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Physiology

TNF-α Promotes Caspase Activation and Apoptosis in Human Fetal Membranes

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***Purpose*:** Increased amniotic fluid tumor necrosis factor (TNF) is a marker of infection when associated with preterm labor and preterm premature rupture of the amniochorionic membranes (PROM). We have noted increased apoptosis in membranes derived from women with PROM. This study examines the role of TNF in promoting fetal membrane apoptosis.
***Methods*:** Amniochorion ($n = 8$), collected at the time of elective repeat cesarean section prior to labor from normal term gestation, were placed in an organ explant system. After 48 h in culture, the membranes were stimulated with recombinant TNF-α (20 ng/mL) for 24 h. Tissue frozen after stimulation was subjected to RT-PCR to study the expression of TNF-induced caspase genes. ELISA assayed the levels of proapoptotic p53 in tissues and cell death related nuclear matrix protein (NMP) in tissue culture supernatants. The activity of caspases in tissue homogenates was measured using substrates specific for caspase 2, 3, 6, 8, and 9. Results were analyzed by using the Wilcoxon nonparametric test for paired samples. A $p < 0.05$ was considered significant.
***Results*:** RT-PCR showed induction of caspases 2, 8, and 9 (caspase cascade initiators) in human fetal membranes after TNF stimulation. Caspases 3 and 6 (effector caspases) expression was constitutive in both TNF stimulated- and control membranes. Caspases, 2, 3, 8, and 9 activity was significantly higher in TNF-stimulated tissues compared with control, whereas, no significant change in caspase 6 activity was noticed. TNF-stimulated tissues released increased levels of NMP (24.03 U/mL) compared with control (13.5U/mL) ($p = 0.03$). TNF also increased p53 levels in the tissues (0.05 ng/mL) compared with control cultures (0.03 ng/mL; $p = 0.02$).
***Conclusions*:** TNF increases proapoptotic p53 levels and caspase activities in fetal membranes. Increased NMP reflects cell death.

KEY WORDS: Amniochorion; caspases; preterm labor; p53; PROM; TNF.

INTRODUCTION

Preterm labor and preterm premature rupture of the amniochorionic membranes (PROM) are major perinatal problems resulting in increased neonatal morbidity and mortality. Although several factors like infection, smoking, and low socioeconomic status are associated with PROM and preterm labor, the exact cause of this problem is still unclear. Subclinical genital tract infection is associated with more than 40% of these cases (1,2). Infection and the subsequent host inflammatory response results in increased amniotic fluid levels of inflammatory cytokines like IL-1, IL-6, and TNF-α (3). Infection also increases bioavailability of matrix metalloproteinases (MMPs) that are capable of degrading the membrane extracellular matrix and predisposing them to rupture (4).

Recently our laboratory has documented a role for apoptosis (programmed cell death) in PROM

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(4,5). Two major apoptotic pathways exist in fetal membranes during PROM: 1) a p53-mediated pathway and 2) the Fas-Fas L/TNF-TNFR p55 (TNFR1) mediated pathway. Activation of both of these pathways has been documented in human fetal membranes after PROM (4,5). PROM is associated with an increased expressions of p53 and p53-associated proapoptotic genes. Similarly, TNF activates genes of the Fas-Fas L/TNF-TNFR pathway. Activation of both of these pathways results in the induction of caspases (cystine protease with aspartic acid substrate specificity). Caspases are proteases that degrade several structural and functional proteins in the cell leading to programmed cell death (6).

This study examines the role of TNF in inducing p53 and activating the caspase cascade in cells leading to cell death. p53-mediated cell death is effected by the collapse of the nuclear matrix and the release of its nuclear matrix protein (NMP). We measured caspase activity in amniochorion after TNF stimulation. We also measured NMP in the culture supernatant as an indication of cell death.

MATERIALS AND METHODS

Culture and Stimulation of Amniochorion With TNF-α

Fetal membranes were collected from women undergoing elective repeat cesarean section with no complications of pregnancy. Amniochorionic membranes were cleansed in phosphate buffered saline, and 6-mm diameter sections ($n = 8$) were placed in an organ explant system as described in our previous works (5,7). After a preincubation for 48 h some membranes were stimulated with recombinant TNF-α (20 ng/mL). This concentration was chosen based on the highest levels of TNF seen in the AF of women with infection-associated pregnancy complications. The 48-h preincubation time was chosen because genes of interests are induced by the physical manipulation in preparation for culture and return to steady state by this time. Tissue samples were collected after a 24-h treatment and frozen at -80°C for mRNA and protein analysis. For protein analysis tissues from three different wells were pooled.

RNA Extraction and PCR

Total RNA was extracted by the Trizol™ (Life Technologies, Bethesda, MD) method and 0.5 μg of total RNA was subjected to oligo-dT-primed reverse transcriptase reaction. cDNA was then subjected to polymerase chain reaction (PCR) using specific primers for caspase 2, 3, 6, 8, and 9. The PCR products were analyzed on a 2% ethidium bromide stained gel.

Protein Extraction and Caspase Activity Assay

Total protein from amniochorionic tissues was isolated using a homogenization buffer consisting of 20 mM Tris-Hcl (pH 7.4), 0.1 M NaCl, 1 mM EDTA, 0.1% Triton, and Protease inhibitor (0.5 M). Homogenization was followed by a centrifugation at 12,000 RPM to precipitate tissue debris. Protein assays were done using the Bio-Rad protein assay (Bio-Rad, Benicia, CA) kit. A colorimetric determination was performed to determine caspase activity (Biosource International, Camarillo, CA). Active caspases present in the tissue after TNF stimulation were expected to cleave synthetic peptides at specific locations. The sequences recognized by the caspases are VDVAD (for caspase 2), DEVD (for caspase 3), VEID (for caspase 6), IETD (for caspase 8), and LEHD (for caspase 9). These synthetic peptides are labeled at their C-termini with *p*-nitroaniline (*p*NA). The *p*NA-tagged cleaved fragments were quantified by using a microplate reader at 405 nm. The caspase activities in tissues after TNF stimulation were compared with activity in unstimulated (control) tissues. For the assay, 100 μg of total protein was mixed with the reaction buffer (containing buffered saline, glycerol, and detergent), DTT (a final concentration of 10 mM), and the substrate (a final concentration of 200 μM). The mixture was incubated at 37°C for 2–3 h in the dark. The change in caspase activity in TNF-stimulated tissues compared with control tissues was determined by spectrophotometry. Background absorbance (determined by control buffer without sample introduction) was deducted from both samples before comparison.

ELISA for p53 and Nuclear Matrix Proteins (NMP)

Concentrations of p53 in the cells and cell death related NMP levels in the culture media were assayed by ELISA. Hundred micrograms of total protein from tissue homogenates was assayed for p53. Undiluted media samples were used for the NMP assay. Manufacturers' instructions were followed for each assay (Oncogene Research Products, Cambridge, MA). Standard curves were developed using duplicate samples of known quantities of recombinant p53 and NMP respectively. Concentrations were determined by relating the absorbance obtained at 450 nm to the

Table I. Caspase mRNA Expression in Fetal Membranes

	Caspase 2	Caspase 3	Caspase 6	Caspase 8	Caspase 9
TNF	+	+	+	+	+
Control	−	+	+	−	−

Table II. Caspase Activity in Fetal Membranes

	TNF (Median, range)	Control (Median, range)	*p*
Caspase 2	1.33; 0.9–1.6	0.9; 0.7–1.4	0.01
Caspase 3	1.49; 0.86–1.8	0.8; 0.7–1.5	0.03
Caspase 6	0.9; 0.87–1.95	0.9; 0.79–1.21	0.4
Caspase 8	1.27; 0.8–1.49	0.7; 0.6–1.0	0.03
Caspase 9	1.09; 1.02–1.95	0.7; 0.8–1.2	0.006

Note. The values are absorbance of the cleaved substrates at 405 nm.

standard curve by linear regression analysis. Assay buffer and plain culture media samples were used as negative controls. The sensitivities of the two assays were 0.01 ng/mL for p53 and 10 U/mL for NMP.

Statistical Analysis

Statistical comparison was made using Wilcoxon nonparametric test for paired samples and a p value of <0.05 was considered significant.

RESULTS

TNF stimulation of the human fetal membranes results in the activation of the caspase genes. Caspases 2, 8, and 9 genes were induced in fetal membranes in response to TNF stimulation. Caspase 3 and 6 expression was seen in both control and TNF-stimulated tissues (Table I).

TNF stimulation resulted in increased levels of p53 in tissues. ELISA documented that TNF-stimulated tissues showed high p53 levels (median 1.69 ng/mL, range 0.2–3.3 ng/mL) compared with control tissues (median 0.25 ng/mL, range 0.1–2.5 ng/mL; $p = 0.014$). Culture supernatant from tissues stimulated with TNF released more NMP cultures (median 20.85 U/mL, range 13–52.2 U/mL) compared with media from control experiments (11.85 U/mL, range 5.1–25.5 U/mL).

Caspase activity was increased in tissues stimulated with TNF-α. Only seven samples were used for this assay since one of the cultures did not document any detectable caspase activity. The mean values for various caspase activities were caspase 2 (TNF, 1.28; control, 0.94; $p = 0.01$), caspase 3 (TNF, 1.42; control, 0.95; $p = 0.03$), caspase 8 (TNF, 1.41; control, 0.86; $p = 0.01$), and caspase 9 (TNF, 1.18; control, 0.77; $p = 0.03$). These activities were significantly increased in TNF-stimulated tissues compared with control amniochorion. Caspase 6 activity was not significantly different in TNF (1.08) and control tissue (0.97; $p = 0.4$), (Table II).

COMMENTS

This study supports our earlier hypothesis that infection or inflammatory mediators may play a major role in apoptosis in human fetal membranes (7). An increase in TNF-α levels in the reproductive biological compartments during pregnancy can cause increased apoptosis in fetal membranes. This is supported in vitro by the increased presence of NMPs in the culture media after TNF stimulation. NMPs constitute the internal structural frame work of the nucleus (8). The nuclear matrix of the cell is the structure that serves to organize the chromatin within the nucleus. NMPs are involved in DNA replication, transcription, and various other nuclear activities (9). Damage to nuclear materials and the detection of NMPs released from dying cells serve as a direct measure of cell death (10,11). TNF stimulation results in the increased release of NMP from cultured human amniochorion, indicating the collapase of nuclear material after TNF stimulation.

Additionally we studied the pathways leading to nuclear collapse and cell death. In a previously published report the induction of Fas and the Fas/TNFR1-mediated apoptotic pathway was documented (see Fig. 1) in fetal membranes. TNF induces Fas expression in fetal membranes, which in turn leads to the induction of caspase 2, 8, 9 (initiator caspases), and 7 (effector caspases) genes. However, this report did not examine fetal membrane caspase activity (7).

An alternate pathway involves the tumor suppressor antigen, p53. Expression of p53 as well as bax (the p53 transactivator protein gene) is increased significantly in fetal membranes from women with PROM. A commensurate downregulation of the anti-apoptotic gene, *Bcl-2* (the *p53* response gene), is noted during PROM (5).

Induction of either the TNF/Fas-mediated pathway or the TNF/p53-mediated apoptotic pathway culminates in the activation of caspases, which normally exist in the cell as inactive proenzymes. Activation of the caspase cascade can result in proteolysis of three major groups of substrates: 1) proteins that play a role in the homeostatic response to stress stimuli, including poly [ADP-ribose] polymerase (PARP), a DNA

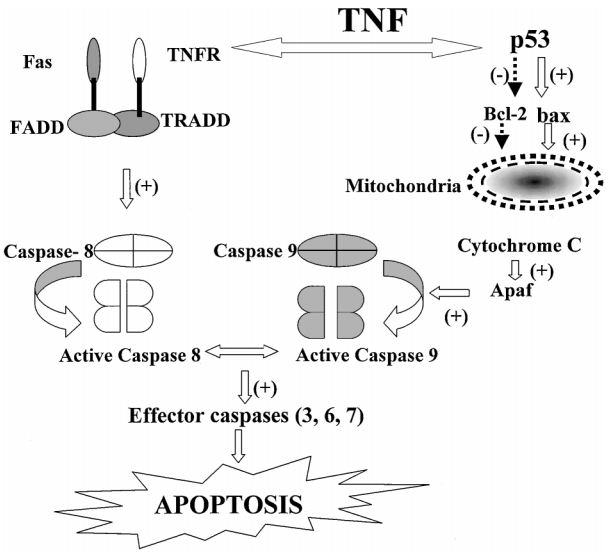

Fig. 1. TNF-mediated apoptotic pathways in fetal membranes. TNF can induce/increase Fas expression or p53 expression in fetal membranes. Fas and TNF receptor (TNFR) mediated pathways recruit Fas- and TNF-associated death domain proteins (FADD and TRADD) which in turn activate caspase 8. p53 transactivates bax (proapoptotic) and downregulates Bcl-2 (anti-apoptotic). Bax causes mitochondrial membrane damage and the release of cytochrome *c*. Cytochrome *c* activates Apaf (apoptotic protein activation factor), which activates caspase 9. At this junction, active caspase 8 or active caspase 9 or both activate caspase 3. This effector caspases can activate other effector caspases leading to proteolysis and programmed cell death (12–16).

damage repair enzyme, and DNA-dependent protein kinases (DNA PKcs); 2) structural proteins that maintain the integrity of the cytoskeleton or nuclear matrix (β-actin, lamin); and 3) several proteins of unknown function (6).

In this study TNF-α stimulation causes the induction of caspase 2, 8, and 9 mRNA expression in human fetal membranes, whereas caspase 3 and 6 were present in both control and TNF-stimulated tissues. The activity of these induced caspases was also high in TNF-stimulated tissues compared with control. TNF stimulation results in the increased activity of caspases that complete apoptosis. This includes caspase 3 although its mRNA expression was seen in both TNF-stimulated and control tissues. An increase in NMP levels is indicative of increased caspase activity.

Both TNF/Fas- and TNF/p53-mediated pathways are activated in fetal membranes during infection-associated PROM. Elevated TNF is capable of inducing both of these pathways and thereby activating caspases. Caspase activation and cell death may lead to membrane weakening and rupture; although the way in which apoptosis interacts with the MMPs to cause PROM still remains to be determined.

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