as previously described (22), and expressed as nanograms of eicosanoid per milliliter of media. Interassay and intraassay variations were 9.1% and 5.3% for hCG and 8.2% and 5.5% for TXB_2, respectively.

Immunocytochemistry. Primary trophoblast cultures were rinsed with PBS, fixed, and permeabilized with methanol (Fisher Scientific, Pittsburgh, PA, U.S.A.) at −20°C for 25 min. To decrease nonspecific binding, we incubated the cells with 2% goat serum in staining buffer (PBS with 0.2% BSA) for 35 min in a humidified chamber at 37°C. Cells were then stained with a mixture of mouse monoclonal anti-human desmosomal antibodies (Sigma Chemical Co.) to label cell surface membranes and with human antinuclear antibody (Antibodies Inc., Davis, CA, U.S.A.) which binds nuclei as previously described (19, 22). An observer blinded to the culture conditions examined eight equidistant fields with a microscope (Zeiss, IM 35, Oberkochen, Germany) equipped with a 63× oil objective and epifluorescence optics. Multinucleated cells with >3 nuclei in a contiguous cytoplasm were identified and quantified as syncytia per field. The assessment of syncytia was confirmed with a BioRad MRC 1024 confocal microscope equipped with Zeiss Axioplasm objectives and Laser Sharp v. 3.2 software (Bio-Rad, Hercules, CA, U.S.A.).

TUNEL staining and morphologic assessment. Cultured cells were washed twice with PBS and fixed in 4% formalin for 10 min. Endogenous peroxidase was quenched with 3% H_2O_2. The Apop Tag kit (Oncor, Gaithersburg, MD, U.S.A.) was used for TUNEL staining according to the manufacturer's instructions. Using bright-field optics at 1000×, two observers blinded to culture conditions quantified TUNEL-stained nuclei as well as the number of total nuclei in eight random fields. The apoptotic index was defined as the percentage of TUNEL-positive nuclei divided by the total number of nuclei. Cells exposed to vehicle alone served as control, and omission of the terminal deoxynucleotidyl transferase step served as a staining control.

In a tandem analysis, we examined cells for morphologic changes typical for apoptosis, including condensed or fragmented nuclei, a low cytoplasm to nucleus ratio (indicative of cell shrinkage), and blebbing of the surface membrane. Cells with apoptotic morphology were expressed as a percentage of the total number of cells counted.

Western blot. Cells were collected in a lysis buffer that contained 10% SDS and were sonicated. Samples with equal amounts of protein (30 μg) were subjected to electrophoresis at 60 V for 2 h in 4°C using 10% polyacrylamide gel slabs, then transferred overnight to polyvinylidene difluoride membranes. Membranes were incubated with 10% skim milk for 1 h, incubated with an anti-p53 MAb (Santa Cruz Biochemicals, Santa Cruz, CA, U.S.A.) for 1 h, washed twice for 10 min with Tris-buffered saline (TBS; 0.75% Tween 20), and then incubated for 1 h with horseradish-peroxidase–linked secondary antibodies (Santa Cruz). The membrane was washed twice for 10 min with TBS, processed for chemiluminescence using an enhanced chemiluminescence kit (Amersham, Pharmacia Biotech, Piscataway, NJ, U.S.A.), and exposed to film for 10 min. Cells exposed to vehicle alone served as controls. The density of the immunoblot bands was quantified using a densitometer and Image Quant version 3.3 software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Statistical analysis. Each measurement was performed in duplicate for each paradigm. Data are presented as mean ± SD. Comparisons were made using *t* test and ANOVA with Bonferroni correction where applicable. A $p < 0.05$ was considered significant.

RESULTS

Influence of prostanoids on trophoblast differentiation. We initially screened the effect of nine prostanoids on media levels of hCG during a 72-h culture period. These prostanoids were selected because they are known to affect differentiation, apoptosis, or proliferation of nontrophoblast cells (23–27). We used 15-deoxy-$\Delta^{12,14}$-PGJ$_2$, known to hinder differentiation and enhance apoptosis, as a positive control (28). Because of the short half-life of TXA_2 (32 s), we used a stable analog of TXA_2, carbocyclic TXA_2, for our experiments (29). Among the prostanoids tested, exposure of cultured trophoblasts to TXA_2 resulted in a significant reduction in hCG release compared with control cells (Fig. 1*A*). The effect of TXA_2 on medium hCG was concentration dependent, with a maximum effect at 10 μM (Fig. 1*B*), and the effect was apparent in the absence or presence of serum. This result indicates that exposure of trophoblasts to exogenous TXA_2 hinders their biochemical differentiation.

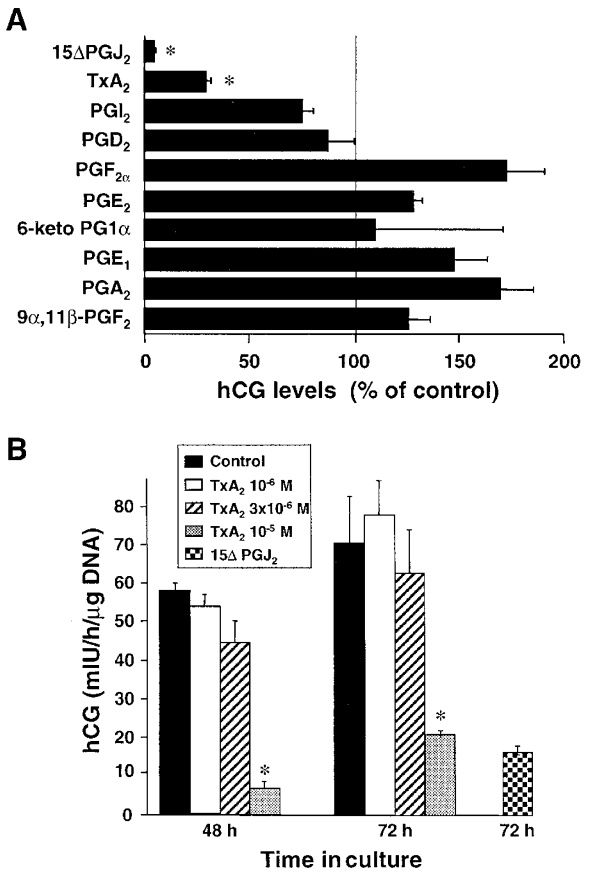

Figure 1. *A*, level of hCG in medium from trophoblasts cultured for 72 h in the presence or absence of nine different prostanoids (all at 10 μM). 15-Deoxy-$\Delta^{12,14}$-PGJ_2, known to diminish hCG production, served as a positive control. Error bars represent ± SD. *B*, level of hCG in medium from trophoblasts cultured in presence of vehicle (control) or increasing concentrations of TXA_2. The result represents five different experiments on different placentas. Levels of hCG (mean ± SD) are shown as milli-International Units per hour per microgram of DNA for the previous 24-h interval. *$p < 0.05$.

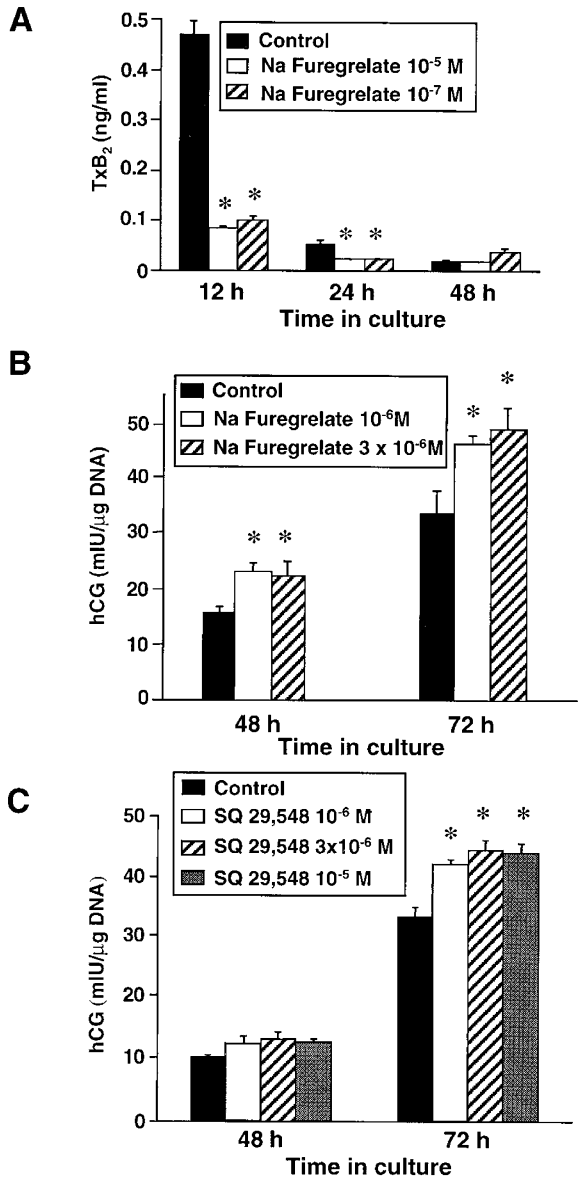

Figure 2. *A*, level of TXB_2 in medium from trophoblasts cultured in the presence or absence of sodium furegrelate. The levels at 12 and 24 h represent accumulation for the preceding 12 h, and the level at 48 h represents accumulation during the preceding 24 h. The result represents three different experiments on different placentas with duplicates. *B*, level of hCG in medium from trophoblasts cultured in the presence or absence of increasing concentrations of sodium furegrelate. The results represent three different experiments on different placentas. *C*, levels of hCG in medium from trophoblasts cultured in the presence or absence of increasing concentrations of SQ 29,548. The results represent three different experiments on different placentas. Levels of hCG (mean ± SD) are normalized to DNA. *$p < 0.05$.

To determine whether endogenous TXA_2 has a similar effect, we determined the effect of either a TX synthase inhibitor or a TX receptor blocker on hCG production. We predicted that addition of either of these agents would result in higher media levels of hCG. We first determined the time course for production of TX by trophoblasts in serum-free media, and found that TX levels were highest in the first 12 h of culture (Fig. 2*A*). Similar results were obtained using serum-containing media. The production of TXA_2 by trophoblast was effectively inhibited by sodium furegrelate (30), a selective TXA_2 synthase inhibitor (Fig. 2*A*). Trophoblasts cultured in the presence of sodium furegrelate showed enhanced levels of hCG at 48 and 72 h, compared with vehicle-treated control cells. Moreover, the effect of the TX synthase inhibitor was concentration dependent (Fig. 2*B*). To support our findings, we also examined the effect of SQ 29, 548, a selective TXA_2 receptor antagonist (31–33), and found that media levels of hCG at 72 h were significantly higher in cultures exposed to the antagonist compared with vehicle-treated controls (Fig. 2*C*). Although maximum TXA_2 levels were present in cultured trophoblast in the first 12 h, we chose 48 and 72 h time points to estimate media hCG levels because biochemical differentiation can be determined in cultures grown >24 h (2). These results indicate that endogenous production of TX hinders biochemical differentiation of cultured trophoblasts.

We determined the effect of TXA_2 on morphologic differentiation of primary trophoblasts using syncytium formation as a marker for differentiation. Qualitative assessment of trophoblast cell morphology indicated that >90% of the nuclei remained as cytotrophoblasts after TXA_2 exposure. In contrast, approximately 50% of the cells exhibited syncytial morphology in control cultures by 72 h (Fig. 3*A*). The number of

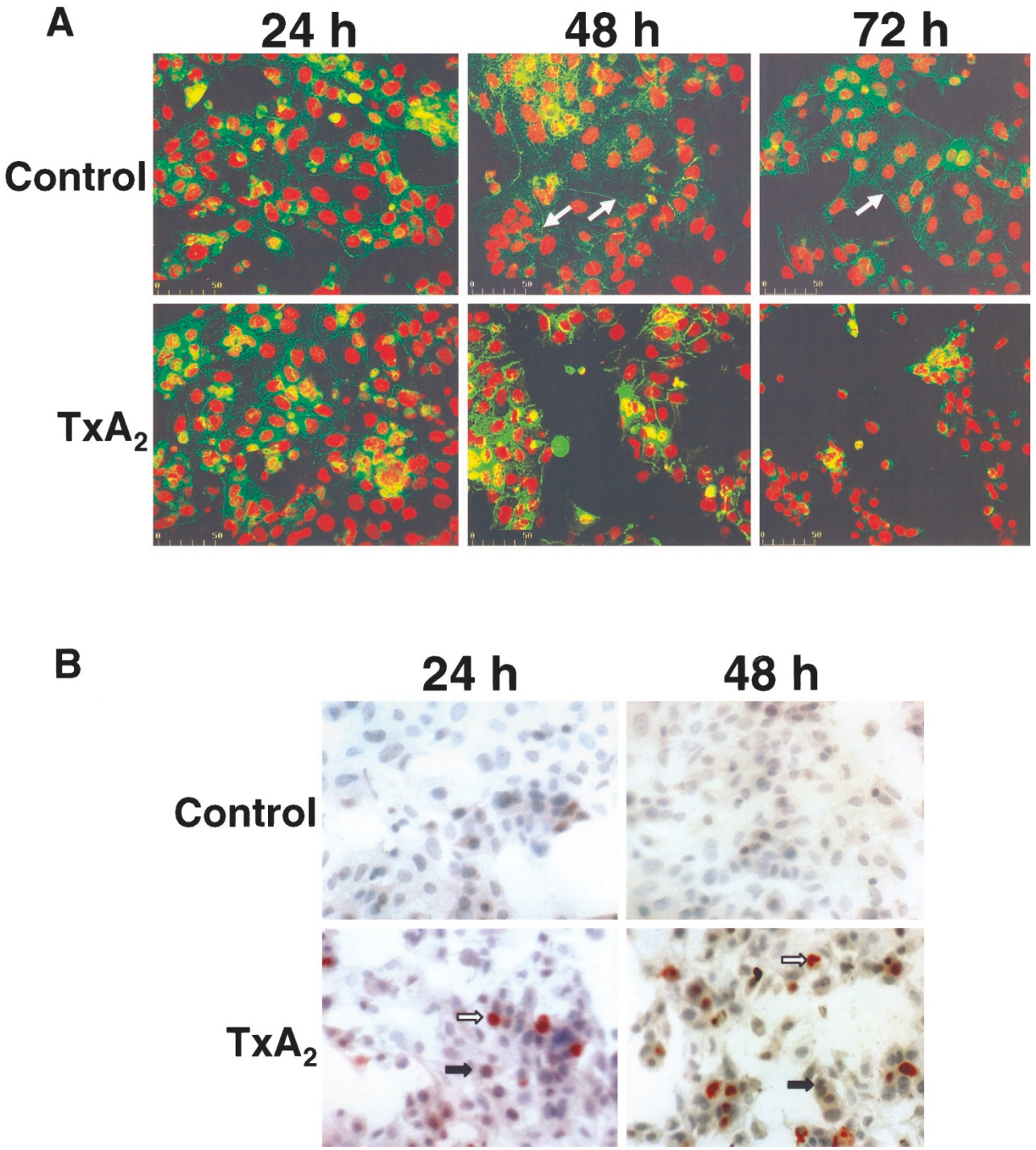

Figure 3. *A*, confocal microscope images of cells cultured for 24 or 48 h in the absence (control) or presence of TXA_2 (10 μM). Green staining illustrates fluorescence detected by cell membrane desmosomal staining, and red-orange represents fluorescence from rhodamine-stained nuclei. Gradations of red, orange, and yellow fluorescence result from overlapping cell borders and nuclei. Cytotrophoblasts cultured for 24 h are predominantly mononucleated with a line of green fluorescence interfacing most nuclei. Multiple syncytia have formed by 48 h (*arrows*) intermixed with cytotrophoblasts. These results contrast with cells cultured in the presence of TXA_2, in which mononucleated cytotrophoblasts dominate throughout the 72 h. A lower cell density was noted in cultures exposed to TXA_2 at 48 and 72 h, compared with control. Bar = 50 μm. *B*, TUNEL staining of trophoblasts in the absence (control) or presence of TXA_2 (10 μM) at 24 and 48 h. Open arrow shows a TUNEL-positive nucleus (red stained) and solid arrow shows apoptotic cells with condensed nuclei that are not TUNEL positive.

syncytia at 24, 48, and 72 h was significantly less in cells treated with TXA_2 when compared with control cells (Fig. 4*A*). These results were reproduced in serum-containing media (data not shown). Taken together, our results indicate that TXA_2 hinders morphologic differentiation of primary trophoblasts.

TXA_2 enhances trophoblast apoptosis. TXA_2 induces apoptosis in several cell types (34, 35). We also noted that addition of TXA_2 to the culture yielded a lower cell number by 72 h. We therefore hypothesized that TXA_2 induced apoptosis in cultured trophoblasts. Using TUNEL staining, we observed a

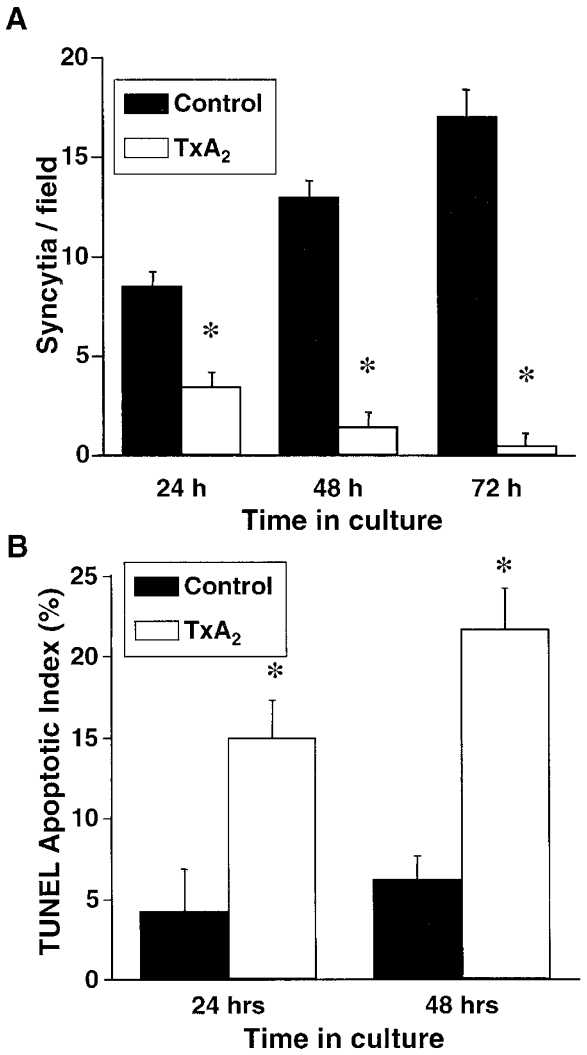

Figure 4. *A*, syncytia formation in trophoblasts in the absence (control) or presence of TXA_2 (10 μM). The result represents five different experiments on different placentas. Results are mean ± SD of syncytia/field as described in Methods. *$p < 0.05$. *B*, apoptotic index of trophoblasts in the absence (control) and presence of TXA_2 (10 μM) as measured by TUNEL staining. The result represents five different experiments on different placentas. Data are mean ± SD. *$p < 0.05$.

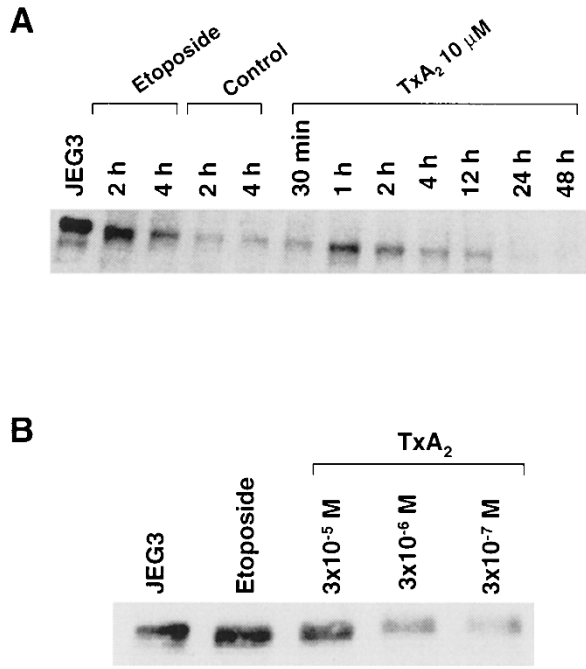

Figure 5. *A*, time course of p53 expression in primary trophoblasts after exposure to TXA_2 (10 μM). JEG3 cells and trophoblasts exposed to etoposide were used as positive controls for p53. Control primary trophoblast cells were treated with vehicle alone. Blot is representative of three different experiments on different placentas. *B*, immunoblot analysis of p53 expression at 90 min in primary trophoblasts after exposure to different concentrations of TXA_2. Blot is representative of two experiments on two different placentas.

significantly higher apoptotic index in cells exposed to TXA_2, compared with control (Figs. 3*B* and 4*B*). We confirmed these results using morphologic criteria of apoptosis for cells cultured with or without TXA_2 (data not shown). These results were reproduced in serum-containing media. These findings indicate that TXA_2 enhances apoptosis in cultured trophoblasts.

Because p53 mediates diverse apoptotic stimuli (36, 37), we examined the affects of TXA_2 on p53 expression. Using densitometry we found that TXA_2 enhanced the expression of p53 by 2.5-fold compared with control. We observed peak p53 expression between 1 and 2 h after exposure to TXA_2 (Fig. 5*A*), which was undetectable by 48 h of culture. Importantly, the induction of p53 by TXA_2 was concentration dependent, with maximal expression at 30 μM TXA_2 (Fig. 5*B*). These results suggest that TXA_2 induces apoptosis in trophoblasts through a p53-dependent mechanism.

DISCUSSION

Villous trophoblasts undergo biochemical and morphologic differentiation (38). This process is regulated *in vitro* through different pathways (38–40). Because prostanoids modulate differentiation, apoptosis, and proliferation of nontrophoblast cells, we examined their influence on trophoblast differentiation. Our analysis focused on nine different prostanoids, based on their known effects in other systems (23–27). Of the prostanoids tested, we found that TXA_2 limits both biochemical and morphologic differentiation of cultured villous trophoblasts derived from term human placentas. Two lines of evidence support this premise. *1*) Addition of TXA_2 hinders trophoblast differentiation in culture. *2*) Inhibition of endogenous TXA_2 production by cultured trophoblast or antagonizing the binding of endogenous TX to its receptor facilitates hormonal differentiation of trophoblasts. We also confirmed earlier observations that the production of TXA_2 by trophoblasts *in vitro* is maximal in the first few hours of culture (10, 21), before syncytium formation (2). These data are supported by the observation that the expression and activity of cyclooxygenase are higher in cytotrophoblast than in syncytiotrophoblast (22). Moreover, syncytiotrophoblasts exhibit a higher expression and activity of PG dehydrogenase (41), which metabolizes prostanoids to inactive products. Nevertheless, the effect of the TX synthase inhibitor sodium furegrelate or the TXA_2 receptor antagonist SQ 29,548 was demonstrated only after 48 h, because significant synthesis of hCG is observed only after trophoblast differentiation into syncytium (2).

We also found that TX enhances trophoblast apoptosis. This was demonstrated using TUNEL staining as well as morphologic criteria for apoptotic cells. The temporal relationship between TX exposure and the enhancement of p53 expression suggests that apoptosis occurs through a p53-dependent mechanism. Moreover, induction of p53 was concentration-dependent, lending further support to our inference. Importantly, TX levels are elevated in the serum (12, 42) and placental villi (8, 11) of women with preeclampsia. Consistent with this observation, the expression of cyclooxygenase-2 (8, 21) and TX synthase (43) are enhanced in placental villi from women with preeclampsia. Because apoptosis is more prevalent in placentas from pregnancies complicated by FGR (7) or preeclampsia (44), it is possible that TX enhances apoptosis in these pregnancy-related diseases. Whether or not the higher level of TX contributes to other aspects of placental dysfunction observed in these diseases, such as maldevelopment of basal plate arteries (45), syncytial knots (6), and diminished amino acid transport (46), remains to be established.

Although trophoblast is normally exposed to marked hypoxia during early embryonic development, intervillous Po_2 in the range of 20–75 mm Hg is observed in the second half of pregnancy (47, 48). We have previously shown that exposure of trophoblasts to hypoxia (oxygen saturation of 1 to 2%, Po_2 of $<$15 mm Hg) *in vitro* hinders differentiation (22) and enhances apoptosis (49). Other investigators have described similar results (50–52). Hypoxia also up-regulates the expression of cylcooxygenase-2, and increases production of TX by trophoblasts (22). These phenotypic changes in trophoblast differentiation are found in placentas from women with preeclampsia and FGR, in which villous hypoxia is commonly found. The mechanisms that control normal and abnormal trophoblast differentiation are poorly understood. Growth factors (epidermal growth factor and transforming growth factor), cytokines (granulocyte macrophage-colony stimulating factor and colony-stimulating factor), and hCG appear to play an important role in this process (53). Our studies indicate that TX also regulates trophoblast differentiation and apoptosis, in addition to the role of this prostanoid in regulation of placental blood flow (54, 55). A similar function of prostanoids, elicited in response to hypoxia, has been demonstrated in several cell types (33, 56–59). Whether or not the influence of hypoxia on trophoblast differentiation is mediated by enhancement of TX production remains to be established. Nevertheless, the role of TX in placental dysfunction suggests that inhibition of TX signaling may limit placental dysfunction in conditions associated with hypoxic trophoblasts.

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