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Increased incidence of apoptosis in non-labour-affected cytotrophoblast cells in term fetal membranes overlying the cervix

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A regional reduction in the cellularity of the cytotrophoblastic and decidual layers occurs in the fetal membranes overlying the cervix in the lower uterine segment prior to labour. Although the mechanism(s) involved are not known it could result from regionally increased apoptosis, the histological manifestation of programmed cell death, or decreased proliferation. Apoptosis was assessed in regionally sampled fetal membranes from women undergoing elective Caesarean section (n = 14) by the presence of apoptotic bodies by light and electron microscopy. Cell proliferation was assessed by immunocytochemical detection of the protein Ki-67. Apoptotic bodies were identified in all regions of the fetal membrane with the highest incidence found within the cytotrophoblast layer. However, this layer in fetal membranes biopsied over the cervix contained significantly more apoptotic bodies (mean ± SD 0.085 ± 0.020%) compared to the layer in fetal membranes obtained from the mid-zone (0.020 ± 0.008%) apoptotic bodies. Isolated Ki-67 positive cells were detected in the cytotrophoblast layer, but no regional differences in their incidence were seen. Fetal membranes also failed to exhibit significant immunoreactivity for BCL-2 but exhibited strong BAX immunoreactivity within the decidual layer. We conclude that the regionally increased incidence of apoptosis in the cytotrophoblastic layer in the membrane overlying the cervix may account for the reduction in its cellularity but not the relative decrease in the decidual layer. Given the consequence of the loss of local function in degrading uterotonins and stabilizing the fetal membrane, the study of the regulation of apoptosis in these cells may have important implications for fetal membrane rupture and parturition.

Key words: apoptosis/fetal membrane/membrane rupture/preterm birth

Introduction

Preterm birth is a major obstetrical problem which is responsible for up to 85% of neonatal deaths and can result in long-term disability in children (Hall *et al.*, 1997). Since a major cause of preterm birth is the pre-labour rupture of the fetal membranes (Keirse *et al.*, 1989), our focus of research has been upon the identification of features that may be uniquely associated with the membranes' ability to rupture, both spontaneously and prelabour. Indeed, structural alterations have been identified in, and restricted to, an area of the fetal membranes located within the rupture line after labour and delivery at term (Malak *et al.*, 1993; Malak and Bell, 1994). These changes are characterized by the marked disruption and swelling of the connective tissue layer, which may be directly linked to fetal membrane strength, and also the pronounced reduction in the thickness of the cytotrophoblastic and decidual layers (Malak and Bell, 1994). The identification of similar but less extensive structural changes in fetal membranes overlying the cervix, prior to labour, suggests that this area represents the site programmed for subsequent rupture during labour (McLaren *et al.*, 1999). These changes have been reported in fetal membranes obtained after preterm birth, which are even more pronounced in those cases preceded by rupture of the fetal membranes (Malak *et al.*, 1993). The appearance of these changes, including the reduction in the cellular layers, may indicate their involvement not only in programming fetal membrane rupture but also associated with parturition itself (Malak and Bell, 1996).

The mechanism responsible for the reduction in the cellularity of the cytotrophoblastic and decidual layers is unclear. Since the fetal membranes cease to grow during the latter half of pregnancy (Parry-Jones and Priya, 1976) it could be anticipated that this is a result of either decreased proliferation with a constant rate of cell death or increased rate of cell death with a constant rate of proliferation. Although microscopical examination does not support the process of widespread necrosis (McLaren *et al.*, 1999), apoptosis, which is the histological manifestation of programmed cell death, could provide a mechanism for the selective regional removal of these cells. Apoptosis is critical to a number of physiological and pathological processes (Williams, 1994) and is a distinct form of cell death, being an active energy-requiring process in which single cells die and are removed by phagocytosis without producing an inflammatory reaction (Savill, 1994). It is characterized by specific morphological features, including DNA fragmentation and the condensation of nuclear heterochromatin and the appearance of dense 'apoptotic bodies' (Kerr and Winterford, 1993; Smith *et al.*, 1997), which are identified by light and electron microscopy. The 'gold standard' for the detection of apoptosis is its characterization by morphological criteria, with the most common sign of apoptosis within tissue sections being the presence of apoptotic bodies (Hall, 1999).

Apoptosis is controlled by the expression of a number of regulatory genes, including *c-myc*, *p53* and *apo-1/fas* (Osborne

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and Schwartz, 1994; White, 1993). One of the most important advances in our understanding of apoptotic cell death has come from studies of the oncogenic *Bcl-2* family. BCL-2 protein promotes cell survival by blocking apoptosis induced by a range of stimuli including growth factor withdrawal (Nunez and Clarke, 1994). It is now clear that the anti-apoptotic properties of BCL-2 depend acutely on its interaction with a number of potent antagonistic proteins (Boise *et al.*, 1993; Kiefer *et al.*, 1995; Yang *et al.*, 1995). In particular, studies have shown that a related protein, BAX, antagonizes the survival-promoting activity of BCL-2 (Oltvai *et al.*, 1993). BCL-2 needs to form heterodimers with BAX in order to function, and it is the ratio of BCL-2 to BAX which predetermines the cell's susceptibility to a given apoptotic stimulus (Oltvai and Korsmeyer, 1994).

Apoptosis is known to occur within both rat amnion (Lei *et al.*, 1996, 1999), human fetal membrane (Runic *et al.*, 1998) and placental trophoblasts (Smith *et al.*, 1997; Uckan *et al.*, 1997), the incidence of which has been determined using the method of terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labelling (TUNEL) to detect DNA fragmentation (Runic *et al.*, 1998). However, the high incidence of TUNEL-positive cells, in the absence of membrane destruction, has cast doubt on the suitability of this method to detect the true incidence of apoptosis in fetal membranes. In addition, the authors failed to determine whether this phenomenon exhibits regional differences in its incidence within fetal membranes, prior to labour. Therefore, in this study we sought to: (i) determine if apoptosis could account for the selective regional reduction of the cellularity of the cytotrophoblastic and decidual cells within the fetal membrane, prior to labour; and (ii) determine the protein localization of the BCL-2/BAX regulatory proteins in fetal membranes at term, prior to labour.

Materials and methods

Patient details

Fetal membranes unaffected by labour or delivery were obtained from women (n = 14) undergoing elective Caesarean sections at 38–39 weeks of gestation for repeat Caesarean section, breech or cephalopelvic disproportion. Infected membranes were excluded following identification of polymorphonuclear infiltration. Membrane samples were obtained within 10 min of delivery from the afterbirth, which would have otherwise gone to waste.

Tissue sampling

Fetal membranes were regionally biopsied from two principal sites according to our previous protocol (Malak and Bell, 1994, 1996). (i) Cervical area: following the delivery of the baby, the fetal membranes overlying the cervix were located and marked by the application of a Babcock clip. The area within the jaws of the clip was not used since light microscopy revealed that this area was too damaged to be included. An area of 2–3 cm^2 around the clip was sampled. (ii) Mid-zone area: half-way between the cervical area and the placental edge. This was usually 10–12 cm from the cervical area. Specimens were then washed briefly in phosphate-buffered saline (PBS) (pH 7.4). Fetal membrane strips were rolled with the amnion innermost and then fixed in buffered (pH 7.6) formalin for 24 h before processing and mounting in paraffin wax.

Apoptosis identification and quantification

Light microscopy

Tissue sections (6–8 μm) were cut from paraffin blocks and then stained with haematoxylin and eosin. Apoptotic cells were identified as those having the presence of characteristic apoptotic bodies (Kerr and Winterford, 1993). These were clearly visible under oil (×1000) magnification and appeared as either single or multiple dense bodies, which maintained a consistent density when viewed through planes. Identification of these as apoptotic bodies was confirmed following electron microscopy of a selection of light microscopic identified cells (see below).

Electron microscopy

Fetal membrane rolls were initially fixed in 2.5% glutaraldehyde in sodium cacodylate buffer pH 7.2, overnight. After fixation the tissue was post-fixed in 1% osmium tetroxide, dehydrated through ascending grades of alcohol, embedded in Epon and sectioned on an ultamicrotome. Thin sections (60 nm) were stained with saturated uranyl acetate and aqueous lead citrate for eventual electron microscopy work.

Apoptotic cell quantification

Quantification of apoptotic cells, within the fetal membrane layers, was achieved following cell counting using the Apple Macintosh Centris Running Image System, version 1.49. For each fetal membrane roll 10–20 fields were viewed for both the amniotic epithelium, connective tissue layer, cytotrophoblast and decidual layers, at a magnification of ×1000. Routinely between 500 and 1000 cells were counted per roll. Each single or group of dense bodies was counted as a single apoptotic cell. The number of apoptotic cells was expressed as a percentage of the total number of cells counted within layer.

Immunohistochemical staining for BCL-2, BAX and Ki-67

Assessment of BCL-2, BAX and Ki-67 proteins was performed on formalin-fixed, paraffin-embedded tissue sections for each antibody in the different regions sampled prior to labour. Serial tissue sections were de-waxed in xylene and hydrated gradually through graded alcohols. These were then microwaved (300 W) in 0.01 mol/l citrate buffer for 30 min, before washing in PBS. Endogenous peroxidase activity was quenched following a 10 min incubation in a 6% solution of hydrogen peroxide. The slides were then treated with a 20% solution of rabbit serum before this was removed and replaced with either 1/40 dilution of mouse monoclonal antibody to BCL-2 (Biogenesis, Poole, UK), BAX (Santa Cruz Biotechnology, Santa Cruz, USA), or Ki-67 (Dako). Positive controls for each antigen were included as well as negative controls in which the primary antibody was omitted and replaced with an irrelevant mouse IgG (Dako). These controls were run in parallel with the fetal membrane samples. The slides were incubated at room temperature for 2 h and then washed in PBS, pH 7.5 for 15 min. A 1/200 dilution of rabbit anti-mouse secondary antibody (Dako) was then added and left for 1 h at room temperature. Following this the slides were washed three times for 15 min and complexed with avidin–biotin peroxidase (Vector, Peterborough, UK) for 30 min. The complex was detected with diaminobenzidine and hydrogen peroxide in 0.1 mol/l Tris–HCl, pH 7.5, before the slides were washed and the colour developed using DAB. Blue/black colouration was achieved following the addition of a Nickel solution.

Statistics

Data were given as means ± SD and were normally distributed, therefore parametric paired Student's t-tests were undertaken to determine statistical differences between cervical and mid-zone regions; this was performed for apoptotic-body-positive cells.

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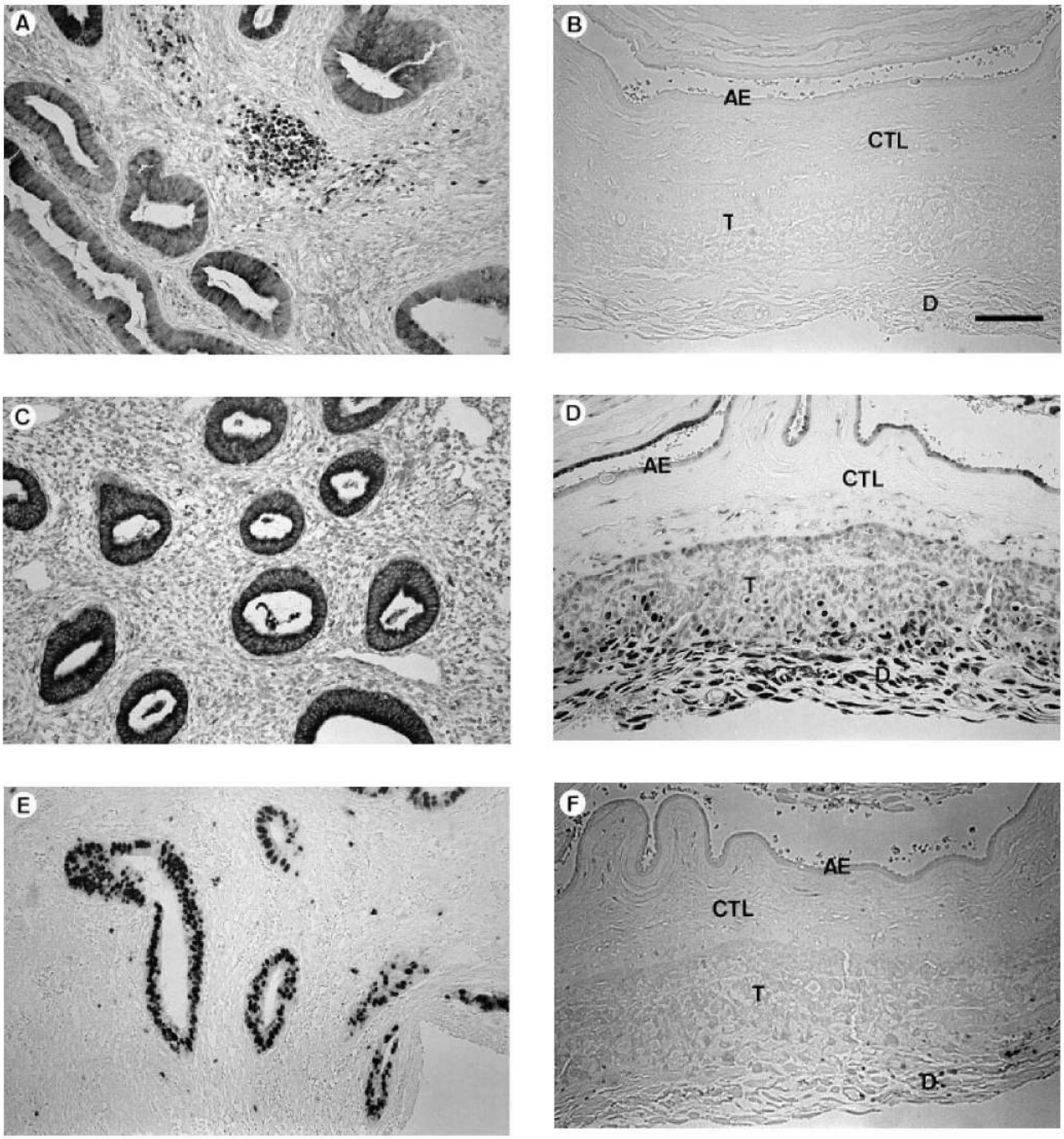

Figure 3. Immunohistochemical staining for BCL-2 (**B**), BAX (**D**) and Ki-67 (**F**) in fetal membranes sampled from over the cervix. Positive controls for BCL-2 (**A**), BAX (**C**) and Ki-67 (**E**) comprise human endometrium. AE = amniotic epithelium; CTL = connective tissue layer (comprising fibroblasts, macrophages and myofibroblasts embedded in extracellular matrix); T = cytotrophoblast cells; D = decidua. Scale bar = 100 μm.

identification of apoptotic bodies. The highest incidence of apoptosis was detected in the cytotrophoblastic cellular layer of the fetal membranes located over the cervix. No such cellular and regional distribution was detected in cell proliferation as evidenced by the proliferation marker Ki-67. The increased incidence of apoptosis may therefore explain the decrease in the thickness of the cytotrophoblastic layer of the fetal membranes overlying the cervix before labour (McLaren *et al.*, 1999), and its even more pronounced decrease in the region of the membranes associated with the rupture site after rupture and delivery (Malak and Bell, 1994). The presence of increased apoptosis in this site supports the suggestion that this area of the fetal membranes in the lower segment over the cervix develops dynamically during late pregnancy and is not a feature present throughout pregnancy. In contrast, no regional difference in the incidence of apoptosis was detected in the decidual layer and it is therefore unlikely that this mechanism accounts for the relative thinness of this layer in the cervical

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area prior to labour and within the 'zone of altered morphology' (Malak and Bell, 1994; McLaren *et al.*, 1999) in the rupture line after labour and delivery. It may be possible that this represents alterations in the plane of cleavage of the fetal membranes in these different locations and conditions.

The lack of immunoreactive BCL-2 in the fetal membranes and any regional variations suggests that it is not involved in the protection of the cells in this tissue from apoptotic stimuli. The strong immunoreactive BAX staining found within the decidual layer may provide evidence for its potential role in decidual cell apoptosis. However, we observed a lack of apoptosis in this layer suggesting that other anti-apoptotic mechanisms could be involved. The absence of BAX staining within the cytrophoblast layer suggests its non-participation in the mechanisms controlling apoptosis in this layer. These results imply that other factors may be involved in the control of this process. Candidates include the other members of the now extensive *Bcl-2* gene family, which can have either pro- or anti-apoptotic regulating activity (Brown, 1996). Alternatively, other potential regulators include Fas receptor and tumour necrosis factor (TNF) receptor 1 (TNFR1) which are cell surface receptors and which mediate apoptosis after binding of Fas ligand and TNFα, respectively. Indeed the presence of Fas ligand and its receptor has been identified throughout the fetal membrane (Runic *et al.*, 1996, 1998) as well as TNFα and TNFR1 (Fortunato *et al.*, 1994). However, their significance as regulators of apoptosis under these conditions is unclear since relative levels of their expression both within the different layers of the fetal membrane and between different regions is unknown. Moreover their presence alone does not guarantee activation of this apoptotic pathway. It is clear that further work is required to clarify the mechanisms involved in the selective regional increase in apoptosis within the cytotrophoblastic populations.

The mechanism(s) responsible for the increased apoptosis in the cytotrophoblastic layer of the membranes overlying the cervix remains to be examined. It is possible that it is the result of a direct para/autocrine induction. Alternatively it may be an indirect effect of extracellular matrix degradation caused by the action of the extracellular matrix degrading matrix metalloproteinase enzymes which also exhibit higher levels in the fetal membranes overlying the cervix prior to labour (McLaren *et al.*, 1998). Indeed recent work, using a rat amnion culture model and cultures of human amnion cells (WISH), has demonstrated that increasing matrix metalloproteinase-9 expression results in an increase in apoptosis and cell detachment (Lei *et al.*, 1999).

The reduction of the cytotrophoblast layer of the fetal membranes overlying the cervix is associated with the swelling and dissociation of the overlying strength-giving connective tissue layers, which are suggested to be associated with susceptibility for membrane rupture at this site (Malak and Bell, 1994; McLaren *et al.*, 1999). It is possible that the cytotrophoblastic cells produce factors favouring extracellular matrix synthesis (So *et al.*, 1992; Vadillo-Ortega *et al.*, 1995), which are lost during its involution, or indeed apoptosis itself could be associated with factors which induce activity of extracellular matrix-degrading enzymes by its constituent cells (Kakinuma *et al.*, 1997). The reduction of the cytotrophoblast layer also occurs in parallel with the preparatory changes in the cervix and myometrium during phase 0 of parturition (Casey and MacDonald, 1993) and therefore may have important implications not only for susceptibility of the membrane to rupture but also for the initiation of parturition. It is well established that membrane stripping from the uterine wall is an effective method of labour induction (Berghella *et al.*, 1996) and may indicate a potential causal relationship between undefined biophysical/biochemical states of the fetal membranes and labour (Malak and Bell, 1994). Indeed the amnion of the fetal membranes is a potent source of uterotonins, e.g. prostaglandins (PG) (Okazaki *et al.*, 1981) and endothelin-1 (Sagawa *et al.*, 1994). The increased importance of this layer in determining the eventual bioavaliability of uterotonins such as PG is highlighted by recent studies which show a lack of regionality in the synthesis of PGE_2 (Sawdy *et al.*, 1999). Yet paradoxically the cells of the cytotrophoblastic layer possess the capacity to degrade these uterotonins by virtue of its expression of an endopeptidase which degrades endothelins (Germain *et al.*, 1994), and 15-hydroxyprostaglandin dehydrogenase (PGDH), the principle enzyme responsible for the metabolism of prostaglandins (Sangha *et al.*, 1994).

We have previously proposed that the regional loss of the cytotrophoblast layer of the fetal membranes overlying the cervix, and hence regional loss of functional capacity, before labour, may allow these factors to escape degradation and to act locally to stimulate the maturation of the cervix and myometrium of the lower segment in preparation for labour (Malak and Bell, 1996). Furthermore the premature and extensive loss of this layer, which we have demonstrated in cases of preterm birth (Malak *et al.*, 1993), raises the possibility of the premature action of uterotonins to induce idiopathic preterm labour. This is supported by, and provides a rationale for, the reports of a subset of patients presenting with preterm labour who have a reduced expression of PGDH in their chorionic cytotrophoblast (Sangha *et al.*, 1994) and the demonstration of reduced prostaglandin catabolism in the fetal membranes from the lower uterine segment (Van Meir *et al.*, 1996, 1997).

Elucidation of the mechanisms involved in the control of apoptosis in the cytotrophoblastic layer therefore may provide insight into the control of both the preparatory phases of parturition, in terms of the cervix and myometrium, and for fetal membrane rupture in term and preterm birth.

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