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Cell Injury, Repair, Aging and Apoptosis

Trophoblastic Oxidative Stress and the Release of Cell-Free Feto-Placental DNA

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Considerable quantities of cell-free fetal DNA circulate in the maternal blood during human pregnancy, but the origin of the DNA remains uncertain. Circumstantial evidence suggests the placenta is the principal source, so we tested the hypothesis that release occurs from the syncytiotrophoblast after the induction of apoptotic changes. Villous explants from normal placentas delivered by elective caesarean section were cultured under normoxic conditions (10% oxygen) for up to 20 hours or exposed to hypoxia (0.5% oxygen) for 1 hour followed by reoxygenation. The concentration of *β-globin* cell-free DNA in the supernatant, measured using real-time polymerase chain reaction methodology, was significantly increased at 20 hours after hypoxia-reoxygenation. Release was associated with increased apoptosis, confirmed by increased activation of caspase-3 on Western blotting, and immunolocalized to the syncytiotrophoblast; necrosis was also evidenced by release of lactate dehydrogenase. Both release of cell-free DNA and apoptosis could be significantly reduced by the addition of antioxidant vitamins C and E to the culture medium. This study provides the first evidence of a mechanistic and quantitative link between placental apoptosis/necrosis and release of cell-free DNA, hence confirming that maternal serum/plasma concentrations of cell-free DNA may act as a biomarker of trophoblast well-being during pregnancy. ***(Am J Pathol 2006, 169:400–404; DOI: 10.2353/ajpath.2006.060161)***

Almost a decade has passed since the discovery that significant quantities of cell-free fetal (cff) DNA circulate in the maternal blood during pregnancy.[1] In that time much progress has been made in assessing the prenatal diagnostic potential of this material, and a number of fetal single gene disorders can now be identified rapidly and noninvasively at an early stage of pregnancy.[2] The origin of the cffDNA has remained uncertain, although evidence suggests that the placenta is the most likely source.[2,3] Transplacental traffic of fetal hemopoietic cells is a potential alternative, but the number of these cells in the maternal circulation is considered to be too small to account for all of the cffDNA released.[2] Equally, in some clinical scenarios, such as preterm labor, there is no correlation between the number of circulating fetal cells and the concentration of cffDNA.[4] Therefore, it is unlikely that intact fetal cell trafficking contributes significantly to the levels of cffDNA in maternal blood.

The human placenta is of the hemochorial type, and maternal blood directly bathes the epithelial covering of the fetal villous tree, the syncytiotrophoblast. A recent study found no correlation between placental volume measured by ultrasonography during the first trimester and the concentration of cffDNA,[5] suggesting that some additional stimulus is required to cause the release of cffDNA. Data from clinical studies support this view, in that elevated concentrations of cffDNA are found in conditions associated with significant placental pathology.[3] A common feature of many of these conditions is placental oxidative stress.

We sought to test the relationship between placental oxidative stress and the release of cffDNA using explants of placental villi from term placentas. Oxidative stress is a powerful inducer of both apoptotic and necrotic changes in many systems. We previously demonstrated that hypoxia-reoxygenation *in vitro* stimulates apoptosis in the syncytiotrophoblast and that the extent of apoptosis can be modulated by the addition of antioxidant molecules.[6] In addition, oxidative stress results in fragmentation of the syncytiotrophoblast apical membrane, allowing release of cytoplasmic contents into the culture supernatant. Here, we report a significant correlation between placen-

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tal apoptosis as a marker of trophoblastic oxidative stress and the release of cffDNA.

Materials and Methods

Samples

Placentas ($n = 11$) were collected from normal term singleton pregnancies delivered by elective caesarean section with informed written consent of the patients and permission of the local research ethics committee. Villous samples were taken midway between the chorionic and basal plates, from the periphery of lobules free of visible infarction, calcification, hematoma, or tears. After a brief rinse in cold phosphate-buffered saline, samples were placed into ice-cold transport medium [TCS large vessel endothelial cell basal medium (TCS CellWorks, Milton Keynes, UK) containing 2% fetal bovine serum, heparin, hydrocortisone, human epidermal growth factor, human basic fibroblast growth factor, 25 μg/ml gentamicin, 50 ng/ml amphotericin B, 1 mmol/L vitamin C, and 1 mmol/L Trolox] that had been equilibrated with 5% O_2/90% N_2/5% CO_2.

Explant Culture

At the laboratory, placental samples were further dissected into small pieces (~5 mm in diameter) in ice-cold culture medium in a glove box under 10% O_2/85% N_2/5% CO_2. After dissection, villous samples were cultured in individual Costar Netwell (24 mm diameter, 500 μm mesh) supports in 4 ml of culture medium (TCS large vessel endothelial cell basal medium, containing 2% fetal bovine serum, heparin, hydrocortisone, human epidermal growth factor, human basic fibroblast growth factor, 25 μg/ml gentamicin, and 50 ng/ml amphotericin B) per well in six-well plates. The samples were then incubated either under normoxic conditions (10% O_2/85% N_2/5% CO_2) throughout or subjected to hypoxia (0.5% O_2/94.5% N_2/5% CO_2) for 1 hour and subsequent reoxygenation at normoxia (10% O_2/85% N_2/5% CO_2) (H/R) for 6 or 19 hours. In all experiments the medium was changed after 1 and 7 hours of incubation. The antioxidant vitamins, vitamin C (2 mmol/L ascorbic acid) and a water-soluble analogue of vitamin E (1 mmol/L Trolox), were added to a subset of samples at the beginning of each experiment and also when medium was changed. At the end of each culture period, the supernatant was aliquoted and frozen, and the villous samples weighed and snap-frozen. All samples were stored at −80°C before analysis.

Measurement of β-Globin DNA

Frozen villous supernatant samples were shipped to Boston, MA, on dry ice via express courier and stored at −80°C. cffDNA was isolated from 400 μl of supernatant using the QiaAMP mini blood kit (Qiagen, Valencia, CA). cffDNA levels were quantified by real-time polymerase chain reaction (PCR) on the Perkin-Elmer Applied Biosystems 7700 sequence detector (Applied Biosystems, Foster City, CA). Amplification of the *β-globin* gene was performed to determine the level of cffDNA in each of the supernatant samples. The sequences of the primers were as follows: forward primer 5′-GTGCACCTGACTCCT-5GAGGAGA-3′ and reverse primer 5′-CCTTGATAC-CAACCTGCCCAG-3′. The dual-labeled fluorescent probe was 5′-FAM-AAGGTGAACGTGGATGAAGTTG-GTGG-TAMRA, in which FAM is 6-carboxyfluorescein and TAMRA is the nonfluorescent quencher. Every reaction was performed in a total volume of 50 μl, containing 20 μl of TaqMan Universal PCR Master Mix (Applied Biosysytems), 100 nmol/L final concentration of primer, and 50 nmol/L final concentration of probe. Thermal cycling was initiated with a 2-minute incubation at 50°C, followed by a 10-minute denaturation at 95°C, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Standard curves of known concentrations of DNA were generated using DNA isolated from male leukocytes.

Each DNA sample was analyzed in triplicate, with the mean result being used for further calculation. The total amount of DNA isolated from the supernatant was calculated and further normalized according to the amount of isolated placental tissue. The end results are shown as pg of DNA per mg of placental tissue.

Western Blotting for Apoptosis

Frozen villous samples were homogenized in ice-cold lysis buffer (1 ml of buffer per 100 mg of tissue) containing 20 mmol/L Tris, pH 7.4, 1 mmol/L EGTA, 0.01% Triton X-100, 1 mmol/L sodium pyrophosphate, 1 mmol/L sodium orthovanadate, 10 mmol/L β-glycerol phosphate, 50 mmol/L sodium fluoride, and a complete mini protease inhibitor cocktail (Roche; Roche Diagnostics, East Sussex, UK). Tissue homogenates were centrifuged at 15,000 × *g*, 4°C for 20 minutes, and the supernatants removed. Protein concentrations were determined using a BCA protein assay kit (Sigma, Poole, UK). Lysates were mixed with 3× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, boiled for 5 minutes, and allowed to return to room temperature. Equal amounts of protein (20 to 30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5 to 15% polyacrylamide, depending on the molecular weight of the target protein), transferred onto nitrocellulose membrane (Invitrogen, Paisley, UK), and subjected to immunoblot analysis. Membranes were blocked for 1 hour at 25°C in 5% milk diluted in Tris-buffered saline and 0.1% Tween 20 and incubated with specific primary antibodies overnight at 4°C. After washing and incubating with secondary antibodies, immunoreactive proteins were visualized by the ECL plus chemiluminescence system following the manufacturer's instructions (Amersham Biosciences, Bucks, UK). Membranes were reprobed with antibody recognizing β-actin to control for protein loading. Protein bands were quantified using Image J software (National Institutes of Health, *http://rsb.info.nih.gov.ij*). The values are expressed as a percentage of the control lysate for each experiment (100%).

Immunohistochemistry

Paraformaldehyde-fixed tissues embedded in paraffin wax were sectioned at 7 μm, dewaxed, and incubated with 3% H_2O_2 for 30 minutes to block endogenous peroxidases. Antigen retrieval was then performed using 0.01 mol/L sodium citrate buffer at pH 6.0 in a pressure cooker for 2 minutes. Sections were then cooled and incubated with nonimmune serum for 20 minutes. The primary anti-cleaved caspase-3 antibody (Promega, Southampton, UK) was applied overnight at 4°C, and binding was detected using Vectastain Elite ABC kits (Vector Laboratories, Peterborough, UK) and SigmaFast DAB (Sigma, Poole, UK), according to the manufacturers' instructions. Sections were then lightly counterstained with hematoxylin. Negative controls were performed by replacement with equal concentration of nonimmune or isotype-matched irrelevant control.

Lactate Dehydrogenase (LDH) Assay

LDH release into culture supernatants was detected by colorimetric enzyme-linked immunosorbent assay, using the cytotoxicity detection kit (LDH) from Roche, according to manufacturers' instructions.

Statistics

Statistical analysis was performed using Statview (SAS Institute Inc., Cary, NC). Concentrations of cffDNA were analyzed using a repeated measures analysis of variance, with the duration of culture and the culture conditions (normoxia, H/R, or H/R + vitamins) as the main effects. To explore the effects of vitamins on concentrations of cffDNA and on apoptosis at each time point, a paired Student's *t*-test was used. In all cases results were considered significant at $P < 0.05$.

Results

The concentration of *β-globin* cffDNA in the supernatant increased significantly with the duration of the culture in the controls and each of the two treatment groups ($P < 0.0001$), although there was considerable sample variability that increased with time (Figure 1). Culture conditions also had a significant influence on cffDNA release ($P < 0.005$), and there was a significant interaction term between the two effects, duration, and culture conditions ($P < 0.0005$). No significant differences were observed between the normoxic controls, the H/R group, and the H/R+ vitamins group after 1 hour in culture. After 7 hours of culture, there was no significant difference in concentrations between the H/R group compared to the normoxic controls. The addition of vitamins, however, significantly reduced concentrations compared to H/R alone ($P = 0.003$). By 20 hours, concentrations were significantly higher in the H/R group compared to controls ($P = 0.013$). The addition of vitamins did not affect concentrations of cffDNA at this stage.

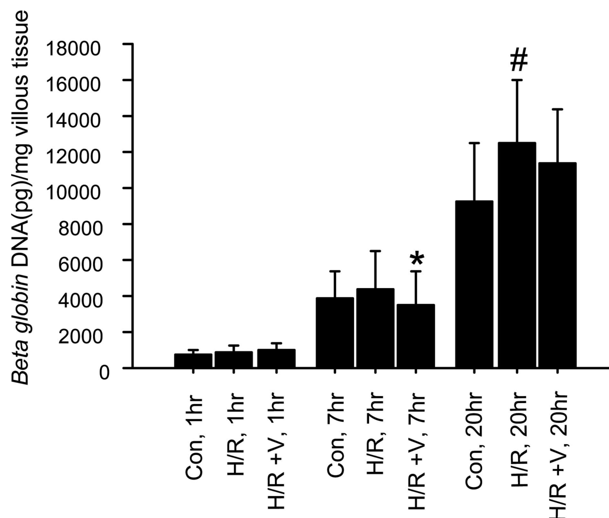

Figure 1. Means ($n = 11$) and SDs for *β-globin* DNA concentration in the culture supernatant for the controls (Con), hypoxia-reoxygenation (H/R) and hypoxia-reoxygenation with vitamins (H/R +V) treatment groups after 1, 7, and 20 hours of culture. A repeated measures analysis of variance confirmed significant effects of time and treatment on the concentration of cffDNA released ($P < 0.0001$ and $P < 0.005$, respectively). *$P < 0.05$ compared to H/R, $^{\#}P < 0.05$ compared to normoxic control.

Apoptosis, as measured by Western blotting of cleaved caspase 3, showed no significant difference between the three treatment groups after 7 hours in culture (data not shown). By 20 hours there was a marked increase in cleaved caspase 3 in the H/R group compared to controls, and this increase was significantly reduced by the addition of vitamins C and E (Figure 2). Immunohistochemistry localized the apoptosis principally to the syncytiotrophoblast layer of the placental villi (Figure 3). The release of LDH into the supernatant was also significantly increased at 7 hours of culture (Figure 4). Unfor-

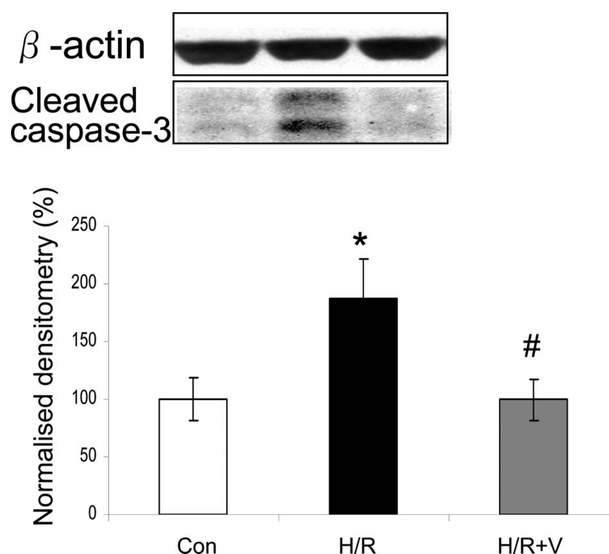

Figure 2. A representative Western blot for cleaved caspase 3 in placental explants after 20 hours in culture. Increased apoptosis is seen after H/R compared to normoxic controls, and this could be suppressed by the administration of the antioxidant vitamins C and E. Quantification of five experimental treatments revealed a significant difference between controls and H/R (*$P = 0.025$) and between H/R with and without vitamins ($^{\#}P = 0.024$).

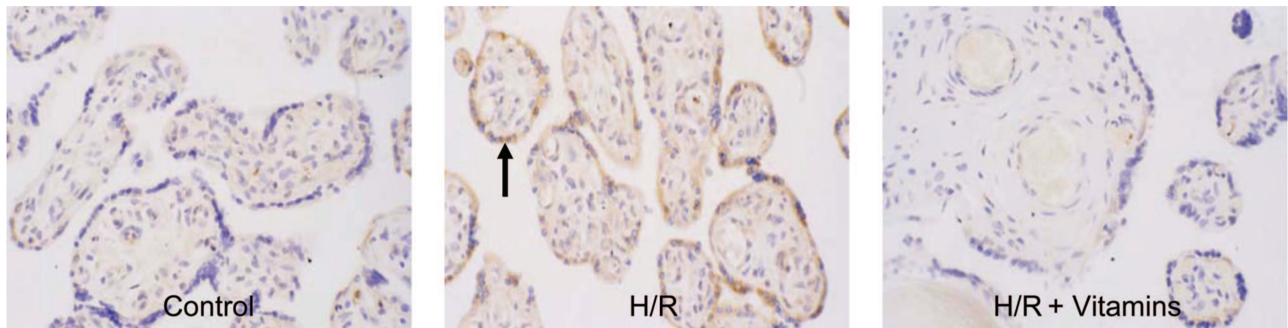

Figure 3. Immunohistochemistry for cleaved caspase 3 revealed that most apoptosis was confined to the syncytiotrophoblast (**arrow**) after H/R.

tunately, the protective effects of vitamins could not be tested because their presence interfered with the colorimetric assay.

Discussion

Our results clearly demonstrate an association between degenerative changes in the syncytiotrophoblast of the villous explants and the release of cffDNA into the culture supernatant. The changes responsible appear to be a mixture of apoptosis and necrosis because there is evidence of both activation of the execution caspase pathway and the release of LDH, a cytoplasmic enzyme, into the supernatant. This is in keeping with our previous findings of increased terminal dUTP nick-end labeling and chromatin condensation in syncytiotrophoblastic nuclei after H/R *in vitro*, but also of fragmentation of the microvillous syncytial surface.[6] In that study we showed that pretreatment with desferrioxamine significantly reduced terminal dUTP nick-end labeling of the syncytiotrophoblastic nuclei and that this was associated with a reduction in the release of cytochrome *c* from mitochondria after H/R, confirming a link between oxidative stress and trophoblastic apoptosis. Desferrioxamine is a powerful antioxidant; by chelating free iron, it prevents formation of the highly reactive hydroxyl radical through the Fenton reaction.[7] The similarity between those findings and the results of administration of vitamins C and E reported here suggests that the vitamins are exerting their protective effects through their radical scavenging properties rather than by interfering with signaling pathways in a nonantioxidant manner.

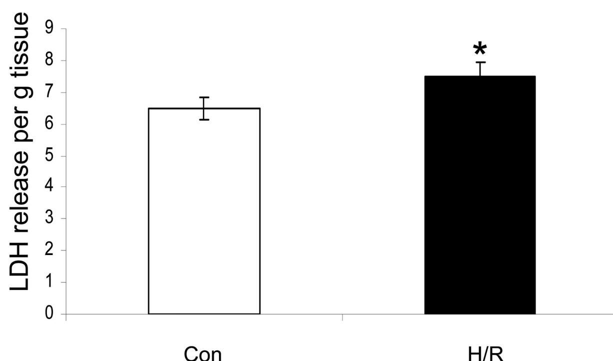

Figure 4. Release of LDH into the supernatant was significantly increased after H/R and 7 hours in culture (*$P = 0.007$). The effects of vitamins could not be tested because their presence interfered with the colorimetric assay.

Whether a cell undergoes apoptosis or necrosis after an insult, or a combination of the two, depends critically on the level of energy available.[8,9] Apoptosis is an ATP-dependent event, and concentrations need to be maintained at a minimum of 50% normal to complete the process.[9] If mitochondrial disruption is severe and the concentration of ATP falls too low to complete apoptosis, then secondary necrosis ensues. The term aponecrosis has been applied to describe this sequence of events.[10,11]

Trophoblast degeneration occurs in all normal pregnancies as part of physiological villous remodeling but may be exaggerated in pathological cases. Thus, during the first trimester the villi over the superficial pole of the gestational sac regress to form the smooth chorion leave. This phenomenon is associated with onset of the maternal circulation to the placenta, which starts in the periphery and subsequently extends centrally. The tissues of the peripheral regressing villi display high levels of oxidative stress and extensive degeneration of the syncytiotrophoblast.[12] At the ultrastructural level, dilation and distortion of the mitochondrial intracristal space and loss of integrity of the apical membrane can be observed, but there is little chromatin condensation within the nuclei. The overall picture is therefore more one of necrosis than of apoptosis. It is intriguing to note that in many of the pregnancies studied longitudinally by Lo and colleagues,[13] a minor peak in maternal cffDNA concentrations was observed at ~10 to 12 weeks when villous regression is maximal. Furthermore, maternal cffDNA levels increase by 21% per week in the first trimester.[14]

Maternal concentrations of cffDNA then rise rapidly during the last few weeks of normal pregnancies,[14,15] and again there appears to be an association with placental apoptosis.[16] The incidence of cells displaying condensed chromatin at term is double that during the first trimester, and trophoblastic nuclei are principally involved.[17] This may reflect rising levels of oxidative stress induced by fluctuations in placental perfusion, caused, for example, by the increasing strength of the Braxton-Hicks contractions.[18] In cases of preeclampsia maternal concentrations of cffDNA may exceed fivefold the normal levels,[3,19,20] and there is conclusive evidence of increased trophoblastic apoptosis, focal syncytial

necrosis, and placental oxidative stress in these pregnancies.[18–23]

Previous clinical studies have suggested a strong association between trophoblast degeneration and the release of cffDNA into the maternal circulation. We believe that this is the first study to demonstrate a mechanistic and quantitative link between the two phenomena. Our results confirm that maternal serum/plasma concentrations of cffDNA may act as a biomarker of trophoblast well being during pregnancy and provide a scientific rationale for the administration of antioxidant vitamins in high-risk pregnancies.

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