Growth Factors, Cytokines, Cell Cycle Molecules

Tumor Necrosis Factor- α and Interleukin-1 β Regulate Interleukin-8 Expression in Third Trimester Decidual Cells

Implications for the Genesis of Chorioamnionitis

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Chorioamnionitis is associated with intense neutrophil infiltration of the decidua. We therefore determined whether chorioamnionitis enhances decidual interleukin-8 (IL-8) expression and examined cytokine-regulated decidual IL-8 expression. Decidua from chorioamnionitis-complicated pregnancies, but not term controls, displayed marked IL-8 immunohistochemical staining and a dense neutrophil infiltrate. Reverse transcriptase-polymerase chain reaction of microdissected decidual cells identified IL-8 mRNA, confirming decidual synthesis of IL-8. Confluent leukocyte-free term decidual cells were primed with 10^{-8} mol/L estradiol (E_2) or $E_2 + 10^{-7}$ mol/L medroxyprogesterone acetate to mimic the steroidal milieu of pregnancy. Compared with cultures maintained in E_2 alone, $E_2 +$ medroxyprogesterone acetate neither significantly affected IL-8 levels nor altered the response to the cytokines. The addition of 1.0 ng/ml tumor necrosis factor- α (TNF- α) or interleukin-1 β (IL-1 β) increased IL-8 secretion levels by 236.6 ± 51.4 - and 1062.6 ± 254.3 -fold, respectively ($n = 8$, mean $\pm$ SEM, $P < 0.05$), as measured by enzyme-linked immunosorbent assay. Concentration-response studies revealed that 0.01 ng/ml TNF- α and IL-1 β elevated IL-8 output by 10- and 100-fold, respectively. Western blotting confirmed these results, and quantitative reverse transcriptase-polymerase chain reaction demonstrated parallel changes in mRNA levels. In conclu-

sion, IL-8 is strongly expressed in term decidua during chorioamnionitis, and TNF- α and IL-1 β enhance IL-8 expression in term decidual cells, suggesting that these cytokines are important regulators of chorioamnionitis-related decidual neutrophil infiltration. (Am J Pathol 2006, 169:1294–1302; DOI: 10.2353/ajpath.2006.060185)

Preterm delivery (PTD) complicates over 12% of live births in the United States and is a primary cause of perinatal morbidity and mortality.^{1,2} Infection of gestational tissues occurs in nearly 50% of early PTDs and is a major antecedent to preterm premature rupture of the membranes (PPROM).³ Intra-amniotic infection as determined by positive cultures for aerobic and anaerobic bacteria and mycoplasma species is demonstrated in 23% of cases of PPRM.^{4,5} However, an additional 19% of PPRM cases without overt infection exhibit elevated amniotic fluid levels of the neutrophil collagenase or matrix metalloproteinase-8 (MMP-8).⁵ This suggests that decidua may be a source of infection and that these microorganisms do not always penetrate into the amniochorion. Other proinflammatory markers in the amniotic fluid linked to PTD include neutrophil elastase, defensins, and calgranulins.^{6,7}

Neutrophil infiltration of inflamed tissues is an early acute response to bacterial infections.⁸ Several different neutrophil chemoattractive and activating chemokines exist. These include interleukin-8 (IL-8 or CXCL8), one of the most potent neutrophil chemoattractants and activa-

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tors.^{9–11} In addition to IL-8, other CXC family members that promote neutrophil trafficking into inflammatory sites include epithelial neutrophil-activating peptide 78 (ENA-78 or CXCL5), growth-related oncogene- α (GRO- α or CXCL1), and granulocyte chemotactic protein-2 (GCP-2 or CXCL6).¹² Amniotic fluid from patients with PTD complicated by intra-amniotic infections contains elevated levels of IL-8^{13–15} as well as ENA-78¹⁶ and GRO- α .¹⁷ Amniotic fluid also has been reported to contain increased levels of the classic proinflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β).^{18,19} The expression of IL-8 is enhanced by TNF- α and IL-1 β in several cell types including leukocytes, endothelial cells, and fibroblasts.^{20–22}

Because chorioamnionitis (CAM) is associated with intense neutrophil infiltration of the decidua,²³ this study evaluated the involvement of decidual-derived IL-8 in CAM. Specifically, laser capture microdissection coupled with reverse transcriptase-polymerase chain reaction (RT-PCR) determined whether decidual cells express IL-8 mRNA and immunohistochemistry (IHC) localized IL-8 and compared its expression in CAM versus control decidua. To elucidate mechanisms underlying inflammation-regulated neutrophil infiltration of the decidua, TNF- α and IL-1 β effects were evaluated on IL-8 production in decidual cells. The decidual cells were passaged until they were free of CD45⁺ cells to eliminate the potential confounding effects of resident leukocytes during experimental incubations. In view of a recent clinical trial demonstrating that 17 α -hydroxyprogesterone caproate administration protects against preterm delivery,²⁴ the current study assessed potential interactions between the progestin, medroxyprogesterone acetate (MPA), and proinflammatory cytokines on IL-8 expression in term decidual cells.

Materials and Methods

Specimens

Eight placentas with CAM, between 33 and 38 weeks of gestation, were selected from the case files of the Department of Human Pathology and Oncology of the University of Siena. Histological diagnosis of CAM was based on the presence of a rich inflammatory infiltrate of polymorphonuclear neutrophils in the membranes, decidua, and cord in the setting of clinical infections. In all infants, biochemical neonatal signs of acute phase response (leukocytosis or leukopenia, c-reactive protein, >1.0 mg/dl) were observed. In four cases, the presence of neonatal infections was confirmed by positive blood cultures for pathogens. All neonates were delivered vaginally and three patients presented with antecedent PPRM. Eight control placentas from uncomplicated pregnancies were obtained following spontaneous delivery at term. Approval for this study was granted by the Human Investigation Committee of the University of Siena. Informed consent was obtained from all women. Placenta sampling for histology included multiple full-thickness placental blocks (10 × 10 mm) and sections of

the umbilical cord at three different levels. For each specimen, a block that best represented the maternal decidua was selected for IHC.

Immunohistochemistry

Sections (4 μ m) of paraffin-embedded placental and attached fetal membrane tissues were cut, deparaffinized, rehydrated, and washed in Tris-buffered saline (TBS: 20 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.6). Tris-buffered saline was used for all subsequent washes and for dilution of the antibody. Antigen retrieval was performed by incubating sections in sodium citrate buffer (10 mmol/L, pH 6.0) in a microwave oven at 750 Watts for 5 minutes. Sections were subsequently rinsed in 3% hydrogen peroxide to block endogenous peroxidase and incubated overnight at 4°C with a goat polyclonal antibody against IL-8 (R&D Systems, Minneapolis, MN) diluted in TBS. Slides were then washed three times with TBS for 5 minutes and incubated with a rabbit anti-goat antibody labeled with peroxidase (Calbiochem, Milan, Italy) at a dilution of 1:2000 for 30 minutes. After washing three times for 5 minutes in TBS, sections were stained with 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO) as chromogen substrate and counterstained with Meyer's hematoxylin. The reaction was stopped by washing the sections in distilled water, and slides were mounted and observed under a light microscope. Negative controls for each tissue section were prepared by substituting the primary antibody with the corresponding pre-immune serum.

Interleukin-8 immunostaining in the decidua was evaluated by one experienced pathologist (P.T.) who was blinded to the origins and clinical outcomes of the patients. A semiquantitative method was used in accordance with the following scoring system: 0, absence of staining/weak staining; 0/+, presence of moderate focal staining; +, moderate staining; and ++, marked staining.

Laser Capture Microdissection

To perform laser capture microdissection (LCM), serial 4- μ m cryostat sections of decidua were mounted on uncoated glass slides, fixed in 70% ethanol, and stained with the HistoGene LCM Frozen Section Staining Kit (Arcturus, Mountain View, CA). Decidual cells were isolated using a PixCell II LCM system equipped with an Olympus microscope (Arcturus). Microdissected decidual cells from two different slides were pooled for subsequent analyses.

Isolation and Culture of Decidual Cells

After receiving written informed consent, placentas and attached fetal membranes were obtained at term from eight nonlaboring patients with uncomplicated pregnancies undergoing repeat Cesarean deliveries at Yale-New Haven Hospital under Human Investigation Committee approval. A small portion of each specimen was formalin-fixed and paraffin-embedded and then examined histo-

logically to exclude underlying acute or chronic inflammation.

The decidua was scraped from the maternal surface of the chorion, minced, and digested in Ham's F-10 + 10% charcoal-stripped calf serum (Flow Laboratories, Rockville, MD) containing 2.5 mg/ml collagenase (200 U/mg) (Worthington Biochemical Corp, Freehold, NJ) in a shaking water bath at 37°C for 30 minutes suspended as 1 g of tissue per 10 ml of solution. After adding 6.25 units of DNase (Sigma-Aldrich) per ml of digestate, the incubation was continued for another 45 minutes. The final digestate was passed through a 23-gauge needle five times to dissociate remaining cell clusters. The isolated cells were centrifuged at 1500 rpm for 5 minutes at 4°C then washed in Ham's F-10. This procedure was repeated three times, and the final cell pellet was resuspended (1 g of tissue/ml) in 20% Percoll (Sigma-Aldrich), layered on a (60:50:40) discontinuous Percoll gradient, and centrifuged at 22,000 rpm for 20 minutes at 4°C. The top cell layer was collected, washed, resuspended in Ham's F-10 without serum, and centrifuged at 1500 rpm for 5 minutes at 4°C. After repeating this procedure, the resulting cell pellet was resuspended in 40% Percoll (1 g of tissue/ml), layered on a discontinuous (55:50:40) Percoll gradient, and centrifuged at 22,000 rpm for 20 minutes at 4°C. The top cell layer was washed twice in serum-free Ham's F-10 and then centrifuged at 1500 rpm for 5 minutes at 4°C. The cell pellet was resuspended in Ham's F-10 + 10% stripped calf serum, and decidual cells were counted in a hemocytometer. Trypan blue exclusion identified >95% of the isolated decidual cells as viable.

Isolated decidual cells (5×10^5 cells/ml) were suspended in basal medium, a phenol red-free 1:1 (v/v) mix of Dulbecco's modified Eagle's medium (Sigma) and Ham's F-12 (Flow Laboratories) with 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone supplemented with 10% stripped calf serum. Decidual cells were seeded onto polystyrene tissue culture dishes coated with 2% type B gelatin (Sigma). The cultures were grown to confluence in a standard 95% air/5% CO₂ incubator at 37°C. Cells were harvested using trypsin/EDTA and analyzed by flow cytometry with anti-CD45 and anti-CD14 monoclonal antibodies (BD Pharmingen, San Diego, CA) to monitor the presence of leukocytes after each passage. After three to four passages, cell cultures were found to be leukocyte-free (<1%). The latter were used for experimental cell incubations. The cultured cells were all vimentin positive and cytokeratin negative on IHC. After five passages, the cells were seeded onto polystyrene tissue culture-treated flasks without gelatin coating.

Experimental Cell Incubations

Confluent decidual cells were primed for 7 days in basal medium supplemented with stripped calf serum containing either 10^{-8} mol/L estradiol (E₂) or E₂ plus 10^{-7} mol/L medroxyprogesterone acetate (Sigma) used in place of native progesterone, which is rapidly metabolized *in vitro*²⁵ with one change of medium. The cultures were

washed twice with Hanks' balanced salt solution to remove residual serum components and switched to a serum-free defined medium (DM) consisting of basal medium plus ITS+ (BD Biosciences, Bedford, MA), 5 µmol/L FeSO₄, 0.5 µmol/L ZnSO₄, 1 nmol/L CuSO₄, 50 µg/ml ascorbic acid (Sigma), and 50 ng/ml epidermal growth factor (BD Biosciences) with corresponding steroid(s) with or without IL-1β or TNF-α (R&D Systems). The manufacturer assures, for both cytokines, a level of endotoxin lower than 1.0 endotoxin units per 1 µg of protein, as determined by the pyrochrome Limulus-Amebocyte-Lysate method. However, to assess the isolated or additive effects of endotoxin on IL-8 output, 0.05 to 5 µg/ml lipopolysaccharide (LPS) (Sigma-Aldrich) were added to select cultures treated with E₂+MPA with or without IL-1β or TNF-α.

After the experimental test period, the cells were harvested in ice-cold phosphate-buffered saline using a cell scraper and then pelleted. Cell proteins were extracted in an ice-cold lysis buffer of TBS with 1% Triton X-100, 1 mmol/L phenylmethanesulfonyl fluoride (Sigma), and Complete protease inhibitor cocktail (Roche, Mannheim, Germany), followed by sonication. The protein content in the cell lysates was determined using a modified Lowry assay (Bio-Rad Laboratories, Hercules, CA). Cell lysates and conditioned DM supernatants were stored at -70°C. In parallel incubations, total RNA was extracted from cultured decidual cells with Tri Reagent (Sigma).

Enzyme-Linked Immunosorbent Assays (ELISAs)

Immunoreactive IL-8, ENA-78, GRO-α, and GCP-2 levels in conditioned DM were measured by specific ELISAs according to instructions provided by the manufacturer (R&D Systems). The sensitivity of the IL-8 ELISA was 3.5 pg/ml. The intra- and interassay coefficients of variation were 4.6 and 6.7%, respectively. The sensitivity of the ENA-78 ELISA was 15 pg/ml, with intra- and interassay coefficients of variation of 5.1 and 9.4%, respectively. The sensitivity of the GRO-α ELISA was 10 pg/ml, with intra- and interassay coefficients of variation of 4.8 and 10.2%, respectively. The sensitivity of the GCP-2 ELISA was 1.6 pg/ml, with intra- and interassay coefficients of variation of 5.4 and 7.4%, respectively. All ELISA results were normalized to total cell protein content.

Western Blotting

Western blot analysis was performed on conditioned DM supernatants, which were diluted 1:1 in reducing sample buffer composed of Laemmli sample buffer and 2-mercaptoethanol (Bio-Rad) and then boiled for 3 minutes. The centrifuged media were electrophoresed on a 10 to 20% SDS polyacrylamide linear gradient gel (BioRad). The gel was electroblotted onto a 0.2-µm nitrocellulose membrane (Bio-Rad). After transfer, the membrane was blocked overnight in phosphate-buffered saline (Sigma) with 1% casein and incubated for 2 hours with a 1:333 dilution of a mouse anti-human IL-8 monoclonal antibody

(R&D Systems). Membranes were rinsed in phosphate-buffered saline and 0.2% Tween 20 before and after incubation with horseradish peroxidase-conjugated anti-mouse IgG (ICN Biomedicals, Aurora, OH). Chemiluminescence was detected with ECL reagents (Perkin Elmer Life Sciences, Boston, MA) and autoradiography film (Amersham Pharmacia, Buckinghamshire, UK) according to the manufacturer's instructions.

RT-PCR and Real-Time-Quantitative RT-PCR

Total RNA from cultured cells was extracted with Tri-Reagent (Sigma-Aldrich). Total RNA from the LCM-captured cells was extracted using the RNeasy micro kit (Qiagen Inc., Valencia, CA) according to the manufacturer's recommendation. RNA was subjected to RT-PCR with a kit from Invitrogen (Carlsbad, CA) on an Eppendorf Mastercycler (Eppendorf, Westbury, NY). For each RNA specimen, a negative control was prepared by omitting the reverse transcriptase.

To perform quantitative real-time RT-PCR for IL-8 mRNA detection in decidual cell cultures, reverse transcription was initially performed with an avian myeloblastosis virus reverse transcriptase (Invitrogen). A quantitative standard curve was created between 1 to 40 ng of cDNA with a Roche Light Cycler (Roche, Indianapolis, IN) by monitoring increasing fluorescence of PCR products during amplification. On establishing the standard curve, quantitation of the unknowns was determined with the Roche Light Cycler and adjusted to the quantitative expression of β -actin from the corresponding unknowns. Melting curve analysis determined the specificity of the amplified products and the absence of primer-dimer formation. All products obtained yielded correct melting temperatures. The following primers were synthesized and gel-purified at the Yale DNA Synthesis Laboratory Critical Technologies: sense primer: 5'-CACAAGAGCC-AGGAAGAAAC-3' and antisense primer: 5'-CTACAAC-AGACCCACACAATAC-3'. The corresponding β -actin sense and antisense primers were 5'-CGTACCACTGG-CATCGTGAT-3' and 5'-GTGTTGGCGTACAGGTCTTTG-3', respectively. The expected sizes of the amplified fragments for IL-8 and β -actin mRNA were 452 and 459 bp, respectively. The identity of the amplified products was confirmed by DNA sequencing.

For IL-8 mRNA detection in LCM-isolated cells, the following primers were obtained from Invitrogen, sense primer: 5'-GTGCAGTTTTGCCAAGGAGT-3' and antisense primer: 5'-CTCTGCACCCAGTTTTCCTT-3', respectively. The expected size of the amplified fragment was 196 bp. PCR product identity was confirmed by restriction analysis. In brief, the product of IL-8 PCR was extracted with phenol/chloroform and precipitated with ethanol. The amplified fragment was digested with *DdeI* (Promega Corporation, Madison, WI) following the manufacturer's suggested conditions. The products were separated by agarose gel electrophoresis, visualized by ethidium bromide staining, and photographed.

Figure 1. Reverse transcriptase-PCR analysis of IL-8 mRNA levels in micro-dissected decidual tissues. Total RNA of two decidua specimens (**lanes 1 and 3**) was reverse-transcribed and amplified in the presence of IL-8 primers. For each specimen a negative control, prepared by omitting the reverse transcriptase, was amplified and loaded onto the gel (**lanes 2 and 4**). Placental RNA was used as a positive control (PC). Forty-five cycles were run for each PCR. The size of the molecular weight makers (lane M; bp) is indicated.

Statistical Analysis

Comparisons of control and the various treatment groups were performed using the Kruskal-Wallis analysis of variance on Ranks test followed by the Student-Newman-Keuls post hoc test with P value < 0.05 representing statistical significance. For IHC, the intensity of staining in control versus CAM cases was compared using the χ^2 test, with significance set at a probability value of $P < 0.05$.

Results

The Effects of CAM on IL-8 Expression in Decidua

The expression of IL-8 mRNA in human decidua was assessed by RT-PCR analysis in cells isolated using LCM. As shown in Figure 1, a band corresponding in size to the IL-8 mRNA product was obtained from the cDNA of the two specimens tested. The identity of the IL-8 PCR product was confirmed by restriction analysis. Digestion of the 196-bp PCR-product with *DdeI* yielded three minor fragments of the expected size (30, 60, and 106 bp; not shown).

The expression of IL-8 protein in human decidua was assessed by IHC in placental specimens from patients with CAM compared with controls. In patients with CAM ($n = 8$), dense decidual neutrophil infiltrates were observed in the amniotic membrane, chorion, and decidua of all cases. By contrast, rare neutrophils were observed in the decidua of six control specimens. In two controls, some neutrophils were identified in association with fibrin deposits (not shown). Decidual cells displayed prominent IL-8 immunostaining in seven CAM cases (typical case shown in Figure 2A) with weaker staining observed in one CAM case. Positive staining for IL-8 is indicated by the brown color. In the controls ($n = 8$), IL-8 immunostaining was not evident in the decidual cells of six specimens (typical case shown in Figure 2B), whereas two specimens displayed weak IL-8 immunostaining in decidual cells proximal to fibrin deposits together with adjacent neu-

Figure 2. IHC analysis of IL-8 expression in CAM. Immunohistochemistry was performed by an indirect peroxidase technique (details in Materials and Methods). **A:** Chorioamnionitis decidua ($\times 100$ original magnification). Positivity is indicated by a brown color. **B:** Control decidua ($\times 100$ original magnification). **C:** Negative control ($\times 100$ original magnification).

trophils. Compared with control placentas, CAM placentas occurred more frequently in categories with more intense immunostaining ($\chi^2 = 16$; $df = 3$; $P = 0.001$).

Figure 3. Effects of E_2 ± MPA, $TNF-\alpha$, and $IL-1\beta$ on IL-8 output by decidual cell monolayers. Confluent, passaged leukocyte-free decidual cells were incubated for 7 days in 10^{-8} mol/L E_2 or $E_2 + 10^{-7}$ mol/L MPA then switched to DM with corresponding steroid(s) ± 1 ng/ml $TNF-\alpha$ or $IL-1\beta$ for 24 hours. IL-8 levels were measured by ELISA in conditioned DM and normalized to cell protein (details in Materials and Methods; $n = 8$, mean ± SEM). * $P < 0.05$ versus E_2 ; ** $P < 0.05$ versus $E_2 + MPA$. Ordinate is log scale.

Regulation of IL-8 Protein Expression in Decidual Cells

Because circulating levels of E_2 and progesterone are elevated during the third trimester, E_2 was used with MPA to mimic the gestational steroidal milieu for the experimental results depicted in Figures 3 to 6. In Figure 3, output of IL-8 in decidual cell monolayers maintained in E_2 alone as measured by ELISA was 1.11 ± 0.24 pg/ml/ μ g protein and 2.45 ± 0.9 pg/ml/ μ g protein in parallel incubations with $E_2 + MPA$ ($P > 0.05$). The addition of either 1 ng/ml $TNF-\alpha$ or $IL-1\beta$ elicited statistically significant increases in IL-8 output. Specifically, in incubations with E_2 , 1 ng/ml $TNF-\alpha$ or $IL-1\beta$ enhanced IL-8 output to 425.8 ± 117.8 and 2779.3 ± 647.1 pg/ml/ μ g protein, respectively (mean ± SEM; $n = 8$, $P < 0.05$). Thus, these increases were 623.5 ± 238.6-fold for $TNF-\alpha$ and 3034.2 ± 731.5 -fold for $IL-1\beta$. Likewise, in parallel incubations with $E_2 + MPA$, $TNF-\alpha$, and $IL-1\beta$ increased IL-8 output to 382.9 ± 115.7 and 1552.9 ± 321.3 pg/ml/ μ g protein by $TNF-\alpha$ and $IL-1\beta$, respectively ($P < 0.05$), which amounted to respective increases of 236.6 ± 51.4 - and 1062.6 ± 254.3 -fold. Inclusion of MPA in the incubation medium did not significantly affect the response to either $TNF-\alpha$ or $IL-1\beta$. By contrast, 0.05 μ g/ml LPS added to cultures treated with $E_2 + MPA$ up-regulated IL-8

Figure 4. Western blot analysis of $TNF-\alpha$ and $IL-1\beta$ effects on IL-8 output by *in vitro* decidual cells. Confluent decidual cells were incubated for 7 days in 10^{-8} mol/L E_2 or $E_2 + 10^{-7}$ mol/L MPA and then switched to a defined medium with corresponding steroid(s) ± 1 ng/ml $TNF-\alpha$ or $IL-1\beta$ for 24 hours. The conditioned medium was then subjected to Western blotting (see Materials and Methods). Results of two independent experiments (**A** and **B**) are shown.

Figure 5. Concentration-dependent effects of $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ on IL-8 output by decidual cell monolayers maintained in E_2 + MPA. Confluent decidual cells were incubated for 7 days in 10^{-8} mol/L E_2 + 10^{-7} mol/L MPA, then switched to DM with the steroids $\pm$ the indicated amount of (0.01 to 10.0 ng/ml) $\text{TNF-}\alpha$ (A) or $\text{IL-1}\beta$ (B). IL-8 levels were measured by ELISA in conditioned DM and normalized to cell protein (details in Materials and Methods; mean $\pm$ SEM, $n = 3$).

protein expression about 56-fold, but no concentration-dependent effects were observed.

Western blot analysis of decidual cell-conditioned medium confirmed the ELISA results. Figure 4 shows the results for two representative experiments in which the conditioned medium contains a single band at the molecular weight of IL-8 (8 kD). Consistent with the ELISA results, Western blotting indicated: 1) 1.0 ng/ml $\text{TNF-}\alpha$ or $\text{IL-1}\beta$ markedly enhanced the magnitude of the band corresponding to IL-8, 2) $\text{IL-1}\beta$ elicited a greater response than $\text{TNF-}\alpha$, and 3) no significant effects for MPA were seen for basal or for cytokine-enhanced IL-8 output.

In view of the absence of a progestin effect on IL-8 output by the term decidual cells, subsequent experiments were performed for incubations with cells that were primed with and then maintained in E_2 + MPA. Both $\text{TNF-}\alpha$ (Figure 5A) and $\text{IL-1}\beta$ (Figure 5B) elicited concentration-dependent increases in IL-8 output over the entire 0.01 to 10 ng/ml concentration range studied, with 0.01 ng/ml $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ elevating IL-8 output by about 10- and 100-fold, respectively. Consistent with its modest effects when added alone, 0.05 $\mu\text{g/ml}$ LPS displayed minimal (< 2 -fold) additive effects when added with either 10 ng/ml $\text{TNF-}\alpha$ or $\text{IL-1}\beta$ (data not shown). Figure 6 indicates that changes in the pattern of IL-8 mRNA corresponded to that of IL-8 protein. Thus, $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ both induced a statistically significant, severalfold increase in IL-8 mRNA levels compared with parallel incubation with E_2 plus MPA alone.

Figure 7 compares the effects of $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ on the secreted levels of ENA-78, GRO- α , and GCP-2 with those of IL-8. These data indicate that the secreted levels of all these cytokines are not only up-regulated by treatment with $\text{TNF-}\alpha$ and with $\text{IL-1}\beta$ but also have the same

Figure 6. Quantitative RT-PCR of effects of E_2 , MPA, $\text{TNF-}\alpha$, and $\text{IL-1}\beta$ on IL-8 mRNA levels in leukocyte-free term decidual cell monolayers. Confluent decidual cells were incubated for 7 days in 10^{-8} mol/L E_2 + 10^{-7} mol/L MPA, then switched to defined medium with corresponding steroid(s) $\pm$ 1 ng/ml $\text{TNF-}\alpha$ or $\text{IL-1}\beta$ for 5 hours. IL-8 mRNA levels were measured by quantitative RT-PCR and normalized to β -actin mRNA levels (details in Materials and Methods; $n = 3$, mean $\pm$ SEM). Ordinate: IL-8 mRNA/ β -actin mRNA. * $P < 0.05$ versus E_2 + MPA.

response pattern. Furthermore, IL-8 is much more abundant in the conditioned media than ENA-78, GRO- α , or GCP-2.

Discussion

Neutrophils are key components of the innate immune system. They are the first cells to arrive at sites of local injury where they phagocytose and kill invading pathogens and promote acute inflammation via release of proteases and reactive oxygen species.²⁶ A subsequent monocyte/macrophage influx promotes chronic inflammation.²⁷ In preparations of highly purified circulating leukocyte subtypes, macrophages, but not neutrophils, produced the Th1 cytokines, $\text{TNF-}\alpha$, and $\text{IL-1}\beta$. By contrast, only neutrophils produced Th2 mediators, such as the cytokine inhibitors TNF soluble receptor type 2 and the IL-1 receptor antagonist,²⁸ suggesting that neutrophils can protect against chronic inflammation and its potential pathological consequences.²⁷

Leukocytes traffic into inflammatory sites by migrating down a chemotactic gradient then interacting with endothelial cell-expressed adhesion molecules.²⁹ Driving this process are members of the CXC chemokine superfamily, including IL-8, ENA-78, GRO- α , and GCP-2. Among these chemokines, IL-8 exerts particularly specific and potent effects on neutrophils. These include shape change, respiratory burst, enhanced adhesion molecule expression, chemoattraction, and activation.^{10,11,30} The current study establishes that microdissected human decidual cells can synthesize IL-8 while demonstrating much more prominent IL-8 immunostaining in decidual cells of placentas complicated by CAM than in normal term specimens. These *in vivo* results were supported by observations in passaged, leukocyte-free term decidual cells. Specifically, $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ were found to markedly up-regulate steady-state IL-8 mRNA levels and to elevate secreted IL-8 levels by about 200- and 2000-fold, respectively. Previously, these cytokines were reported to enhance IL-8 output by only two- to threefold in unpassaged term human decidual cells.³¹ However, our finding that approximately 15% of cells in such cultures are CD45 positive³² suggests that the latter may blunt cyto-

Figure 7. Effects of $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ on IL-8, ENA-78, GRO- α , and GCP-2 output by decidual cell monolayers maintained in E_2 + MPA. Confluent, passaged leukocyte-free decidual cells were incubated for 7 days in 10^{-8} mol/L E_2 + 10^{-7} mol/L MPA then switched to DM with corresponding steroid(s) $\pm$ 1 ng/ml $\text{TNF-}\alpha$ or $\text{IL-1}\beta$ for 24 hours. Secreted levels of IL-8, ENA-78, GRO- α , and GCP-2 in conditioned DM were measured by ELISA and normalized to cell protein (details in Materials and Methods; $n = 7$ or 8 (for IL-8), mean $\pm$ SEM). * $P < 0.05$ versus E_2 + MPA.

kine-induced IL-8 expression in the decidual cell monolayers and raises the possibility that a similar phenomenon may occur *in vivo*.

Infection of the cervix, decidual-chorionic tissues, or the amniotic cavity by such microorganisms as group B *Streptococcus* (GBS), *Escherichia coli*, *Neisseria gonorrhoeae*, or *Chlamydia trachomatis* are known to cause CAM.³³ Contact between bacteria or their toxic products; ie, LPS, lipid A, or lipoteichoic acid, triggers a signaling cascade in both immune and nonimmune cells that induces local proinflammation.³⁴ Recently, in an elegant study, LPS and GBS were each found to enhance $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ expression in full thickness amnion-choriode-

cidua membranes-mounted in Transwell chambers in which the upper and lower chambers are physically separated. The choriodecidua proved more responsive than the amnion.³⁵

Observations that LPS targets decidual cells and decidual macrophages to secrete $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ ^{33,35–37} constitute the initial steps of the scheme of infection-induced PPRM depicted in Figure 8. The current study provides the next step in the proposed scheme by demonstrating that both cytokines enhance IL-8 expression in term decidual cells. The positive feedback of $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ to induce decidual cells to secrete IL-8 probably would promote neutrophil infiltration of the decidua, thereby triggering release of neutrophil elastase, neutrophil MMP-8, and MMP-9. Neutrophil elastase is a serine protease stored in azurophilic granules that degrades phagocytosed proteins and extracellular matrix proteins,⁶ whereas MMP-8 preferentially degrades fibrillar collagens and MMP-9 targets basement membrane associated collagens IV and V.^{38,39} Synergy among these proteases would effectively degrade the extracellular matrix of the decidua and fetal membranes and promote PPRM. Furthermore, Figure 8 indicates that neutrophil trafficking into the decidua is reinforced by neutrophil-derived MMP-9. This MMP-9 cleaves IL-8 to a truncated form [IL-8 (7-77)] that is 10- to 30-fold more active than full-length IL-8 in promoting neutrophil activation, MMP-9 secretion, and chemotaxis.⁴⁰ Although MMP-9 activates IL-8, it either inactivates or has no effect on monocyte/macrophage chemoattractants and activators such as macrophage chemoattractant protein-2 (CCL8), regulated on activation normal T cell expressed and secreted (CCL5), GRO- α , and PF-4 (CXCL4).⁴⁰ This selective action of MMP-9 likely would promote infiltration of neutrophils, but not monocytes/macrophages, into the decidua during CAM.

In summary, the current study provides evidence that during CAM, neutrophil infiltration of the decidua stems at least in part from $\text{TNF-}\alpha$ - and $\text{IL-1}\beta$ -induced production by the decidual cells of neutrophil activating and recruiting chemokines. These include predominantly IL-8, as well as to a lesser extent ENA-78, GRO- α , and GCP-2. Interpreted in terms of the absolute amount of cytokine

Figure 8. Scheme of infection-induced PPRM.

secreted, these data strongly support a main role for IL-8 in regulating neutrophils trafficking in the human decidua. This increase in the neutrophil population likely contributes to the onset of PPRM, leading to PTD. Extrapolation of results obtained with cultured term decidual cells to term decidua *in vivo* suggests that protection against PTD associated with progesterin administration²⁴ does not involve inhibition of decidual IL-8 expression once an active inflammatory process has been initiated. Thus, despite our previous demonstration that term decidual cells express functional progesterone receptors,^{41,42} the current study found that incubation with MPA did not significantly inhibit TNF- α - or IL-1 β -induced IL-8 expression in these cells.

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