Histologic Chorioamnionitis, Amniotic Fluid Interleukin 6, Krebs von den Lungen 6, and Transforming Growth Factor β_1 for the Development of Neonatal Bronchopulmonary Dysplasia

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ABSTRACT

BACKGROUND: Chorioamnionitis (CAM) is an important risk factor for the development of bronchopulmonary dysplasia (BPD) in preterm infants.

OBJECTIVES: To evaluate the effects of CAM on the development of BPD using interleukin 6 (IL-6), Krebs von den Lungen 6 (KL-6), and transforming growth factor β_1 (TGF-β_1) in the amniotic fluid as markers for inflammation, lung injury, and fibrosis/remodeling, respectively.

METHODS: Amniotic fluid concentrations of IL-6, KL-6, and TGF-β_1 were measured with enzyme-linked immunosorbent assay or electro-chemiluminescence immunoassay.

RESULTS: Of the 36 preterm infants, 18 were exposed to histologically confirmed CAM. Of these, 12 were later diagnosed as having BPD. The IL-6, KL-6, and TGF-β_1 levels in the amniotic fluid significantly increased with increasing histologic severity of CAM. Moreover, these markers were higher in the BPD group with histologic CAM than those without.

CONCLUSIONS: Our study suggests that CAM is likely to induce inflammatory, injury, and remodeling processes in the fetal lung.

KEYWORDS: HCAM, IL-6, KL-6, TGF-β_1, BPD

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Introduction

Although neonatal respiratory management has made great progress, bronchopulmonary dysplasia (BPD) remains the most common serious pulmonary morbidity in premature infants. Infants with BPD continue to have significant impairment and deterioration in lung function into late adolescence.[1,2] In addition, recent data have shown that BPD affects not only pulmonary outcomes but also neurodevelopmental outcomes.[3,4] Understanding the pathophysiology and optimal management of BPD is essential for intact survival of preterm infants.

The term *BPD* was first described by Northway et al in 1967 as a lung injury from oxygen therapy and mechanical ventilation in preterm infants with respiratory distress syndrome (RDS) showing radiographic evidence of abnormal chest at 36 weeks postmenstrual age.[5] Since then, the definition and pathology of BPD have evolved over several decades of discussion.[6,7] Unlike the original BPD reported by Northway et al, the new form often develops in preterm infants without RDS who may have required little or no ventilator support. Histologically, the lungs of these infants show more diffuse patterns and poor lung alveolar and vascular development.[7-10]

Bronchopulmonary dysplasia is often associated with a variety of underlying factors, such as lung immaturity, RDS, barotrauma, volutrauma, oxygen toxicity, sepsis, inflammation, patent ductus arteriosus, and intrauterine infection such as chorioamnionitis (CAM).[7] Chorioamnionitis is one of the most important factors associated with disturbance in normal lung maturation and growth.[11] Relationships between intrauterine infection and BPD have been researched by measuring inflammatory/proinflammatory cytokines and by culture or polymerase chain reaction to detect pathogens in the serum, cord blood, bronchoalveolar lavage fluid, urine, and amniotic fluid.[12] Watterberg et al[13] reported that the incidence of chronic lung disease was higher in infants born to mothers with CAM. Some studies have shown an increased risk of BPD in infants exposed to CAM, whereas others have reported conflicting results.[14-16] Thus, the association between BPD and CAM is yet to be understood.

Krebs von den Lungen 6 (KL-6) is an extracellular sialylated sugar chain antigen that exists on a type of mucin called MUC1, a transmembrane glycoprotein expressed on the surface of epithelial cells. It is a specific marker of interstitial lung diseases, and its level is significantly elevated in active diseases. In humans, KL-6 is found in type II pneumocytes, respiratory bronchiolar epithelial cells, and bronchial gland cells. Krebs von den Lungen 6 is shed into the alveolar lining fluid in a small quantity in the normal lung; in interstitial lung disease, however, shedding increases due to hyperplasia of type II pneumocytes. Therefore, KL-6 is the most sensitive and specific marker of pulmonary interstitial injury.

Transforming growth factor β_1 (TGF-β_1) is a type of cytokine produced in lung epithelial cells and vascular endothelial cells. In the wound healing process, TGF-β_1 promotes synthesis and deposition of extracellular matrices to facilitate wound repair. In the normal tissues, the production of TGF-β_1 is transient, but repeated injury to the lung results in overexpression. Thus, it has been used as a marker of fibrosis and remodeling.

The purpose of this study was to evaluate the effects of CAM on the development of BPD. To this end, we measured interleukin 6 (IL-6) as an inflammatory cytokine, KL-6 as a marker of lung injury, and TGF-β_1 as a marker of fibrosis and remodeling in the amniotic fluid and compared them among infants with or without CAM, funisitis, and/or BPD.

Methods

Written informed consent to participate was obtained from the parents. The protocol was in accordance with Declaration of Helsinki and approved by the institutional committee on human research. Among the infants admitted to the neonatal intensive care units of Osaka City General Hospital between January 2006 and August 2013, preterm infants who were born at or before 32 weeks of gestation and from whose mothers amniotic fluid samples were obtained during cesarean delivery were enrolled. The reasons for cesarean delivery were fetal indications (nonreassuring fetal heart rate, breech presentation; n = 20), maternal-fetal indications (placental abruption, placenta previa; n = 5), and maternal indications (preeclampsia; n = 11). Infants with major congenital malformations (eg, congenital heart disease, multiple malformations, and chromosomal anomaly) and infants who died within 28 days of birth were excluded. Demographic and clinical characteristics were retrospectively evaluated from medical records. Bronchopulmonary dysplasia was defined as an abnormal chest radiograph and requirement of oxygen therapy at 28 days of age.

Amniotic fluid samples were obtained from the mothers during cesarean delivery by amniocentesis and centrifuged at 18 000*g* for 10 minutes at 4°C, and the supernatants were frozen and stored at −80°C until analysis. Interleukin 6 concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Chemicon, Temecula, CA, USA). Krebs von den Lungen 6 concentrations were determined using an electrochemiluminescence immunoassay kit (EIDIA, Tokyo, Japan). Transforming growth factor β_1 concentrations were assessed by an ELISA kit (R&D Systems, Minneapolis, MN, USA).

Placenta samples were obtained from the mothers during cesarean delivery. Histologic chorioamnionitis (HCAM) was defined as the presence of polymorphonuclear leukocytes in the fetal membranes. Histologic chorioamnionitis was classified into 3 stages using the Blanc classification.[17] Briefly, stages 1, 2, and 3 were defined as subchorionic, chorionic, and full-thickness inflammation of both chorion and amnion, respectively. Funisitis was diagnosed based on the presence of neutrophil infiltration in the umbilical vessel walls.

Statistical analyses were performed using SPSS version 22.0. The concentrations of IL-6, KL-6, and TGF-β_1 were compared among infants with or without CAM, funisitis, and/or BPD by the Kruskal-Wallis test. The Dunn test was used for subgroup analyses. Differences between 2 groups were assessed by the Mann-Whitney *U* test. The Pearson test was used for correlation analysis. In all analyses, $P < .05$ was considered statistically significant.

Results

In total, 36 infants were included. Patients' clinical characteristics are summarized in Table 1. Of those, 18 (50%) were born to mothers with HCAM (6 in stage 1, 3 in stage 2, and 9 in stage 3). Six of those 18 infants also experienced funisitis. Of all infants, 26 (72%) developed BPD. Of these, 12 (33%) had mothers diagnosed as having HCAM. Four infants (11%) were negative for both HCAM and BPD. The median amniotic concentrations (range) of IL-6, KL-6, and TGF-β_1 were 0.854 (0.0025-68) ng/mL, 298 (72-3350) U/mL, and 119.1 (12.9-1924) pg/mL, respectively. None of these concentrations correlated with gestational age (IL-6: $r = -.093$, $P = .595$; KL-6: $r = -.232$, $P = .173$; TGF-β_1: $r = -.222$, $P = .201$) or birthweight (IL-6: $r = .063$, $P = .721$; KL-6: $r = -.105$, $P = .542$; TGF-β_1: $r = -.090$, $P = .606$).

Amniotic IL-6, KL-6, and TGF-β_1 levels increased with increasing histologic severity of CAM (Figure 1). In particular, the values in stage 3 HCAM were significantly higher than those without CAM (IL-6: $P < .005$; KL-6: $P = .001$, TGF-β_1: $P = .001$). Amniotic IL-6, KL-6, and TGF-β_1 levels were significantly higher with funisitis than without (IL-6: $P = .016$; KL-6: $P = .013$; TGF-β_1: $P = .009$) (Figure 2). In addition, IL-6, KL-6, and TGF-β_1 levels were significantly higher in the presence of both BPD and HCAM than in the presence of BPD without HCAM or the absence of both (Figure 3). None of these levels were associated with the need for oxygen therapy (IL-6: $P = .511$; KL-6: $P = .791$; TGF-β_1: $P = .884$) or abnormal chest radiographic findings at 28 days of age (IL-6: $P = .195$; KL-6: $P = .689$; TGF-β_1: $P = .637$).

Discussion

In this study, IL-6, KL-6, and TGF-β_1 concentrations in the amniotic fluid of mothers delivering preterm were measured to investigate the impact of CAM on the development of BPD. We found that IL-6, KL-6, and TGF-β_1 levels increased with the histologic severity of CAM and that infants who experienced CAM and later developed BPD had significantly higher IL-6, KL-6, and TGF-β_1 levels than those who developed BPD in the absence of exposure to CAM or those who did not experience either CAM or BPD. These findings indicate that CAM is likely to initiate fetal lung inflammation, injury, and

Table 1. Clinical characteristics of patients (n = 36).

	NO CAM (N = 18)	CAM (N = 18)	*P* VALUE
Gestational age, wk	28 (24–31)	28 (22–32)	.894
Birth weight, g	770 (441–1708)	1047 (438–2092)	.146
Apgar score (1 min)	5 (1–8)	5 (2–9)	.247
Apgar score (5 min)	8 (4–9)	8 (5–9)	.891
Oxygen therapy, d	52 (8–118)	57 (4–230)	.987
Mechanical ventilation, d	10 (0–58)	3 (0–76)	.351
Antenatal steroid use, n	8/18 (44%)	7/18 (39%)	.892
Funisitis, n	0/18 (0%)	6/18 (33%)	.010
BPD (oxygen use at day 28), n	14/18 (78%)	12/18 (67%)	.527

Abbreviations: BPD, bronchopulmonary dysplasia; CAM, chorioamnionitis.
Values are median (range) or numbers.

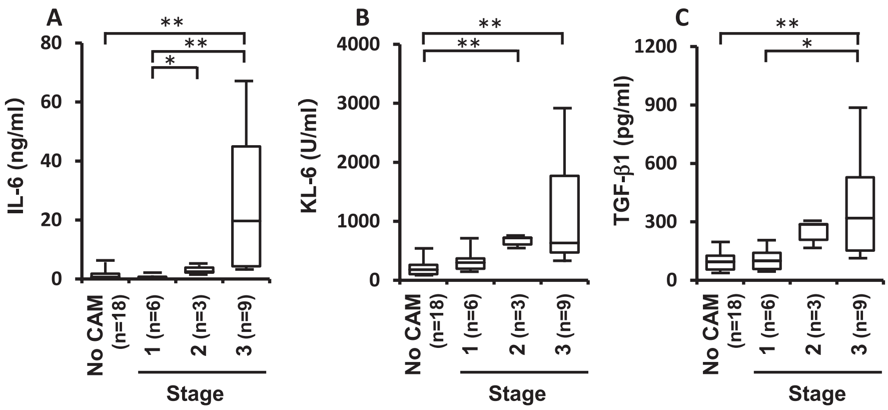

Figure 1. Relationships between IL-6, KL-6, and TGF-β_1 concentrations in amniotic fluid and histologic stage of CAM. (A) IL-6 (P = .000), (B) KL-6 (P = .002), and (C) TGF-β_1 (P = .004) concentrations in amniotic fluid were elevated with increasing severity of CAM. Subgroup comparison also showed significant difference. Especially each concentration of stage 3 CAM was significantly higher than that of no CAM. Box plots show the median values with interquartile range, and whisker plots show the 10th and 90th percentiles. *P < .05; **P < .005. CAM indicates chorioamnionitis; IL-6, interleukin 6; KL-6, Krebs von den Lungen 6; TGF-β_1, transforming growth factor β_1.

remodeling during pregnancy. And this may be the initial trigger resulting in neonatal BPD.

The association between CAM and BPD has frequently been investigated in clinical studies.[8,11,13,18-23] The development of BPD was first attributed to CAM in 1996 by Watterberg et al[13] based on the level of IL-1β in the bronchoalveolar lavage fluid measured within the first 24 hours of life. In the following year, Yoon et al[24] found that amniotic fluid concentrations of proinflammatory cytokines, IL-6, tumor necrosis factor α, IL-1β, and IL-8 were higher in infants who later developed BPD than in those who did not. Their findings indicate that fetal lung inflammation is already present before birth and that the management of BPD should begin in the uterine. The inflammatory effects of HCAM on fetal lungs and the increased risk of developing BPD in infants born to mothers with HCAM have also been described in many other studies.[11,25] Some other studies, however, reported that HCAM might promote lung maturation and reduce the subsequent development of BPD or found no association between HCAM and BPD.[26,27] A systematic review published in 2012[28] not only showed a significant association between CAM and BPD but also found strong evidence of publication bias. Thus, the association between CAM and BPD is controversial.

Fetal inflammatory response syndrome is a concept proposed to describe a fetal inflammatory condition characterized by hypercytokinemia and multiple organ dysfunctions as a result of fetal response to intrauterine inflammation.[29,30] Chorioamnionitis-associated inflammatory cytokines can reach the fetus and result in systemic inflammation involving the lung, intestine, nervous system, and other organs via cytokines and reactive oxygen species. It is well established that fetuses are exposed to inflammatory cytokines and have elevated concentrations of plasma cytokines during CAM.[30] However, the effects of CAM on the fetal lung and subsequent development of BPD have been less studied. In a sheep model, intrauterine injection of *Escherichia coli* endotoxin increases

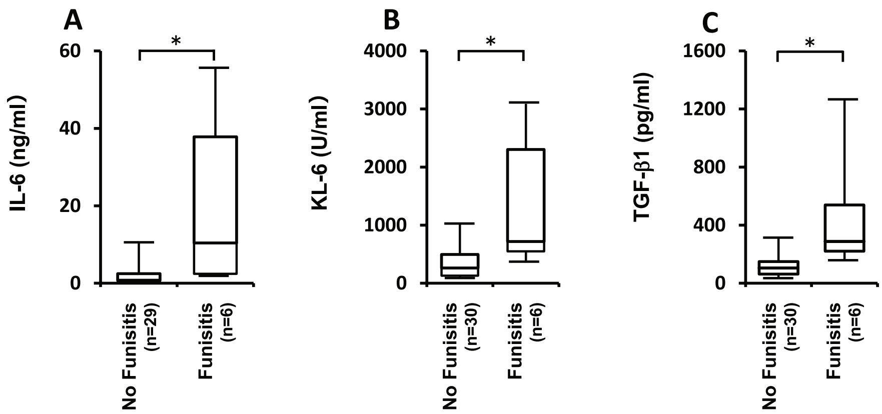

Figure 2. Relationships between IL-6, KL-6, and TGF-β_1 concentrations in amniotic fluid and the presence or absence of funisitis. (A) IL-6 (P = .016), (B) KL-6 (P = .013), and (C) TGF-β_1 (P = .009) concentrations in amniotic fluid were significantly higher in funisitis group than no funisitis group. Box plots show the median values with interquartile range, and whisker plots show the 10th and 90th percentiles. *P < .05; **P < .005. CAM indicates chorioamnionitis; IL-6, interleukin 6; KL-6, Krebs von den Lungen 6; TGF-β_1, transforming growth factor β_1.

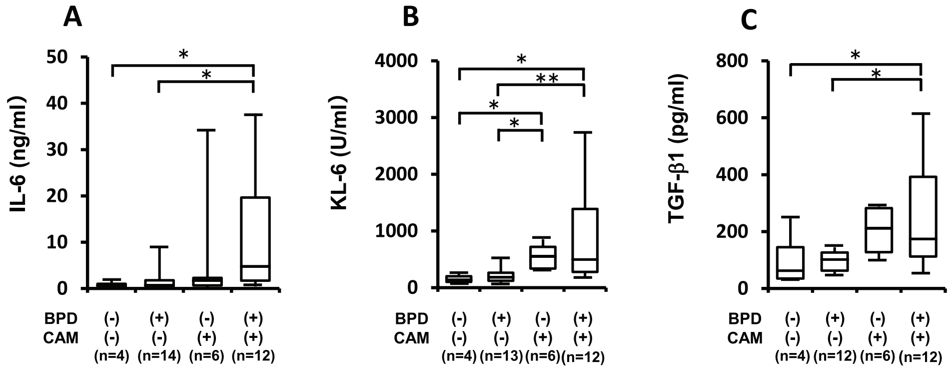

Figure 3. Relationships between IL-6, KL-6, and TGF-β_1 concentrations in amniotic fluid and the presence or absence of histologic CAM or BPD. Group comparison in amniotic fluid (A) IL-6 (P = .042), (B) KL-6 (P = .007), and (C) TGF-β_1 (P = .041) concentrations showed significant difference. Subgroup comparison also showed significant difference. Especially each concentration was significantly higher in BPD with HCAM than BPD without HCAM or the absence of both. Box plots show the median values with interquartile range, and whisker plots show the 10th and 90th percentiles. *P < .05; **P < .005.

surfactant protein and lung compliance while resulting in decreased alveolar number, decreased expression of vascular endothelial growth factor, and increased arteriolar smooth muscle thickness.[31] These results indicate that intrauterine infection and inflammation may not only promote fetal lung maturation but also cause alveolar and microvascular simplification. In our study, we used inflammation, injury, and fibrosis and remodeling markers to assess the effects of HCAM on the fetal and neonatal lungs.

Krebs von den Lungen 6 is a specific marker of pulmonary injury that has been demonstrated to be a good predictor of severe BPD.[32,33] Transforming growth factor β level is elevated in the bronchoalveolar lavage fluid of preterm infants who have developed BPD.[34] In vitro studies using fetal lung fibroblasts and alveolar epithelial cells exposed to tracheal effluents from premature infants have shown that TGF-β_1 may worsen BPD by inducing fibrosis,[35] whereas an increased amniotic TGF-β_1 level is a risk factor for the development of BPD.[36]

In this study, amniotic IL-6, KL-6, and TGF-β_1 levels were not correlated with gestational age or birthweight, indicating that these levels reflect the extent of fetal inflammatory exposure inside the uterus regardless of gestational age. The levels of these 3 biomarkers increased with increasing histologic severity of CAM and were significantly elevated with funisitis and in the presence of both CAM and BPD. These findings suggest that the fetal lung has undergone inflammation, injury, and remodeling processes before developing CAM-associated BPD. It should also be noted that, however, not all infants born to mothers with CAM developed BPD. Furthermore, amniotic IL-6, KL-6, and TGF-β_1 levels were not associated with the need for oxygen therapy or the severity of BPD at 36 weeks postmenstrual age. This is probably because various confounding factors, such as the severity of intrauterine infection, antenatal steroids, gestational age, postnatal ventilation management, pneumonia, and sepsis, are involved in the development of BPD.[25] In the most recent study, the incidences of RDS, BPD, intraventricular hemorrhage, and retinopathy of prematurity increased incrementally with increased stage of CAM, but these increases were found to be nonsignificant when adjusted for gestational age.[37]

The limitation of this study was the small number of subjects. Univariate analysis used in this study ignores several other important variables. However, the small sample number made it difficult to use multivariate regression model to include all confounders. Second, antenatal steroids data were excluded from statistical analysis due to the lack of detailed information. Steroids have been safely used in mothers with intrauterine infection and are expected to mitigate the effects of inflammation on the fetal lung.[27,38,39] In addition, the rate of BPD is fairly high. This is probably due to the very immature population of this study.

In conclusion, the results of this study suggest that fetuses exposed to CAM undergo lung inflammation, injury, and remodeling processes during intrauterine life. Chorioamnionitis is certainly one of the major risk factors, if not the only one, for the development of BPD. Monitoring amniotic IL-6, KL-6, and TGF-β_1 may help understand the pulmonary conditions of fetuses exposed to CAM and allow proper maternal health management, timely delivery, early and more accurate prediction and prevention of BPD, and optimal postnatal care.

Author Contributions

HM and HI designed the study and wrote the manuscript. HM, SO, and MS performed assays. HM, HI, SO, and MS collected and analyzed data. HS critically reviewed the manuscript and supervised the whole study process. All authors read and approved the final manuscript.

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