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Profiles of inflammatory cytokines in bronchoalveolar lavage fluid from premature infants with respiratory distress disease

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In view of cytokine's effects in promoting or inhibiting inflammation, the objective of this study was to explore the characteristics of the proinflammatory cytokine, interleukin-8 (IL-8), and the inhibitory cytokine, interleukin-10 (IL-10), in the bronchoalveolar lavage (BAL) fluid of premature infants suffering from respiratory distress disease. Eighteen premature neonates with respiratory distress disease with gestational age (GA) ranging from 24 to 37 weeks were recruited for study. BAL fluids were collected following endotracheal intubation during an episode of hypoxemia or dyspnea. A series of BAL samples were obtained on day 1, 2, 4 and 7 after intubation for measuring IL-8 and IL-10 levels. The results indicate that premature infants with GA ranging from 24 to 32 weeks had a higher level of IL-8 ($p = 0.029$), but not level of IL-10 ($p = 0.109$), in the BAL obtained during the first intubation compared to premature infants with GA ranging from 33 to 37 weeks. The administration of exogenous surfactant did not influence the profiles of IL-8 and IL-10, as compared to those in-patients without treatment. Levels of IL-8 were correlated with IL-10 levels ($r = 0.613$, $p = 0.007$) in BAL fluid samples obtained on the day of intubation. The level of IL-8, but not IL-10, was significantly correlated with the duration of intubation. IL-8 and IL-10 levels in BAL fluid samples collected on the day of intubation were correlated with the development of chronic lung disease (CLD). The results suggest that extreme prematurity tends to have increased IL-8 and IL-10 levels in BAL fluid compared to premature infants with older GA, and that these increased levels are associated with the development of CLD.

Key words: Interleukin-8 (IL-8), interleukin-10 (IL-10), bronchoalveolar lavage (BAL), chronic lung disease (CLD)

There are a variety of mechanisms which contribute to the development of respiratory distress diseases in premature infants. Lung immaturity, hypoxemia, oxygen radicals, barotrauma from mechanical ventilation and infection have been suggested as major factors triggering neonatal lung injury and inflammation [1-3]. Recent progresses in neonatal intensive care and surfactant replacement therapy have enhanced the survival of premature infants suffering from respiratory distress syndrome (RDS). However, the mechanisms responsible for the development of subsequent complications such as chronic lung disease (CLD) and brain deficits remained to be determined [4,5]. Extremely premature neonates may be at a higher risk of lung injury due to the immaturity of their immune system, and the deficit of surfactant and antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) as compared to term infants [6-9]. Significant structural changes may occur in the developing lung, starting from 28 weeks through 40 weeks of gestational age (GA) [10]. Extrauterine existence during this period carries an increased risk of development of lung diseases for premature infants. Infants with CLD can be distinguished from those without CLD by an ongoing inflammatory process that can augment or perpetuate lung damage [11]. In premature infants, an influx of leukocytes into the lung can take place after giving oxygen and mechanical ventilation [11-14]. Among infants with RDS, a significant reduction in pulmonary neutrophils may occur in those who achieve complete recovery after the first week, as compared to those who progress to CLD [11-14,28]. Persistence of an abnormally large number of pulmonary neutrophils is a source of continuing lung damage, regardless of curtailed treatment with oxygen and positive airway pressure [11]. Activated neutrophils may contain or generate several species of oxygen radicals, proteases (e.g. elastase), phospholipases, and several inflammatory mediators, which can recruit and

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activate more neutrophils, creating a positive feedback loop [11,14]. As neutrophils play an important role in premature lung injuries, preventing or reducing neutrophil infiltration in premature lungs may be an optimal way to prevent CLD in premature infants. This study was conducted to explore the characteristics of the neutrophil chemotactic cytokine, IL-8, and the anti-inflammatory cytokine, IL-10, in bronchoalveolar lavage (BAL) fluid obtained from premature infants with different stages of RDS. IL-8 and IL-10 were selected for study because the former is a potent and specific neutrophil chemoattractant, and the latter is an anti-inflammatory cytokine that acts to reduce the expression and function of proinflammatory cytokine signals. Our hypothesis was that different profiles of IL-8 and IL-10 in the premature lungs might contribute to different disease progress and outcome.

Since the introduction of exogenous surfactant replacement therapy, the survival of preterm babies with RDS has improved. However, the developments of CLD and brain deficits have not been significantly affected by this therapy [4,5]. The present study also assessed levels of the neutrophil activating cytokine, IL-8, and the neutrophil suppressing cytokine, IL-10, before and after the initiation of surfactant replacement therapy in premature infants with respiratory distress disease. In addition, the kinetic change of IL-8 and IL-10 was also assessed in BAL fluid obtained from premature infants with respiratory distress disease with and without surfactant therapy.

Materials and Methods

Study population

Neonates who required intubation were recruited after obtaining written informed consent from the parents in our neonatal intensive care unit. All patients were intubated due to respiratory distress or tachypnea. They were ventilated with a time cycled, pressure-limited, continuous flow neonatal ventilator, which was set with the same protocol.

Bronchoalveolar lavage

Collection of BAL fluid was performed on the first, second, fourth and seventh days of intubation. The collection on day one was made after initial stabilization of the intubated infant and before initiation of surfactant therapy. Subsequent collections were performed at the time of clinically indicated tracheal suctioning. With the baby lying supine and head at the midline, the ventilator was disconnected. A 6- or 8-Fr suction catheter connected to a syringe that contained 1 mL/kg of saline solution was advanced through the end porthole of the endotracheal tube until resistance was felt [15,16]. The catheter was then withdrawn 1 cm and saline fluid was instilled. A small amount of air was instilled to clear the dead space, and lavage fluid was aspirated into a syringe. Three aliquots of the saline were instilled and the ventilator was reconnected after each aliquot withdrawal. Additional oxygen was given to maintain the oxygen saturation at 90% to 95% as measured by an oximeter. The heart rate and respiratory rate were monitored and allowed to stabilize during the suctioning procedure. The samples were sent to the laboratory immediately. Supernatants from BAL were separated by centrifugation (1,500 *g* for 3 min), aliquoted into 0.21 mL per Eppendorf tube after filtering the supernatant with a 0.45 μm filter, and stored at -70 °C until batch analysis.

IL-8 and IL-10 assays

The concentrations of IL-8 and IL-10 in the BAL samples were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis). The detecting sensitivity of the ELISA kits is in the picogram/mL range. Cytokine quantification was expressed as pg/mL of BAL fluid after equalizing the total protein content using protein detection kits (Bio-Rad, California).

Protein assay and normalization of IL-8 and IL-10 in BAL

Protein concentrations in the BAL fluid were quantitatively assayed with Protein Assay Dye (Bio-Rad, California). Since each lavage may have a different diluting effect on the concentration of BAL fluid contents, concentrations of IL-8 and IL-10 in the BAL fluid samples were all normalized to protein concentrations in the BAL fluid samples to correct for dilution during the sampling procedure [17].

Statistical analysis

Student's t test, paired t test and Spearman's correlation coefficient were used to analyze the significance of differences between groups. A p value of less than 0.05 was considered to be statistically significant.

Results

Demographic data of patients

Eighteen neonates born with a GA ranging from 24 to 37 weeks were recruited for this study; 12 of the 18 (group 1) were 24 to 32 (mean 28.67 ±0.67) weeks of GA, and the remainder six (group 2)

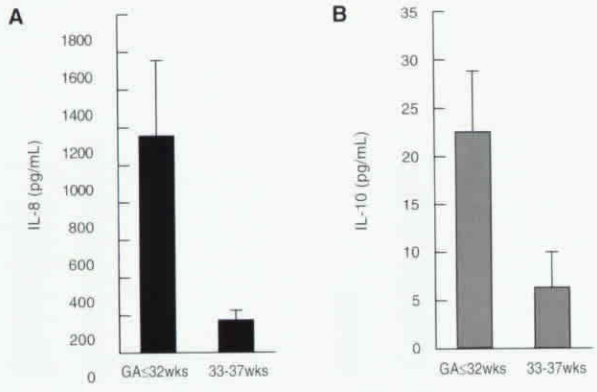

Fig. 1. Higher cytokine levels in the BAL fluid collected from extremely premature neonates. **A.**, Extremely premature neonates (GA < 32 weeks) had higher IL-8 levels ($p = 0.029$) in BAL fluid upon intubation than those with later GA (GA > 32 weeks). **B.**, No significant difference was found in IL-10 levels from the BAL fluid collected upon intubation between the two groups ($p = 0.109$).

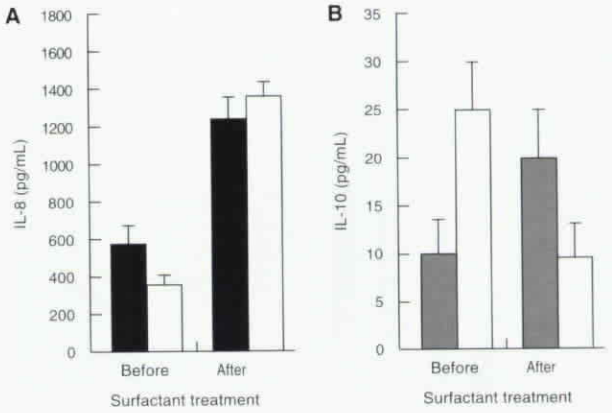

Fig. 2. Administration of surfactant did not suppress IL-8 levels (**A**) or IL-10 levels (**B**) in BAL fluid samples collected upon intubation, (■ and ■ : with treatment, □ : without treatment).

were 33 to 37 (mean 35.33 ±0.76). Birth body weight of the patients ranged from 730 g to 2,910 g (mean 1618.89 g ±672.95 g). The mean birth weight of group 1 was 1292.42 g ±131.26 g and of group 2 was 2271.83 g ±230.02 g. There were eight male infants in group 1 and three in group 2. Four out of the 12 in group 1 and three out of six in group 2 were born via Cesarian section delivery. RDS developed in 11 in group 1, and in none of the patients in group 2. Surfactant (Survanta, Ross Laboratories) replacement therapy was given in eight patients in group 1, and two in group 2. Intraventricular hemorrhage (IVH) and other brain deficits were noted in six patients in group 1, and one in group 2. CLD (seven in group 1) was defined as ventilator requirement or supplementary oxygen at the postconceptional age of 36 weeks and pulmonary changes on chest x-ray compatible with CLD. None of the patients in group 2 had a diagnosis of CLD. Three patients in group 1 and two in group 2 died during the study course.

During this study, all the patients tolerated the sequential BAL fluid samplings well and no adverse reaction was noted. There was no apparent changes in vital signs, oxygen saturation and ventilator requirements associated with the sampling procedure.

IL-8 and IL-10 levels in the BAL of premature infants with CLD

During the course of this study, sequential BAL samples collected from 18 intubated premature patients were evaluated for cytokine expression, with f ive patients having completed the whole course of BAL sampling. The initial samples from both groups were taken on the first day upon intubation, with the initial sample time ranging from 0.5 to 12 h after birth. The IL-8 levels in the first BAL samples in group 1 were significantly higher compared with group 2 (1158.58 ±394.14 pg/mL vs. 161.83 ±58.05 pg/mL, $p = 0.029$), as shown in figure 1A. The IL-10 levels (20.85 ±7.88 pg/mL vs. 6.44 ±2.93 pg/mL, $p = 0.109$) were not significantly different between the two groups, as shown in figure 1B. There were 10 prematures who received surfactant treatment. Administration of surfactant did not cause a significant decrease in IL-8 levels in the BAL fluid samples, $p = 0.196$ (Fig. 2A). Prematures who required surfactant treatment appeared to have lower initial IL-10 but higher IL-10 levels after therapy, although this result did not reach a significant level ($p > 0.05$) (Fig. 2B). Interestingly, an increase in IL-8 levels was noted with progression of intubation time (Fig. 3A). No progressive increase of IL-10 level was found with duration of intubation (Fig. 3B). In the first BAL fluid samples, levels of IL-8, but not IL-10, were significantly correlated with GA ($r = -0.538$, $p = 0.021$). The IL-8 levels were also significantly correlated with IL-10 levels ($r = 0.613$, $p = 0.007$) in the first BAL fluid samples. IL-8 levels in the first BAL fluid samples obtained from premature infants who subsequently developed CLD were significantly higher than that obtained from those in the non-CLD group ($p = 0.017$), as shown in figure 4A. Similarly, IL-10 levels in the first BAL were significantly higher in the CLD than that in the non-CLD group ($p = 0.026$), as shown in figure 4B. Persistent low levels of IL-10 were observed in the favorable outcome group. Exclusively high levels of IL-10 in the last BAL were detected in a patient who

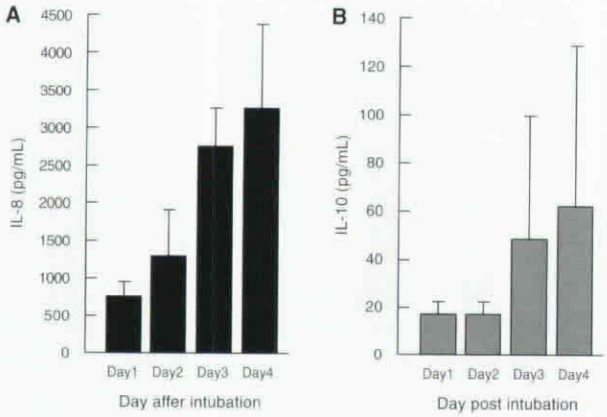

Fig. 3. Changes of cytokine levels in BAL fluid after intubation. **A.**, A significant increase in the IL-8 levels was correlated with the duration of intubation. **B.**, No significant change of IL-10 levels was found with the duration of intubation.

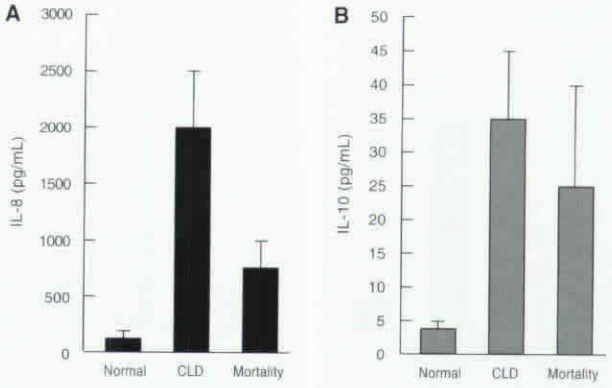

Fig. 4. Correlation of cytokine levels with outcomes. **A.**, A significantly higher IL-8 level ($p = 0.017$) in BAL fluid samples collected upon intubation was correlated with the development of CLD. **B.**, Significantly higher IL-10 level ($p = 0.026$) in BAL fluid samples collected upon intubation was correlated with the development of CLD.

died after 7 days of age. Correlation of IL-8 or IL-10 with brain deficit was not observed in this study.

Discussion

In this study, we found that the IL-8 levels in BAL fluid samples obtained on the first day of intubation were significantly higher in premature infants with GA ranging from 24 to 32 weeks than that in those with GA ranging from 33 to 37 weeks. This result is inconsistent with the findings of a study by Jones *et al* [18], who reported lower levels of IL-8 in premature infants. This difference in findings may have been due to the small number of patients in their study and because we divided premature infants into extremely premature (GA of 24 to 32 weeks) and premature (GA of 33 to 37 weeks) groups. Several studies have demonstrated early expression of the proinflammatory cytokines, TNFα, IL-1ß and IL-8 in cells obtained from BAL fluid in the lungs of premature infants with RDS [18-21]. In addition, previous studies have demonstrated an increase in IL-8 levels in BAL fluid samples of premature infants who subsequently developed bronchopulmonary dysplasia (BPD) [22]. In the present study, a significant correlation was found between the IL-8 levels in BAL samples obtained on the first day of intubation and subsequent development of CLD. This finding may suggest that recruitment of leukocytes by IL-8 is a major mechanism in the RDS of premature infants who subsequently develop CLD.

It is uncertain why significantly higher IL-8 levels in the first BAL were observed in the extremely premature infants in this study. It may have been that extreme prematures received higher concentrations of oxygen therapy resulting in more oxygen radical production, which is known to promote IL-8 expression [23]. In addition, neutrophils have been shown to generate oxygen free radicals [24] and with their depletion, reduced edematous lung injury caused by hyperoxia and oxygen free radical was noted [25]. Carvalho *et al* [26] showed that anti-interleukin-8 monoclonal antibody reduced free radical production in rabbits. If this mechanism is demonstrated to exist in humans, it may indicate the use of anti-IL-8 antibody to reduce free radical production in the lungs of premature infants with RDS in order to reduce lung injuries due to free radicals.

No significant difference was found in the IL-8 levels of BAL samples from prematures (GA ranged 24 to 37 weeks) with and without surfactant replacement therapy. This finding suggests that surfactant replacement therapy may improve oxygen exchange rather than prevent inflammation. Arnon *et al* [27] reported that surfactant therapy did not induce any change in the neutrophil profile during the first week of life. In addition, many studies have reported a significant reduction in pulmonary air leak after the institution of surfactant replacement therapy. Unfortunately, the major morbidities of CLD and brain deficits were not reduced significantly [5].

In the present study, levels of IL-8 in BAL fluid samples from premature infants revealed a significant increase with duration of intubation, as shown in figure 4A. This finding is consistent with results reported by Kwong *et al* [20] that IL-8 levels in BAL fluid were highest on the twelfth day of age and then declined

gradually to the lowest level at the age of 28 days. In addition, the study of Kotecha [19] revealed that the highest level of IL-8 was found in BAL fluid samples from the CLD group at the age of 10 days followed by gradual reduction thereafter till the age of 21 days.

Kwong *et al* [28] reported low levels of IL-10 in the sequential BAL samples from premature infants with RDS. Their *in vivo* and *in vitro* results demonstrated that lung inflammatory cells, predominantly of neutrophils and macrophages, did not produce IL-10 mRNA in the lungs of infants with RDS during the first month of life. However, these cells, when cultured *in vitro*, were capable of a response to rIL-10, in which down-regulation of IL-1ß and IL-8 were noted. Studies have shown that lung inflammatory cells from premature infants are capable of activating proinflammatory mediators, TNFα, IL-1ß and IL-8. These cells exhibited a reduced or absent ability to produce the counter-inflammatory cytokine, IL-10. Based on these observations, it was hypothesized that the ongoing lung inflammation resulted in lung tissue injury. Thus, abrogation of the pathogenesis of CLD may be possible through gene and peptide analog therapy [28]. In this study, IL-10 production in BAL fluid samples of premature infants obtained upon intubation showed no significant difference between the extremely premature and premature groups. The IL-10 levels were absent or reduced in the favorable outcome group, as compared to the CLD and mortality group. However, IL-10 levels in the BAL fluid samples obtained upon intubation were significantly higher in the CLD group than those obtained from in the non-CLD group. Additionally, a correlation of IL-8 levels with IL-10 levels in BAL samples obtained upon intubation was also observed in our study. These observations indicate that premature lungs are not only capable of producing IL-10 but also that there is an interaction between IL-8 and IL-10 production.

In conclusion, predisposition to the development of CLD in the present study was not associated with a reduction or absence of expression of IL-10. Further studies are needed to clarify the mechanism of CLD development. In addition, further studies on whether anti-IL-8 antibody or IL-8 antagonist might decrease premature lung injuries are needed.

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