OBSTETRICS

IL-18, a Product of Choriondecidual Cells, Increases During Premature Rupture of Membranes but Fails to Turn on the Fas–FasL-Mediated Apoptosis Pathway

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Problem: IL-18 is a novel cytokine, which promotes inflammation and apoptosis. This study examines its expression pattern, site of production, and levels in the amniotic fluid (AF) during pregnancy complications such as preterm labor and preterm premature rupture of membranes (pPROM). The ability of IL-18 to induce the Fas–Fas ligand (FasL)/caspase-mediated apoptotic pathway is also studied.

Methods: Amniochorion collected at term was placed in an organ explant system. IL-18 mRNA expression was studied by RT-PCR. IL-18 mRNA and peptide were localized by in situ hybridization and immunohistochemistry. IL-18 in the AF of women with pPROM, with preterm labor with no rupture of membranes, and at term was measured using ELISA. Multiplex PCR was used to study the expression pattern of proapoptotic genes such as Fas, FasL, caspase 8, and Fas-associated death domain (FADD). ELISA was also used to measure the release of soluble Fas from IL-18-stimulated amniochorion in culture media.

Results: IL-18 is a constitutively expressed gene in human chorion and decidua but not in human amnion and increases in the AF of women with pPROM [2.9 ± 3.3 ng/ml (SD)] compared to women with preterm labor (1.1 ± 0.67 ng/ml; $P < 0.05$) and term (0.9 ± 0.73 ng/ml; $P < 0.05$). IL-18 induces

Fas expression, whereas FADD is a constitutively expressed gene in human fetal membranes. IL-18 failed to induce FasL or caspase 8 expressions. Soluble Fas release from amniochorion was increased after IL-18 stimulation.

Conclusion: Chorion and decidua are a source of IL-18, whose concentrations are increased in the AF during pPROM. IL-18 induced Fas expression in amniochorion; however, it failed to turn on other genes in the Fas–FasL apoptosis pathway including FasL and caspase 8.

KEY WORDS: IL-18; apoptosis; Fas–FasL; PROM; amniotic fluid; fetal membranes.

INTRODUCTION

Interleukin (IL)-18 or interferon γ inducing factor is a novel, proinflammatory and proapoptotic cytokine that is produced mainly by immunocytes (1). It shares sequence similarities with other IL-1 family members, and like IL-1 β it is secreted as a precursor lacking a signal peptide and requires cleavage by proteases for biological activity. The pro-forms of IL-18 are activated by IL-1 β converting enzyme (ICE/caspase1)(2,3). It does not bind to the IL-1 receptor, as it uses IL-1-related receptor, protein for signal transduction (4).

IL-18 increases the inflammatory process by stimulating the production of tumor necrosis factor (TNF), IL-1 β , and IL-6. Its proinflammatory properties are attributed to its ability to promote nuclear translocation of nuclear factor- κ B (Nf- κ B). Nf- κ B is a transcriptional activator DNA binding protein that

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Fig. 1. Proposed IL-18-induced Fas-FasL apoptotic pathway: IL-18 can increase cell membrane-associated FAS expression and induce FasL, which will bind to Fas. This binding will turn on intracellular signaling events recruiting Fas-associated death domain protein (FADD), a docking protein that will facilitate the activation of pro-caspase 8 to its active form. Active caspase 8 then activates pro-caspase 3, which in turn will activate several other caspases (effector caspases). These caspases are aspartic acid-specific cystine proteases that will cause proteolysis of several of the homeostatic and structural proteins (actin, lamin etc.). This proteolysis will lead to programmed cell death or apoptosis.

binds to the κ B motif in the promoter regions of the inflammatory cytokine genes (5). IL-18 can also promote apoptosis (programmed cell death) of cells by increasing Fas expression and by inducing Fas ligand (FasL) expression. Fas (also known as CD95 or APO 1) is a member of the TNF receptor family and promotes apoptosis in a variety of cells when bound by FasL (6,7). The known pathway of Fas-FasL-mediated programmed cell death is described in Fig. 1.

The proinflammatory and proapoptotic properties of IL-18 are relevant in complications of pregnancy such as preterm labor and preterm premature rup-

ture of membranes (pPROM). These conditions are associated with elevated amniotic fluid (AF) levels of the proinflammatory cytokines (IL-1, TNF, IL-6, and IL-8) (8,9). We have recently documented a role for apoptosis in pPROM (10). Apoptosis may lead to matrix degrading enzyme activation, promoting membrane degradation and rupture (10). In this study we examine the expression pattern of IL-18 and the site of its production in placental membranes. We also study the changes in IL-18 concentration during preterm labor and pPROM as well as its ability to turn on the Fas-FasL-mediated apoptotic pathway in human fetal membranes.

METHODS

Organ Culture. Fetal membranes ($n = 8$) were collected from women at term undergoing elective repeat cesarean delivery with no complications of pregnancy. Investigation of IL-18 mRNA expression and localization of its peptide were done on fetal membranes placed in an organ explant system for 4 days (see our earlier publications for details of methodology) (10,11). Tissue sections were frozen at the time of collection (0 hr in culture) and after each 24-hr period in culture and stored at -70°C until analysis. Women with a history of pregnancy complications (previous preterm labor, PROM, PIH, gestational diabetes), with infection, and on antibiotic treatment prior to C-section including prophylaxis to Group B streptococcal infection were excluded from this study. Tissue sections used for organ culture were subjected to histologic evaluation to rule out histologic chorioamnionitis (>3 or 4 polymorphonuclear leukocytes/HPF). Sections with histologic chorioamnionitis were excluded from this study. Tissue viability was tested at the end of the culture period by RT-PCR for glyceraldehyde 3-phosphate dehydrogenase expression.

RNA Extraction and RT-PCR. Total RNA was extracted by the Trizol (Life Technologies, Bethesda, MD) method, and 0.5 μg subjected to oligo(dT)-primed reverse transcriptase reaction. cDNA was then subjected to PCR using IL-18 specific primers (12) for 30 cycles using the following parameters: 3 cycles of pre-PCR were performed with denaturation at 90°C for 5 min, annealing at 60°C for 5 min, and extension at 72°C for 1 min. This was followed by 35 cycles of PCR with denaturation at 90°C for 1 min and extension annealing at 55°C for 1 min. A final annealing was done at 72°C for 4 min. The PCR products were analyzed on ethidium bromide-stained 2% agarose gels.

In Situ Hybridization and Immunohistochemistry. In situ hybridization was performed with 3' and 5' biotinylated sense and antisense probes, respectively, for IL-18 (the same as those used for PCR). The detailed protocols can be found in our earlier publications (11). Hybridization was performed at 50°C for 12 hr and color development was done for 3 hr. Sense strand was used as a negative control. Immunohistochemistry was performed on paraffin-embedded sections using goat anti-human IL-18 primary antibody (0.5 $\mu\text{g}/\text{ml}$; R&D Systems, Minneapolis, MN). Incubation at 37°C for 3 hr with primary antibody was followed by overnight incubation at 4°C . Biotinylated mouse anti-goat secondary antibody

and streptavidin–horseradish peroxidase was used for detection (Zymed, San Francisco, CA). The manufacturer's instructions were followed to detect biotin–streptavidin–HRP complex using AEC reagents. If peptide for IL-18 were present, this would give a red staining reaction. The sections were lightly counterstained with hematoxylin and mounted using gel mount (Zymed). Antibodies to Type IV collagen and HLA-DR were used as positive and negative controls, and phosphate-buffered saline was used as reagent control.

ELISA for IL-18. A cross-sectional study was designed to evaluate the changes in the concentrations of IL-18 in the AF during pPROM, during preterm labor, and at term. AF samples from eligible women were selected from our bank of previously collected and frozen samples. This study included women with preterm pPROM ($n = 20$), which was defined as spontaneous amniorrhexis before labor and before 37 weeks of gestation. Membrane rupture was diagnosed using positive identifiers such as the nitrazine test, the fern test, pooling, and oligohydramnios on ultrasound examinations. The second group ($n = 24$) consisted of women with preterm labor and no rupture of membranes. The third group was composed of women at term with no history of pregnancy complications. Women with other medical or obstetrical complications were excluded from this study. All AF samples were collected by transabdominal amniocentesis under continuous ultrasonographic guidance or through uterine incisions during cesareans. Samples were centrifuged at 1700g for 10 min and the supernatants were stored at -70°C until ready to use.

IL-18 levels were measured using ELISA. A 1:10 dilution of the AF samples with assay diluent was necessary to bring the IL-18 concentration to the linear portion of the standard curve. Manufacturers' instructions were followed for the assay. Standard curves were developed using duplicate samples of known quantities of recombinant human IL-18. Concentrations were determined by relating the absorbance to the standard curve by linear regression analysis. Assay buffer was used as negative control.

IL-18 Stimulation of Amniochorion. After a 48-hr incubation in culture, membranes were stimulated with 15 ng/ml recombinant IL-18 (Biosource International, Camarillo, CA). The concentration was chosen based on the highest level of this cytokine seen in the AF in the previous part of this study. The time of stimulation was chosen based on our prior work with cytokines (11). Tissue samples and media were collected, frozen, and stored until analysis.

EISA for Soluble Fas. ELISA was used to measure release of Fas (soluble FAS) from IL-18-stimulated amniochorion (Oncogene Research Products, Cambridge, MA). The manufacturer's instructions were followed for the assay. (Also, see above standard curve preparation protocol).

Multiplex PCR (MPCR). MPCR was used to study the expression pattern of proapoptotic genes such as Fas, FasL, caspase 8, and Fas-associated death domain in IL-18-stimulated amniochorion. In multiplex PCR multiple target genes are amplified simultaneously in the same reaction tube (BioSource International). Multiple primers are used to amplify different target genes at the same time. Formation of primer dimers and masking of low-copy number target genes by high-copy number genes are prevented in this assay by optimizing the buffers and also by selecting primer pairs with similar temperatures of melting so highly stringent conditions are used for all primers. In this assay Fas, FasL, caspase 8, tumor necrosis factor receptor-associated death domain, and Fas-associated death domain gene expression was studied. GAPDH was used as the control gene.

Statistical Analysis. The unpaired nonparametric, two-sample Mann-Whitney *U* test and Tukey-Kramer multiple-comparison test were used to analyze the statistical significance of ELISA data. ($P < 0.05$ significant).

RESULTS

RT-PCR revealed a constitutive expression of IL-18 in all human fetal membranes at the time of collection (0 hr; $n = 8$) and at various time points in culture up to 3 days ($n = 8$) (Fig. 2). A total of eight tissues section from each cultures was subjected to in situ hybridization and immunohistochemistry. In situ hybridization localized IL-18 mRNA to chorion cells (both laeve and cytotrophoblasts) (Fig. 3a), while amnion cells were negative (Fig. 4a). We also placed

Fig. 2. Representative gel picture of RT-PCR showing IL-18 expression in human fetal membranes at various time points in culture [zero hour (at the time of collection) and 24, 48, and 72 hr].

some fetal membranes with attached decidua in culture and found that, along with chorion, decidua is also a source of IL-18 mRNA ($n = 3$). The localization was intense at the choriodecidual junction (Fig. 3). No cells in the extracellular matrix region showed any signal for IL-18 mRNA. Sense strand to IL-18 showed no staining, as would be expected for a negative control. The staining was limited to a very few cells of the chorion and decidua, and this pattern was consistent in all the sections tested. Similarly IL-18 peptide was also localized mainly in the choriodecidual junction, where both chorion and decidual cells showed positive staining for the IL-18 protein. In agreement with the in situ hybridization data, protein localization of IL-18 was limited to a very small population of choriodecidual cells, with other portions of the membrane acting as an internal negative control (Figs. 3b and 4b). Amnion remained negative for IL-18 peptide, supporting the absence of IL-18 mRNA in those cells. Antibody to Type IV collagen (positive control) stained the basement region of the chorion as well as the region between chorion cells. No nonspecific staining was seen with antibody to HLA-DR and phosphate-buffered saline. Data were consistent for both in situ hybridization and immunohistochemistry in all the tissue samples tested.

A total of 63 AF samples was tested for IL-18. IL-18 was detectable in all the AF samples tested. The mean gestational age for the preterm pPROM group was 30.7 weeks (SD, ± 4.1 weeks), and that for the preterm labor group was 32.0 weeks (SD, ± 3.09 weeks; $P = \text{ns}$). The mean gestational age for the term group was 37.3 weeks (SD, ± 1.36 weeks; $P = 0.001$ vs preterm labor and pPROM groups). In the pPROM group 35% (7/20) experienced preterm labor and three samples (3/7) showed positive AF culture results for microorganisms (Group B β hemolytic streptococci). The rest of the pPROM samples were culture-negative and did not experience preterm labor (13/20). A total of 24 samples was assayed in the preterm labor group, with 4 samples showing positive AF culture results (2 Group B β hemolytic streptococci, 1 *Staphylococcus aureus*, and 1 *Candida*). A total of 19 AF samples was assayed in the term group, of which 9 experienced active labor.

IL-18 was significantly elevated in the AF of women with pPROM compared to the other two groups. The levels were 2.9 ng/ml (median, 1.86 ng/ml; range, 0.3–15.2 ng/ml) in the pPROM, 1.14 ng/ml (median, 0.92 ng/ml; range, 0.1–2.7 ng/ml; $P < 0.05$) in the preterm labor, and 0.92 ng/ml in the term (median, 0.16 ng/ml; range, 0.1–1.2 ng/ml; $P < 0.05$) groups.

Fig. 3. (a) Chorion (C) and decidua (D) show positive staining reactions after hybridization with IL-18 antisense strand. (b) Immunohistochemical analysis with IL-18 specific antibody identified the protein in chorion and decidua cells that were positive for IL-18 mRNA. Arrowheads indicate positive staining reactions.

Fig. 4. Amnion cells show negative staining (a) for IL-18 mRNA and protein and (b) for IL-18 protein. Original magnification, $\times 40$.

There was not a statistically significant difference between the preterm labor and the term groups.

We also compared the levels of the culture-positive fluids and those experiencing preterm labor within the pPROM group. Women with pPROM (in the absence of preterm labor and infection; $n = 13$) had higher concentrations of IL-18 compared to those with pPROM with infection and preterm labor ($n = 7$). pPROM in the absence of labor or infection had a mean level of 4.1 ng/ml IL-18 (median, 3.1 ng/ml; range, 1.08–15.2 ng/ml), compared to 1.1 ng/ml (median, 0.7 ng/ml; range, 0.3–0.9 ng/ml; Mann–Whitney $P < 0.003$) in pPROM with preterm labor and infection. No statistically significant change was observed when the preterm labor group was compared internally as culture-positive (4/24) and culture-negative (20/24), which may also be attributed to the small sample size of culture-positive fluids. No significant difference was noted between the term active labor ($n = 9$) and the term not in labor groups ($n = 10$).

MPCR showed induction of Fas expression in IL-18-stimulated tissues compared to controls, where expression was not seen in any other cultures (Fig. 5). Fas-associated death domain gene expression was constitutive in both stimulated and control tissues, whereas FasL and caspase 8 expression was never seen. TNF receptor-associated death domain (TRADD) gene expression was seen in some control and IL-18-treated samples. Although weak, tumor necrosis receptor-associated death domain gene expression seems to be constitutive. GAPDH expression was seen in all the samples tested. A cDNA sam-

ple obtained from HeLa cells undergoing apoptosis was used as a positive control, which showed the expression of all the genes under investigation, confirming the specificity of the amplification.

ELISA on culture media ($n = 8$) from tissue sections stimulated with IL-18 documented an increased release of soluble Fas from human fetal membranes. The mean level of soluble Fas in IL-18-stimulated tissues was 4.2 U/ml (median, 3.6 U/ml; range, 0.25–10 U/ml) compared to 0.92 U/ml (median, 0.6 U/ml; range, 0.1–2.3 U/ml; Mann–Whitney $P < 0.04$).

DISCUSSION

In human fetal membranes chorion cells are the source of IL-18 production, along with maternal decidua. Amnion cells do not produce IL-18 mRNA or peptide. IL-18 expression was documented throughout the culture period as well as in tissues collected at the time of cesarean delivery (0 hr), suggesting that IL-18 expression is constitutive in human fetal membranes at term. It was interesting to note that IL-18 concentrations were significantly elevated in the AF obtained from women with preterm pPROM. This is the first inflammatory cytokine studied that seems to be elevated in pPROM independent of labor or infection. pPROM in the presence of infection and preterm labor showed a drop in the levels of IL-18 in the AF compared to the pPROM-alone group. This is the third set of evidence produced by our laboratory documenting that the pPROM and preterm labor pathways diverge and proceed along

Fig. 5. Representative gel picture of multiplex PCR shows expression of Fas (321 bp), Fas-associated death domain (205 bp), and glyceraldehyde 3-phosphate dehydrogenase (500 bp; control-housekeeping gene) in human fetal membranes. Caspase 8 (405 bp) and FasL (250 bp) expression was not seen. Cells undergoing Fas–Fas-mediated apoptosis (positive control) show the expression of all the genes tested. MW, molecular weight marker.

different routes. Previously we have documented an increased expression of the proapoptotic genes p53 and bax along with DNA fragmentation (sign of apoptosis) in human fetal membranes during pPROM (10). These markers of apoptosis were decreased or absent during preterm labor, and Bcl-2, an antiapoptotic gene, was increased in preterm labor. Matrix metalloproteinase gene expression is increased to a greater degree in pPROM than in preterm labor, and active matrix metalloproteinase 9 (gelatinase A) is increased in the AF of women with pPROM (10,13,14).

IL-18 is involved in cell-mediated immunity and its actions are synergistic with IL-12 and IL-15 (15). We have seen increased IL-15 concentrations in the AF during preterm labor compared to term but its levels were not increased during infection (16). We have not been able to demonstrate the presence of IL-12 in human fetal membranes (unpublished data). This may indicate a lack of coordinated cell-mediated immunity during preterm labor or during infection-associated preterm labor. The role of IL-18 and the factors that elevate its levels in pPROM are yet to be elucidated. The elevated levels of IL-18 during pPROM were thought to suggest that it might induce apoptosis in fetal membranes through the Fas–FasL pathway. Runic has already documented Fas expression by amnion cells (17). Maymon *et al.* have reported increased Fas–FasL levels in the AF in pPROM (18). Herein, we tested both of these findings. Our MPCR data suggest that the IL-18-induced Fas–FasL-apoptosis pathway is incomplete in fetal membranes. IL-18 induced Fas expression and Fas associated death domain was constitutively expressed in amniochorion; however, IL-18 failed to turn on FasL and caspase 8, the initiator of the Fas–FasL-mediated caspase-apoptotic pathway. This agreed with similar findings by Payne *et al.* in trophoblasts in which induced Fas expression did not result in FasL-mediated apoptosis (19).

The increased release of soluble Fas from IL-18-stimulated human fetal membranes may explain in part the absence of the Fas–FasL-mediated apoptosis pathway. Soluble Fas is a fragment released from the cell surface, which binds FasL and neutralizes it, preventing it from binding to the cell membrane-associated Fas. Although amniochorion is not a source of FasL, IL-18-induced soluble Fas may neutralize FasL produced by other cell types (immunocytes) in the AF during pPROM. It is possible that the role of IL-18 in pPROM is protective for fetal membranes in that it reduces the risk of the Fas–FasL-mediated apoptosis pathway. This does not rule out other alternative pathways of apoptosis during pPROM. We

have already shown a role for p53 and p53-responsive genes in pPROM (bax and Bcl-2) (10). TNF can also cause apoptosis by the same Fas–FasL pathway of genes. Research is under way in our laboratory to study the role of TNF and the lipopolysaccharide-mediated apoptosis pathway in fetal membranes.

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