Interleukin-8 Expression by Fetal and Neonatal Pulmonary Cells in Hyaline Membrane Disease and Amniotic Infection

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ABSTRACT

IL-8, a chemokine with striking neutrophil-activating properties, is important in the pathogenesis of various disorders of the adult lung. Little is known about its production and possible role in fetal and neonatal lung disorders. We therefore examined IL-8 expression by immunohistochemistry in lung tissue from neonates with hyaline membrane disease, from fetuses with amniotic infection, and from a fetal control group with noninflammatory diseases. In the majority of cases with hyaline membrane disease, intense IL-8 immunoreaction was seen in fetal and neonatal neutrophils and in almost half of these cases, in epithelial cells of the terminal airways as well as in the connective tissue cell compartment. In contrast, in the amniotic infection group, strong IL-8 immunostaining was almost exclusively seen in maternal aspirated neutrophils. Little or no IL-8 signal was seen in the control cases in all cell types examined. Also, no IL-8 production by fetal lung cells was detected in fetuses <18 wk of gestation.

The marked presence of IL-8 in all cell types of the lung in hyaline membrane disease cases indicates a role for IL-8 in the pathobiology of hyaline membrane disease possibly similar to that in adult respiratory distress syndrome. It further suggests that the cytokine network of the fetal lung is already well developed by the second trimester of pregnancy. (*Pediatr Res* **48: 299–303, 2000**)

Abbreviations
HMD, hyaline membrane disease
ARDS, adult respiratory distress syndrome
TNF-α, tumor necrosis factor-α
RDS, respiratory distress syndrome
LPS, lipopolysaccharide
BAL, bronchoalveolar lavage

IL-8 belongs to a family of structurally related proinflammatory cytokines designated as C-X-C-chemokines (1–3). It is produced by an array of normal cells, including monocytes (4), neutrophils (5), and cells of the human placenta (6). In the lung, alveolar macrophages (7), epithelial cells (8), fibroblasts (9), microvascular endothelium (10), and smooth muscle cells in arterial walls (11) are sources of IL-8.

During pregnancy, IL-8 is constitutively produced by human placenta, and its expression is enhanced in intrauterine infections (6). In various pulmonary disorders of children and adults, IL-8 seems to be critical for direct recruitment of neutrophils, for example, in ARDS (12, 13), asthma (14), idiopathic pulmonary fibrosis (15), and bacterial pneumonia (16). The migration of leukocytes to the pulmonary interstitium or air space in inflammatory processes is dependent on several cytokine mediators, including TNF-α, IL-1, and chemokines (1, 17). In lung tissue, IL-8 appears to be the major neutrophil chemotactic factor (18). The rapid and marked production of IL-8 is part of a cascade-like interaction between various cells of the alveolar-capillary wall. Alveolar macrophages can respond *in vitro* to initial stimuli (LPS, bacteria, immune complexes) by producing different proinflammatory cytokines such as IL-1, TNF-α, and IL-8 (7, 17). IL-1 and TNF-α then induce the release of additional IL-8 by alveolar macrophages, type II pneumocytes, fibroblasts, and endothelial cells. This cascade might lead to the brisk accumulation of IL-8 followed by the migration of neutrophils to the pulmonary interstitium or air spaces (17). In addition, neutrophils produce IL-8 in response to a variety of inflammatory stimuli, such as LPS, IL-1, and TNF-α, which suggests that neutrophils amplify their own recruitment in an autocrine loop (5). Neutrophils also produce IL-1 and TNF-α (19) and may thus in turn enhance the production of IL-8 by pulmonary epithelial and stromal cells.

Several previous reports suggest a key role for IL-8 in inflammatory reactions of fetuses and newborns. *In vitro*, fetal

mononuclear cells produce IL-8 in response to LPS in chorio-amnionitis (20), and neonatal pulmonary fibroblasts have been identified as a source of IL-8 (21). In *in vivo* cell cultures of BAL, exogenous anti-IL-1β antibody resulted in a marked inhibition of IL-8 production by lung inflammatory cells (22). *In vivo*, increased levels of IL-8 were found in BAL obtained from premature infants with chronic lung diseases (23). Furthermore, abundant expression of IL-8 mRNA in BAL was seen in newborns who either recovered from RDS or developed chronic lung disease (24).

To gain more insights into the role of IL-8 in fetal and neonatal pulmonary disorders, we have investigated the expression of IL-8 by immunohistochemical staining of postmortem lung specimens from newborns with HMD, fetuses with amniotic infections, and fetuses with noninflammatory conditions leading to premature delivery or termination of pregnancy.

METHODS

Tissue specimens. Lung tissue specimens obtained at autopsy of 72 fetuses and neonates were classified into three groups: HMD, amniotic infections, and controls. The postmortem examination was performed within a range of 4 to 120 h (mean, 32 h). The mean time interval between death and autopsy was not different among the three groups. The HMD group was defined by HMD, gestational age >25 wk, and postnatal survival. The amniotic infection group was defined by amniotic infection, gestational age ≤25 wk, and no postnatal survival. Amniotic infection, which usually occurs after premature rupture of membranes, was diagnosed by histologic examination of the placenta. The control group included lung tissue specimens from fetuses without amniotic infection or inflammatory conditions who had died or where pregnancy had been terminated (*e.g.* because of dysmorphic syndromes or chromosomal abnormalities). In this group, the gestational age was ≤25 wk. No age-matched controls are available for the HMD because at that age almost all infants die with signs of lung pathology.

Antibodies and reagents. Monoclonal mouse anti-human IL-8 antibody (clone 6217.11, IgG, neutralizing) was from R&D Systems Europe Ltd. (Abingdon, UK). The primary antibody was chosen after testing several antibodies for cross-reactions against other CXC/CC-chemokines (ELISA); no cross-reactions were detected (results not shown). Bridging antibody and alkaline phosphatase anti-alkaline phosphatase complexes (APAAP) complexes were from DAKO (Glostrup, Denmark). The antibody was tested for its reactivity for IL-8 on cytospin preparations of HL-60 cells known to produce IL-8 protein (25). Jurkat cells, which do not produce IL-8 (25), were used as negative control. Human recombinant IL-8 was from Sandoz AG (Vienna, Austria).

Immunohistochemical staining. Tissue specimens were fixed in 4% buffered formalin for 24 h and embedded in paraffin. Tissue sections (5 μm) were prepared on poly-L-lysine–treated slides, deparaffined with xylene, and rehydrated through isopropanol and graded concentrations of ethanol. For antigen unmasking, the slides were treated with microwave heat in 0.01 M citrate buffer for 10 min at medium power (26). Immunostaining was performed according to the APAAP method (27). Briefly, the slides were incubated with the MAb [~20 μg/mL in 0.05 M Tris-buffered saline (TBS)/0.5% bovine serum albumin] for 2 h, followed by a rabbit anti-mouse antibody (Z-259; DAKO) and the APAAP-complex (D-651; DAKO). After each incubation step, the slides were vigorously washed in TBS. To enhance the signal, incubation with the second antibody and the APAAP complexes were repeated once. The slides were developed with a mixture of naphthol AS-MX sodium salt, Fast Red TR, and levamisole (Sigma Chemical Co., St. Louis, MO, U.S.A.), all in veronal acetate buffer. After counterstaining with Mayer's hematoxylin, the slides were cover-slipped using an aqueous mounting medium. No specific signals were seen when the first antibody was omitted or by incubation with mouse control serum (Ig G_1, X-931; DAKO). As an additional control experiment, preabsorption with recombinant human IL-8 was performed, which inhibited IL-8-specific staining.

For immunohistochemical analysis of IL-8 production, tissue sections were divided into five cellular compartments: epithelium of bronchioli, epithelium of alveoli, fetal and neonatal neutrophils, maternal aspirated neutrophils, and connective tissue. These cells were defined morphologically. Cells exhibiting an intense signal were considered as IL-8 positive, and cells with no or very weak staining as negative for IL-8. The percentage of IL-8-positive bronchial and alveolar epithelial cells was assessed by counting 500 cells; the percentage of IL-8 expression in aspirated maternal neutrophils and neonatal and fetal neutrophils was determined by examining 100 cells. Maternal and fetal neutrophils were distinguished by morphology. Fetal neutrophilic granulocytes are predominantly located within the vasculature of the lungs, and their nuclei are oval or indented. In contrast, maternal neutrophils are seen within alveolar spaces and they display highly segmented nuclei. The specimens were classified into three groups according to the percentage of IL-8-positive cells: 51 to 100% positive cells (denoted as + + in Table 1); 11 to 50% positive cells (denoted as +); and 0 to 10% positive cells (denoted as −).

RESULTS

Patients' characteristics and diagnostic groups are shown in Table 2. Of the 72 fetal and neonatal autopsy lung specimens studied, 22 were classified as HMD, 30 as amniotic infection, and 20 were in the control group. The mean gestational age in the HMD group was 28.3 wk (range, 26 to 40 wk) as calculated from the first day of the last menstruation, and the mean postpartal survival was 2.2 d (range, 0.01 to 9 d). In the amniotic group, mean gestational age was 20.3 wk (range, 13 to 25 wk), and in the control group, the mean gestational age was 20.1 wk (range, 17 to 24 wk).

All lung specimens from fetuses with IL-8 immunopositive fetal cells had a gestational age >18 wk. Six patients of the amniotic infection group had a gestational age <18 wk, and the youngest fetus examined had a gestational age of 13 wk.

In HMD, IL-8 protein was seen within neonatal cells in 17 of the 22 patients examined (Table 1). Five specimens were

Table 1. *Expression of IL-8 by different cells in 72 fetal and neonatal lung autopsy specimens*

| | Cellular compartments (Number of cases with IL-8 positive cells)* | | | |
| | Fetal/neonatal cells | | | Maternal cells |
Diagnosis	Epithelium of bronchioli	Epithelium of alveoli	Neutrophils	Neutrophils
HMD (*n* = 22)	9 (4+/5++)	5 (0+/5++)	17 (4+/13++)	3 (0+/3++)
AI (*n* = 30)	2 (1+/1++)	2 (2+/0++)	10 (0+/10++)	23 (0+/23++)
Controls (*n* = 20)	2 (2+/0++)	All cases (−)	2 (0+/2++)	All cases (−)

Abbreviation: AI: amniotic infection.

(++) specimens with >50% IL-8-positive cells.

(+) specimens with 11–50% IL-8-positive cells.

(−) specimens with 0–10% IL-8-positive cells, which were considered as IL-8 negative.

Table 2. *Characteristics of patients (n = 72): gestional age and diagnostic groups*

Gestational age (wk)	HMD (*n* = 22)	Amniotic infection (AI) (*n* = 30)	Controls (*n* = 20)
13	–	1	–
14–16	–	2	–
17–19	–	10	8
20–22	–	6	9
23–25	–	11	3
26–28	16	–	–
29–31	3	–	–
32–34	1	–	–
35–37	1	–	–
38–40	1	–	–
Mean age (range)	28.3 (26–40)	20.3 (13–25)	20.1 (17–24)

negative for neonatal IL-8 expression. All 17 positive specimens showed marked IL-8 protein staining in neonatal neutrophils. In nine of these specimens, IL-8 expression was also noted in bronchial epithelial cells, and in five of those, both bronchial and alveolar epithelial were positive für IL-8. The latter five infants also showed IL-8 positivity in adjacent connective tissue cells, such as endothelial cells, fibroblasts, and smooth muscle cells of vessels as well as chondrocytes (Fig. 1, *A* and *B*).

The HMD specimens were also analyzed with regard to the presence of bacteria, pneumonia, and amniotic infection. Interestingly, bacteria were found only in the five specimens with marked IL-8 protein expression in all tissue compartments. Two of these infants also had pneumonia. No bacteria were found in the remaining patients. Signs of pneumonia were also found in three other specimens, which were positive for IL-8 in bronchial epithelial cells but negative in alveolar cells. Only five of 22 infants showed concomitant amniotic infection. In these patients, no association was seen between amniotic infection and IL-8 immunoreactivity.

The mothers of nine of the 22 HMD patients received cortisone treatment for induction of fetal lung maturation. No association was seen between levels of IL-8 protein and steroid therapy.

In contrast to HMD, only two of 30 specimens of the amniotic infection group showed IL-8 immunoreactivity in the epithelium of alveoli and bronchioli and in a few scattered connective tissue cells. However, in 23 of the 30 specimens, a strong IL-8 immunostaining was seen in up to 100% of abundantly present maternal neutrophils, which were located in alveoli and bronchioli (Fig. 1*C*). In a third of all specimens with amniotic infection, not only the aspirated maternal neutrophils but also the fetal neutrophils were positive for IL-8. No association between levels of IL-8 expression and factors for amniotic infection (*e.g.* increased granulopoiesis in the liver, occurrence of bronchus-associated lymphatic tissue in the lungs, maternal leukocyte count, C-reactive protein, time interval between premature rupture of membranes and birth) or treatment of mothers with steroids was found.

In the control group, notable IL-8 production was found only in two of 20 specimens in neutrophils, in epithelial cells of bronchioli but not of alveoli, and in connective tissue cells (Fig. 1*D*). No maternal neutrophils were seen.

DISCUSSION

We examined IL-8 production by immunohistochemical staining in normal and pathologic fetal and neonatal lung tissue. In patients with HMD and amniotic infections, IL-8 expression was observed in all cell types of the lung (epithelial cells, fetal, neonatal, and maternal neutrophils, connective tissue), although to various degrees and in different patterns. In contrast, no IL-8 signal was seen in the great majority of the control specimens.

Deficiency or dysfunction of surfactant leads to alveolar collapse, hypoxia, and acute lung injury, with severe epithelial and endothelial lesions in HMD of premature neonates as well as in ARDS (28). ARDS and HMD also show similar pathologic features, with damage of the alveolar-capillary wall, development of hyaline membranes, and alveolar collapse. This implies similar pathogenic mechanisms for the formation of hyaline membranes in adults and neonates (29). The role of IL-8 in ARDS is well established. Elevated IL-8 levels were found in bronchioalveolar fluid from ARDS patients (13); moreover, high IL-8 levels were associated with a poor prognosis in ARDS (12). Similarly, increased IL-8 production was detected in alveolar macrophages, neutrophils, and exfoliated epithelial cells in BAL from neonates suffering from RDS (24). Our findings of strong IL-8 immunostaining in all neonatal lung cell compartments in nine of 22 patients with severe HMD confirm and extend these previous studies by demonstrating that in cases with extensive HMD, not only inflammatory cells in the air spaces but also epithelial cells of the terminal
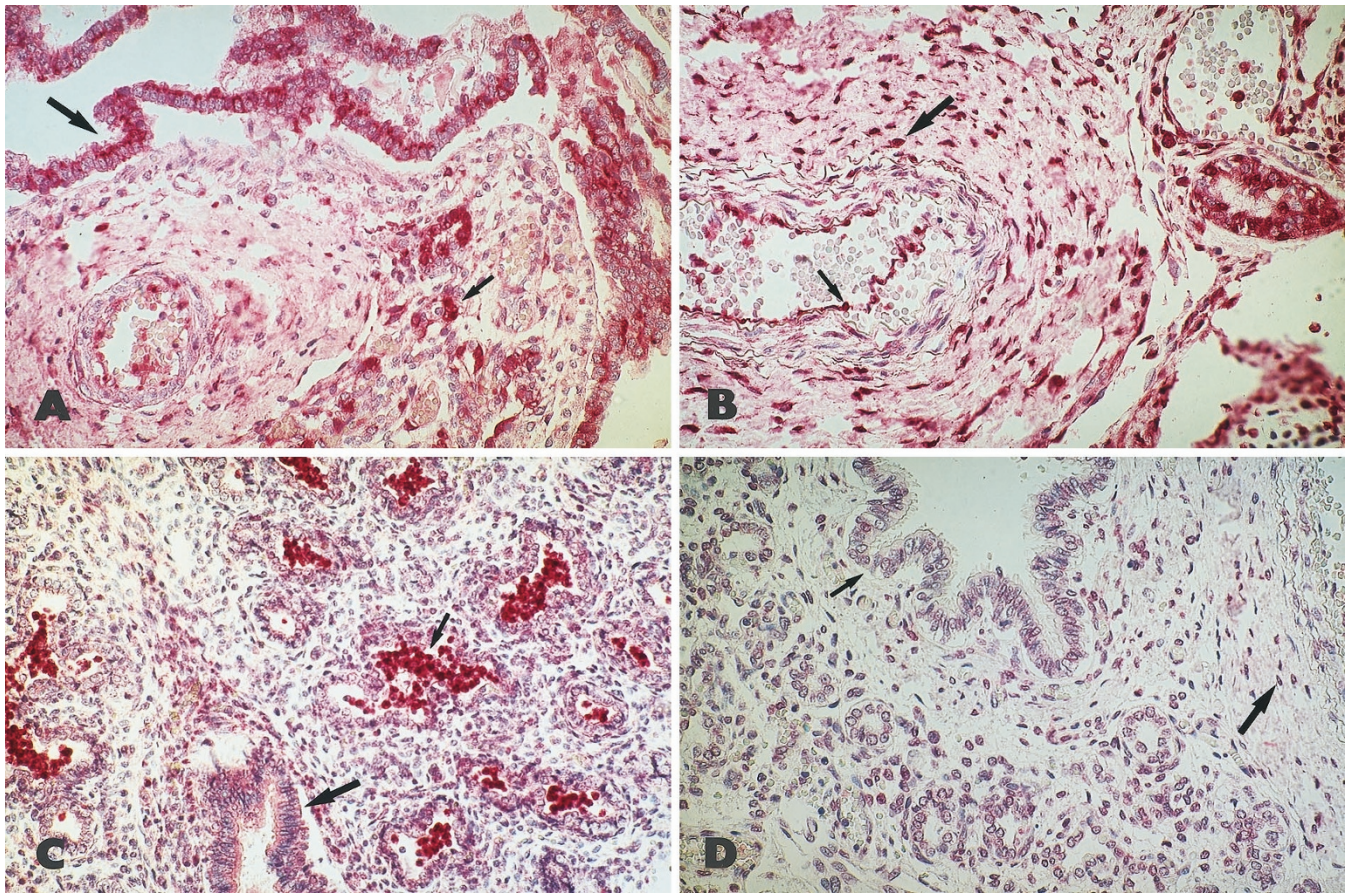
Figure 1. *A,* IL-8 protein in a neonatal lung with HMD (29 wk of gestational age). IL-8-positive alveolar cells (→) and immunoreactive bronchial cells (▲) are seen in the area of epithelial ulcerations (anti-IL-8, APAAP; ×200). *B,* IL-8 protein in a neonatal lung with HMD (27 wk of gestational age). Intense IL-8 signal is seen in the endothelium (→) and smooth muscle cells of the vessels (▲; anti-IL-8, APAAP; ×200). *C,* IL-8 protein in a fetal lung with aspiration of maternal neutrophils during amniotic infection (19 wk of gestational age). Numerous IL-8-positive aspirated neutrophils (→) are present, whereas the epithelial cells of the bronchioli and alveoli (▲) are negative (anti-IL-8, APAAP, ×200). *D,* IL-8 protein expression in a fetal lung from the control group (22 wk of gestational age). Epithelium of the bronchioli (→) and the alveoli as well as cells of the connective tissue (▲) are IL-8 negative (anti-IL-8, APAAP; ×200).

airways, as well as connective tissue cells, produce IL-8. Bacteria were found in blood vessels or in tissue in five of the 22 children of the HMD group. Interestingly, these five infants showed the strongest IL-8 expression in all tissue compartments examined. These findings suggest that IL-8 might play an important role in HMD, in particular in neonates suffering from RDS associated with sepsis. Also, in HMD associated with pneumonia, IL-8 positivity was not restricted to the areas with pneumonia, which indicates that neighboring tissue may also be damaged by increased IL-8 production.

In five patients with HMD, immunoreactivity for IL-8 was weak or absent in all cell types. This might be explained by the limited sensitivity of the method used, sampling errors, or limited postpartal survival. Our finding of widespread IL-8 production in all cellular compartments in neonatal lungs with severe HMD nevertheless suggests that IL-8 production might be part of a cascade-like interaction between different cells of the neonatal lung similar to the cytokine network operating in the adult lung. Early expression of the C-C chemokine macrophage inflammatory protein-1α was found to be associated with later development of fibrosis and a poor outcome in RDS (30). Our study implies that IL-8 might be one important factor in the development of HMD, and in agreement with previous reports (30, 31), our study confirms an inflammatory response in neonatal RDS beginning within the first day of life.

Amniotic infections often lead to inflammation of the fetal lung by aspiration of bacteria-containing amniotic fluid. Detection of neutrophils in aspirated material represents an early stage of fetal lung infection (32). In contrast to HMD, in fetuses with amniotic infection, strong IL-8 immunoreactivity was mostly confined to maternal aspirated neutrophils as well as fetal neutrophils. This finding is consistent with previous reports, which demonstrated high levels for IL-8, IL-1, and TNF-α in amniotic fluid from fetuses with amniotic infection (6, 33, 34). One might perhaps have expected an induction of IL-8 in fetal pulmonary epithelial and connective tissue cells. The near absence of IL-8 positivity in these cell types in our patients with amniotic infection may primarily be explained by their early gestational age. Indeed, fetuses with amniotic infection and a gestational age of ≥18 wk revealed IL-8 expression in their granulocytes. This is in agreement with the finding that mRNA for other proinflammatory cytokines, such as IL-1 and TNF-α, has been detected in fetuses >20 wk of gestation, suggesting maturity of the fetal cytokine response by the second trimester of pregnancy (35).

High IL-8 production in all cellular compartments of the neonatal lung in cases with HMD suggests that IL-8 might contribute to the development of HMD. In contrast, in amniotic infections the fetal lung seems to be mostly affected by maternal IL-8-releasing neutrophils.

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