BASIC SCIENCE: OBSTETRICS

Natural antimicrobial production by the amnion

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OBJECTIVE: The purpose of this study was to determine the expression of natural antimicrobials in primary cultured amnion epithelial cells and to examine their regulation by interleukin-1 beta (IL-1β).

STUDY DESIGN: Primary amnion epithelial cells were cultured from samples that were obtained at prelabor cesarean section (n = 12) and stimulated with IL-1β. Natural antimicrobial messenger RNA expression was determined by real-time quantitative polymerase chain reaction, and protein was measured by enzyme-linked immunosorbent assay. Data was analyzed by analysis of variance.

RESULTS: Primary amnion epithelial cells express messenger RNA for human beta defensin (HBD) 1 to 3, secretory leukocyte protease inhibitor and elafin, but not HBD4. IL-1β 10 ng/mL stimulates HBD2 messenger RNA in a biphasic pattern, with a 51-fold increase at 6 hours and a 67-fold at 12 hours ($P < .001$). HBD2 protein production is significantly increased by 24 hours ($P < .05$).

CONCLUSION: The amnion produces potent natural antimicrobials that may help protect the pregnancy from infection. HBD2 production is dramatically upregulated by the labor-associated inflammatory cytokine IL-1β.

Key words: amnion, defensin, infection, natural antimicrobial, pregnancy

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Labor is an inflammatory process that is characterized by leukocyte invasion of the uterine tissue, and increased cytokine and prostaglandin production.[1] Intrauterine infection in pregnancy can stimulate this process prematurely and is responsible for approximately one third of all cases of preterm labor.[2] Prevention of ascending infections is paramount in maintaining a healthy pregnancy.

Natural antimicrobials are peptides that are essential components of the innate immune system that provide broad-spectrum protection against bacteria, yeasts, and some viruses.[3] The human beta defensins (HBDs) are a major family of vertebrate natural antimicrobials; HBD1-4 are expressed widely at mucosal surfaces.[4] In addition to their antimicrobial activity, HBDs have chemoattractant properties that suggest that they interact between the innate and adaptive immune systems.[5,6] A related family of molecules with important antimicrobial activities is the antileukoproteinases.[7] This group includes elafin (skin-derived antiproteinase) and secretory leukocyte protease inhibitor (SLPI).

Natural antimicrobial proteins are expressed throughout the nonpregnant female reproductive tract.[8,9] In pregnancy, natural antimicrobials are found in the amniotic fluid[10-13] and have been localized in the placenta, decidua, and fetal membranes.[14-18]

The amnion is positioned critically between the normally sterile amniotic cavity and the contaminated extrauterine environment, but amniotic production of natural antimicrobials has not been elucidated. The bacterial product lipopolysaccharide has been shown to stimulate HBD3 messenger RNA (mRNA) in so-called amnion-derived FL cells.[17] However, this cell line is derived from cervical He-La cell contaminants[19] (www.atcc.org), thus the validity of this finding is unclear. The current study examined natural antimicrobial expression and regulation in primary cultured amnion cells. We also compared the expression with that in the contaminated FL cell lines and the similarly contaminated WISH cell line[20] (www.atcc.org) to determine whether they are a suitable model for the study of amniotic innate immune responses.

METHODS

Samples

The Lothian Local Research Ethics Committee approved this study, and written informed consent was obtained from all participants. Fetal membranes were collected from 12 women who underwent elective cesarean section at 39 weeks of gestation. All of the women had uncomplicated singleton pregnancies, with no signs of labor or infection. Amnion for culture was stripped from underlying chorion, washed, and transported to the

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TABLE 1
Sequences of primers and probes for Taqman polymerase chain reaction

Variable	Forward primer	Reverse primer	Probe
HBD1	*TCAGCAGTGGAGGGCAATG*	*CCTCTGTAACAGGTGCCTTGAAT*	*TCTATTCTGCCTGCCCGATCTTTACCAA*
HBD2	*CTGATGCCTCTTCCAGGTGTTT*	*CTGGATGACATATGGCTCCACTCT*	*AAGGCAGGTAACAGGATCGCCTATACCACCA*
HBD3	*CAGAGGCGGCCGGTGT*	*CGAGCACTTGCCGATCTGTT*	*CTGTGCTCAGCTGCCTTCCAAAGGA*
HBD4	*GGCAGTCCCATAACCACATATTC*	*TGCTGCTATTAGCCGTTTCTCTT*	*TGTCCAATTCAAATTCGCTTCTCACTGGA*
SLPI	*GCATCAAATGCCTGGATCCT*	*GCATCAAACATTGGCCATAAGTC*	*TGACACCCCAAACCCAACAAGGAGG*
Elafin	*TGGCTCCTGCCCCATTATC*	*CAGTATCTTTCAAGCAGCGGTTAG*	*ATCCGGTGCGCCATGTTGAATCC*

laboratory in sterile phosphate-buffered saline solution (Sigma, Poole, Dorset, UK).

Three endometrial samples were used as positive controls for natural antimicrobials. Because individual natural antimicrobials are expressed differentially throughout the menstrual cycle,[21] an endometrial sample from the period of maximal expression for each natural antimicrobial was used (menstrual sample for elafin and HBD2; proliferative sample for HBD4; mid-secretory sample for HBD1, HBD3, and SLPI). They were collected from women who underwent gynecologic procedures for benign conditions. All of the women had regular menstrual cycles (28-35 days) and had not received any hormonal treatments for 3 months before biopsy collection. Menstrual cycle stage was determined from the date of the patient's last menstrual period, histologic dating, and circulating serum estradiol and progesterone concentrations. Endometrial biopsy specimens were immersed in Tri reagent (Sigma) for RNA extraction. A portion was also fixed in 10% neutral buffered formalin overnight at 4°C, stored in ethanol, and then wax embedded for subsequent histologic examination, which was normal in all cases.

Cell culture

Amnion epithelial cells were isolated with a method adapted from Bennett et al.[22] Amniotic membrane was washed and steeped in EDTA (0.5 mmol/L; Sigma) for 15 minutes. Cells were dissociated by incubation in a solution of Dispase (Gibco, Paisley, UK) for 45 minutes at 37°C, and released by agitation in Roswell Park Memorial Institute 1640 medium (Sigma).

Primary amnion cells were plated in 6-well plates (Nunc; Gibco) at a density of 1.5 to 2×10^6cells/mL. FL, WISH, and He-La cells (ATCC, Manassas, VA) were plated at a density of 0.5×10^6cells/mL. Cells were cultured in Roswell Park Memorial Institute 1640 medium that was supplemented with 10% fetal calf serum (Mycoplex; PAA Laboratories, Teddington, UK), penicillin (50 μg/mL; Sigma), streptomycin (50 μg/mL; Sigma,) and L-glutamine (2 mmol/L; Sigma) and was maintained at 37°C in 5% Co_2 and 95% air.

After 24 hours of culture, cells were washed, and fresh medium was added. After 72 hours, when confluent, medium was changed to serum-depleted (2% fetal calf serum) for 20 hours before treatments were added; 2% fetal calf serum was used because preliminary experiments showed decreased cell viability after 48 hours of culture in the absence of serum. The epithelial origin of primary cultured cells was confirmed by immunocytochemistry, with >95% of cells positive for the epithelial cell marker pan cytokeratin (Dako, Ely, Cambs, UK).

Treatments

Cells were incubated with recombinant human interleukin (IL)-1β (Peprotech, London, UK) to stimulate natural antimicrobial production. Initial experiments used a dose of 10 ng/mL and treatment times of 6 and 12 hours, because these were the optimal conditions in a previous study.[23] Subsequent experiments investigated the effects of dose or treatment time by using doses of 0.1-100 ng/mL or treatment times of 1, 2, 3, 6, 12, 16, 24, or 48 hours, after which time the effects diminished. All experiments were performed in quadruplicate, with 1 set of duplicates being used to determine mRNA expression by real-time TaqMan quantitative polymerase chain reaction (PCR) and 1 set being used for protein analysis by enzyme-linked immunosorbent assay. Media from the second set of duplicates were stored at –20°C. Treatments had no significant effect on cell numbers or viability at 48 hours, as determined by trypan blue exclusion.

RNA extraction and quantitative PCR

RNA was extracted from amnion with RNeasy minispin columns (Qiagen, Crawley, West Sussex, UK), and from endometrium with the use of Tri-reagent (Sigma), according to manufacturer's protocols. RNA quantity and quality were assessed by the Agilent 2100 bioanalyzer system in combination with RNA_{6000}nano chips (Agilent Technologies, Cheshire, UK). Only RNA that displayed intact 18S and 28S peaks was reverse transcribed to complementary DNA for real-time PCR.

RNA samples were reverse transcribed with the use of random primers (Taqman RT-PCR kit; Applied Biosystems, Foster City, CA) and amplified by ABI Prism TaqMan 7900 (Applied Biosystems), according to standard protocols. Target mRNA was quantified in relation to 18S ribosomal RNA abundance in each sample. Negative controls were: i) a negative reverse transcribed-sample (RNA template with no reverse transcriptase enzyme); ii) reverse tran-