Magnesium regulates hypoxia-stimulated apoptosis in the human placenta

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ABSTRACT

Apoptosis (programmed cell death) in the human placenta is likely to play a major role in determining the structure and function of that organ. Fetal growth restriction (FGR) has been shown to be associated with increased levels of placental apoptosis. Altered regulation of apoptosis may play an important pathophysiological role in FGR. As reduced placental perfusion and reduced oxygenation are features of FGR, one aim of this study was to determine the effects of hypoxia on apoptotic activity, as assessed by DNA laddering, of placental tissue in vitro. In addition, levels of placental apoptosis may be affected by pharmacological agents routinely used in obstetric patient management. Thus an additional aim of this study was to determine the effects of several relevant pharmacological agents on the levels of DNA laddering during in vitro incubation of human placentae under hypoxic conditions. Incubation of normal placental explant tissue at 37 °C for 1–2 h under hypoxic conditions significantly increased placental DNA laddering compared with that in non-incubated tissue, whereas levels of DNA laddering during incubation for up to 2 h under normoxic conditions were not significantly higher than those in non-incubated tissue. The DNA laddering activity of placental explants after 2 h of incubation under hypoxic conditions was significantly increased with increased concentrations of magnesium, but remained unchanged by the inclusion of pethidine, aspirin, nifedipine, dexamethasone, heparin or indomethacin in the incubation mixture. These results suggest that hypoxia may stimulate apoptotic activity in cultured human placental tissues, and that hypoxia-stimulated placental apoptosis may be further increased by increasing the extracellular magnesium concentration.

INTRODUCTION

Apoptosis or programmed cell death is a normal process that is important for regulating developmental changes and maintaining tissue homoeostasis. Apoptosis involves highly co-ordinated structural changes driven by internal molecular interactions that result in cell death. Apoptosis occurs when factors that determine the susceptibility of the cell to apoptosis interact with apoptosis-initiating stimuli [1]. This process is distinctly different from cell necrosis, which involves cell death caused ‘accidentally’ by external factors. Apoptosis is characterized by isolated cell and nucleus shrinkage, condensed chromatin and membrane blebbing. In contrast, necrosis involves more extensive numbers of cells, cell swelling, lysis and cytokine-mediated inflammatory responses [2]. A unique biochemical feature of apoptosis is the internucleosomal cleavage of DNA by an activated Ca²⁺- and Mg²⁺-dependent endogenous endonuclease [3]. The cleaved DNA, which is in multiples of 180–200 bp, can be detected as characteristic ladder-like patterns on agarose-gel electrophoresis [4].

Placental development depends on cell proliferation and remodelling. The number of epithelial plates

Key words: apoptosis, hypoxia, magnesium, placenta.

Abbreviations: FGR, fetal growth restriction; LDH, lactate dehydrogenase; \( P_{O_2} \), partial pressure of oxygen.

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increases in the placenta during pregnancy. The increase in epithelial plates increases the surface area of effective materno–fetal exchange units [5]. Developmental remodelling of tissue structure is mediated by the active physiological killing and removal of cells [3]. Apoptosis has been shown to occur within normal placental tissue during early pregnancy (5 and 7 weeks) and during the third trimester [6,7]. The majority of apoptotic cells were identified as trophoblastic, as well as lesser numbers of endothelial and stromal cells. Thus apoptosis in the human placenta is likely to play a major role in determining the structure and function of that organ.

Evidence suggests that pregnancies complicated with fetal growth restriction (FGR) are associated with increased placental apoptosis [8]. Abnormalities in the regulation of apoptosis may deplete the syncytiotrophoblast population of the placenta, leading to impairment of placental materno–fetal exchange functions [9]. Attenuation of the materno–fetal exchange functions will retard fetal growth and development [5]. Therefore altered regulation of apoptosis may play an important pathophysiological role in FGR.

As reduced placental perfusion and reduced oxygenation are features of FGR, one aim of the present study was to determine the effects of hypoxia on the apoptotic activity, as assessed by DNA laddering, of placental tissue in vitro. In addition, levels of placental apoptosis may be affected by pharmacological agents used in obstetric patient management. Thus an additional aim of this study was to determine the effects of several relevant pharmacological agents on the levels of DNA laddering during in vitro incubation of human placentae under hypoxic conditions. The pharmacological agents of interest were magnesium sulphate, nifedipine, heparin, indomethacin, pethidine, dexamethasone and aspirin.

**METHODS**

**Drugs and chemicals**

Tergitol-type Nonidet P-40, glycerol, citric acid, acetylsalicylic acid (aspirin) and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NaCl, NaHCO$_3$, sodium acetate, KCl, CaCl$_2$, d-glucose, Na$_2$HPO$_4$, ethanol and Bromophenol Blue were obtained from BDH Ltd. (Poole, Dorset, U.K.). The Cell Death Detection ELISA kit, Tris, RNase A, proteinase K and ethidium bromide were obtained from Boehringer Mannheim Pty. Ltd. (Castle Hill, New South Wales, Australia). Tris/borate/EDTA (TBE) was obtained from Amresco (Solon, OH, U.S.A.). pGem and agarose were obtained from Promega Corp. (Madison, WI, U.S.A.). Dexamethasone and heparin were obtained from David Bull Laboratories (Mulgrave, Victoria, Australia). Nifedipine (Adapine 20) was obtained from Amrad Pharmaceuticals Pty. Ltd. (Kew, Victoria, Australia). Pethidine was obtained from Astra Pharmaceuticals Pty. Ltd. (Abbotsford, Victoria, Australia). Potassium phosphate monobasic was obtained from ICN (Costa Mesa, CA, U.S.A.). Nitrogen, carbon dioxide and oxygen gas mixtures were supplied by BOC Gases (Preston, Victoria, Australia). Polaroid 665 film was from Polaroid Corp. (Cambridge, MA, U.S.A.).

**Tissue collection**

Experimental procedures used in this study were approved by the Royal Women’s Hospital Research and Ethics Committees, in accordance with the National Health and Medical Research Council of Australia guidelines. Written, informed consent was obtained from all patients participating in the study. Term placentae (37–40 weeks’ gestation) were collected at Caesarean section or normal vaginal delivery from clinically normal patients, and used within 30 min.

**Explant incubation**

The technique of placental villous explant culture, which has been used successfully to study a number of placental functions [10–13], was used in the present study to investigate changes in placental DNA laddering activity. Small pieces of villous tissue were cut at random from beneath the basal plate of the placenta until about 20 g of tissue had been collected. This was cleaned of any blood clots, calcification or large vessels, chopped finely with scissors and then separated into 2 mm³ explants. The explants were washed three times in ice-cold modified Krebs buffer (97 mmol/l NaCl, 24.4 mmol/l NaHCO$_3$, 3 mmol/l KCl, 1.2 mmol/l KH$_2$PO$_4$, 1.89 mmol/l CaCl$_2$, 1 mmol/l MgSO$_4$, 5.5 mmol/l glucose, pH 7.35). After blotting excess moisture, 0.4–0.7 g was weighed into tissue cassettes. For zero incubation time points, 0.2 g explants were placed immediately into 4 ml of ice-cold 70% (v/v) ethanol before homogenization.

Modified Krebs solution (50 ml) was placed into a sterile container with two holes drilled in the lid. The required gas mixture was fed in through a neonatal feeding line inserted through the lid into the Krebs solution. Duplicate solutions were prepared for each condition. Solutions were gassed at 37 °C for 30 min prior to incubating the explants. Gas analysis of the Krebs solution was carried out using a blood gas analyser (CIBA Corning 278 Blood Gas System), taking care not to introduce O$_2$ into the solution during the analysis. Normoxic conditions were generated by using a gas mixture of 90% nitrogen, 5% carbon dioxide and 5% oxygen, which gave a partial pressure of oxygen (P$_{O_2}$) of 60–80 mmHg. Hypoxic conditions were generated by using a gas mixture of 95% nitrogen and 5% carbon dioxide, which gave a P$_{O_2}$ of 20–40 mmHg. Cassettes were placed into pre-gassed Krebs solution at 37 °C, and gassing was continued for the duration of the experiment. Explant tissues were incubated under...
either normoxic or hypoxic conditions for various time intervals up to 2 h. At the end of the incubation period, the explants were briefly blotted and 0.2 g tissue was weighed out. This was placed immediately into 4 ml of ice-cold 70% (v/v) ethanol. Explants were homogenized on ice using an Ultra Turrax (T25) blade homogenizer (3 x 10 s). The volume of each tube was made up to 10 ml with 70% (v/v) ethanol, mixed well then left to fix at −20 °C for at least 48 h before extracting the DNA. Homogenates were stored at −20 °C for up to 2 weeks. The viability of the incubated placental explant tissue was assessed by measurement of the release of the intracelluar enzyme lactate dehydrogenase (LDH) into the medium and by measurement of glucose utilization. Levels of both were found to remain constant over the course of the 2 h incubations, and have been shown previously not to change significantly during incubations of up to 24 h. The level of LDH in the medium of placental explant cultures, which reflects the state of cellular membrane integrity, has been found previously to be consistently less than 10% of total tissue LDH [12].

For experiments that investigated the effects of magnesium or other drugs on placental DNA ladder- ing, placental explant tissue was incubated for 2 h in the absence or presence of the appropriate drug or in the presence of a range of Mg concentrations (0.5–10 mmol/l MgSO$_4$). The final concentrations of drugs used in the Krebs solution were: 1 µg/ml nifedipine, 0.5 µg/ml dexamethasone, 3 i.u./ml heparin, 1 µg/ml pethidine, 10 µg/ml acetylsalicylic acid and 3.6 µg/ml indomethacin [14]. These concentrations were chosen to be at or above those that occur with maximum therapeutic dosing.

**DNA extraction**

Homogenates were spun at 800 g for 10 min. The supernatant was decanted and pellets were left to dry for 15 min. The pellets were resuspended by gentle mixing with 200 µl of phosphate/citrate buffer (192 mmol/l Na$_2$HPO$_4$, 4 mmol/l citric acid, pH 7.8). These were incubated at 37 °C for 60 min, then placed at 4 °C for 2–4 h. The extracts were reheated to 37 °C for 15 min before centrifugation at 1000 g for 10 min. Portions of 200 µl of the supernatants were transferred to 1.5 ml microcentrifuge tubes and then evaporated to dryness in a Jouan centrifugal evaporator (24–48 h). Samples were stored at −20 °C for up to 4 weeks. Samples were resuspended in 5 µl of 0.25% Tergitol-type Nonidet P-40 diluted in water. Then 5 µl of 1 g/litre RNase A, also diluted in water, was mixed in by gentle vortexing, and the extract was incubated at 37 °C for 30 min. (The RNase had been made DNase-free by preparing a 10 g/l solution in 0.01 M sodium acetate pH 5.2 and heating to 100 °C for 15 min. After slow cooling to room temperature, the pH of the RNase solution was adjusted by adding 0.1 vol. of 1 mol/l Tris, pH 7.4.) After the addition of 5 µl of a 1 g/litre solution of proteinase K diluted in water, the samples were incubated for an additional 30 min at 37 °C. Samples were allowed to cool, microfuged briefly and then mixed. Purity and yield were determined by spectrophotometry at 260 and 280 nm of a 2 µl aliquot diluted with 198 µl of water. The mean 260/280 nm absorbance ratio for 151 DNA preparations was 1.81 ± 0.006. The remainder of the sample was stored at 4 °C for up to 2 weeks. Each experiment was processed as a single batch, such that the control and test samples were always analysed together. In this way, any effects of storage on the samples were randomized across the groups.

**Agarose-gel electrophoresis**

After the addition of 6 µl of loading buffer [2.5 g/l Bromophenol Blue and 30% (v/v) glycerol in water], samples were loaded on a 2.2% (w/v) agarose gel in 0.5 × TBE (45 mmol/l Tris base, 45 mmol/l borate, 1 mmol/l EDTA) containing 300 µg/l ethidium bromide. Horizontal agarose-gel electrophoresis was performed at 10 V/cm for 2.5 h. The ethidium bromide-stained characteristic DNA laddering patterns in the gel were visualized under UV light and photographed with Polaroid 665 film. Densitometry was performed on the negative image. To quantify the pixel scores for each lane, a uniform rectangle of predetermined area was analysed between the positions corresponding to 1198 and 179 bp of the pGem markers, which were run on every gel.

**Photometric enzyme immunoassay**

In a number of samples, apoptotic activity was also assessed using the Cell Death Detection ELISA (Boehringer Mannheim), which detects apoptotic cytoplasmic histone-associated DNA fragments. Explant tissues were homogenized in 70% (v/v) ethanol as described above. The homogenates were centrifuged (800 g, 10 min) and the dried pellets were resuspended in 500 µl of the lysis buffer from the kit. After centrifugation (800 g, 10 min), the supernatant was collected and a range of dilutions (neat, 1:5, 1:20 and 1:100) were assayed according to the manufacturer’s instructions.

**Statistics**

Data were presented as means ± S.E.M. Significant differences between means were determined using the Kruskal–Wallis test for non-parametric data followed by Dunn’s multiple-comparison test. A P value of less than 0.05 was taken as significant. Statistical analysis was undertaken using GraphPad Prism Version 2.01 (GraphPad Software Inc.).

**RESULTS**

Low-molecular-mass DNA extracted from placental explant tissue showed oligosomal DNA laddering patterns on gel electrophoresis, consistent with apoptosis
In vitro incubation of placental explant tissue for 1–2 h under hypoxic conditions or normoxic conditions significantly increased placental apoptosis compared with that in non-incubated control tissue. Values are means ± S.E.M. Significant increases relative to the non-incubated control tissue are indicated by * \( P < 0.05 \) (Kruskal–Wallis test). Regression analysis determined that there was a linear relationship between the amount of DNA loaded on the gels and the pixel score determined by densitometry, up to a concentration of 150 \( \mu \text{g} \) of DNA per well (\( r^2 = 0.927 \), slope \( P < 0.0005 \), \( n = 8 \) samples). Incubation of placental explant tissue for 1–2 h under hypoxic conditions significantly increased placental DNA laddering compared with that in non-incubated tissue (\( P < 0.0001 \)). On the other hand, levels of DNA laddering during incubation up to 2 h under normoxic conditions were not significantly higher than those in non-incubated tissue (Figures 1 and 2).

Apoptotic activity of the placental explant tissues incubated under normoxic and hypoxic conditions was also assessed by the Cell Death Detection ELISA. Similar results as in the DNA laddering assay were obtained. Activity (expressed as a percentage of that in the non-incubated tissue) was significantly greater during incubation under hypoxic conditions (1 h, 286 ± 48%; 2 h, 832 ± 184%; \( n = 8 \)) compared with that in incubations under normoxic conditions (1 h, 214 ± 28%; 2 h, 267 ± 55%; \( n = 4 \)).

The DNA laddering activity of placental explants after a 2 h incubation under hypoxic conditions was related to the concentration of Mg in the Krebs solution (Figure 3). DNA laddering activity was significantly increased compared with the control (1 mmol/l Mg) when the Mg concentration was raised to 5 and 10 mmol/l (\( P < 0.0001 \)). In addition, at concentrations less than 5 mmol/l, regression analysis showed that there was a correlation between DNA laddering activity and the log of the magnesium concentration (\( r^2 = 0.859 \), slope \( P < 0.0001 \)), indicating a concentration–response relationship. DNA laddering activity, however, remained unchanged on inclusion of pethidine (1 \( \mu \text{g/ml} \)), aspirin (10 \( \mu \text{g/ml} \)), nifedipine (1 \( \mu \text{g/ml} \)), dexamethasone (500 ng/ml), heparin (3 i.u./ml) or indomethacin (3.6 \( \mu \text{g/ml} \)) in the incubation mixture compared with that in controls without the addition of the drug (Table 1).

**DISCUSSION**

This study has confirmed the presence of patterns of DNA characteristic of apoptosis in placental villous tissue. Characteristic oligosomal DNA laddering patterns were observed on agarose-gel electrophoresis of placental DNA extracts. These results are consistent with other studies that have demonstrated apoptosis in normal human placental tissue. In situ fragmented DNA characteristic of apoptosis [6,7,15], as well as oligosomal DNA laddering [16], have been reported in both first- and third-trimester placentae. In addition, light and electron microscopic techniques have been used to describe morphological features, including condensation of chromatin along the periphery of the nucleus and nuclear shrinkage, that are indicative of apoptosis in human placenta [7,17,18]. Further, recent studies have demonstrated the presence of both pro- and anti-apoptotic oncoproteins in human placental tissue [19–21]. Thus apoptosis is likely to play a role in the normal development of the placenta.

It has been reported previously that the incidence of apoptosis, quantified by light microscopy, was significantly increased in placentae from pregnancies characterized by FGR compared with that in normal third-trimester placentae [8]. These results suggested that apoptosis may play a role in the pathophysiological mechanisms of FGR. For example, apoptosis...
may mediate the degeneration of placental villi that occurs in pregnancies with FGR. Reduced placental perfusion and oxygenation are features of FGR. The present study has determined that a decrease in P\text{O}_2 from 60–80 mmHg to 20–40 mmHg markedly increased DNA laddering activity in placental tissue during incubation \textit{in vitro}, suggesting that placental apoptotic activity was increased. Similar effects of changes in P\text{O}_2 were obtained when placental apoptosis was assessed using a photometric enzyme immunoassay which measures cytoplasmic histone-associated DNA fragments. Hypoxia has been shown previously to stimulate apoptosis in various tissues \textit{in vivo}, as well as in a number of cultured cell models \textit{in vitro} [22–25]. Thus hypoxia may play a role in the apoptosis-related placental events associated with FGR.

The present study used hypoxic culture of placental explant tissue as a model to study the pharmacological regulation of apoptosis in the human placenta. The effects of a number of drugs of clinical relevance to obstetrics on hypoxia-stimulated DNA laddering in human placental tissue were investigated. Several mechanisms were proposed by which the drugs of interest might affect apoptotic activity. Salicylates and indomethacin inhibit the synthesis of the pro-apoptotic autacoid thromboxane A\textsubscript{2} [26]. Opioids have been shown to stimulate apoptosis in human lung cancer cells [27] via specific opioid receptors. Heparin can induce apoptosis in human neutrophils [28], and may suppress it in cultured rat hepatocytes [29]. Calcium-channel blockers, including nifedipine, have been widely studied for their anti-apoptotic activities [30], and may block calcium signals that trigger apoptosis. Dexamethasone suppresses apoptosis in human gastric cancer cells through modulation of \textit{bcl-x} gene expression [31], and induces apoptosis in multiple myeloma cells [32]. Nevertheless, \textit{in vitro} incubation of placental tissue in the presence of each of the above drugs at concentrations at the upper limit of those that might be achieved \textit{in vivo} failed to significantly affect DNA laddering activity. Thus no evidence was obtained that any of these drugs may affect apoptosis in the placenta. The possibility that multiple exposure to any of the drugs \textit{in vivo} over a longer time frame might affect placental apoptosis, however, cannot be discounted.

We found that magnesium stimulated DNA laddering in human placental explants, suggesting that placental apoptotic activity was increased. Increasing the concentration of MgSO\textsubscript{4} in the incubation medium from 1 mmol/l to either 5 or 10 mmol/l caused significant increases in placental DNA laddering. In addition, at concentrations less that 5 mmol/l there was a significant concentration–response relationship between magnesium and placental DNA laddering activity. The mechanism by which magnesium might affect placental apoptosis is not known. Magnesium can affect calcium entry into cells and may modulate apoptosis in this way. In addition, endonucleases involved in the cleavage of DNA during apoptosis are magnesium-dependent [3], and intracellular magnesium levels, which may be affected by extracellular levels, may modulate apoptosis.

In conclusion, the present study has found that both hypoxia and magnesium stimulated placental DNA laddering \textit{in vitro}, consistent with increased placental apoptosis. Placental hypoxia may be associated with conditions involving reduced placental perfusion, such as during FGR. Magnesium sulphate is used clinically for the treatment of pre-term labour and for the prevention or control of seizures associated with eclampsia or severe pre-eclampsia. In the light of the results of this study, further consideration may need to be given to the possible effects of increased magnesium levels during clinical therapy on placental apoptotic activity and placental function, in particular during situations where oxygenation might be reduced.

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**Figure 3** DNA laddering activity of placental explant tissue incubated under hypoxic conditions for 2 h with increasing concentrations of MgSO\textsubscript{4}

The P\text{O}_2 of the incubation was 20–40 mmHg. DNA laddering activity is expressed as a percentage of that following a 2 h incubation with 1 mmol/l MgSO\textsubscript{4}. Values are means ± S.E.M. Significant increases relative to the control (1 mmol/l MgSO\textsubscript{4}) are indicated by *P < 0.05 (Kruskal–Wallis test).

**Table 1** Effects of pharmacological agents on the DNA laddering activity of placental explant tissue

DNA laddering activity is expressed as a percentage of that in controls in the absence of the test drug. All incubations were carried out for 2 h under hypoxic conditions and in the presence of 1 mmol/l Mg.

<table>
<thead>
<tr>
<th>Drug</th>
<th>DNA laddering activity (%)</th>
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<tbody>
<tr>
<td>Nifedipine (1 μg/ml)</td>
<td>106 ± 10 (n = 4)</td>
</tr>
<tr>
<td>Indomethacin (3.6 μg/ml)</td>
<td>91 ± 17 (n = 6)</td>
</tr>
<tr>
<td>Dexamethasone (0.5 μg/ml)</td>
<td>102 ± 10 (n = 4)</td>
</tr>
<tr>
<td>Heparin (3 i.u./ml)</td>
<td>92 ± 8 (n = 4)</td>
</tr>
<tr>
<td>Pethidine (1 μg/ml)</td>
<td>84 ± 11 (n = 9)</td>
</tr>
<tr>
<td>Aspirin (10 μg/ml)</td>
<td>122 ± 7 (n = 4)</td>
</tr>
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