

# Transforming Growth Factor-Beta 1 in Humidifier Disinfectant-Associated Children's Interstitial Lung Disease

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**Summary.** Background: Humidifier disinfectant-associated children's interstitial lung disease has an unpredictable clinical course with a high morbidity and mortality. Objectives: To evaluate the differences in clinical findings between survivors and non-survivors of humidifier disinfectant-associated children's interstitial lung disease. To evaluate dynamic changes in serum cytokines related to inflammation and fibrosis in lung injury, and to determine whether these changes are predictive of survival in this disease. Methods: We evaluated 17 children with humidifier disinfectant-associated children's interstitial lung disease, from whom serum samples were obtained weekly during hospitalization. The severity of chest tomographic and lung pathologic findings was scored. Levels of several cytokines were measured in the serial serum samples. Results: Seven of the 17 children were survivors. Compared to survivors, non-survivors had greater ground-glass attenuation on follow-up chest tomography, higher admission neutrophil counts, and more macrophages on pathologic findings. Transforming growth factor-beta 1 persisted at an elevated level (1,000–1,500 pg/ml) in survivors, whereas it decreased abruptly in non-survivors. At the time of this decrease, non-survivors had clinical worsening of their respiratory failure. Transforming growth factor-beta 1 was positively correlated with PaO<sub>2</sub>/FiO<sub>2</sub> ( $r = 0.481$ ,  $P < 0.0001$ ). Conclusions: Non-survivors exhibited more inflammatory clinical findings than survivors. Transforming growth factor-beta 1 remained elevated in survivors, suggesting that it affected the clinical course of humidifier disinfectant-associated children's interstitial lung disease. The prognosis of this lung disease may depend more on controlling excessive inflammation and repairing damaged lung than on fibrosis, and transforming growth factor-beta 1 may play a key role in this process. **Pediatr Pulmonol.** 2016;51:173–182. © 2015 Wiley Periodicals, Inc.

**Key words:** lung disease; interstitial; child; prognosis; wound healing; transforming growth factor beta1.

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## INTRODUCTION

A severe interstitial lung disease (ILD) intractable to any treatment has affected Korea every spring since approximately 2006, and a similar ILD led to the death of several pregnant women in 2011.<sup>1-5</sup> Based on the results of an epidemiological study and an animal study, the Korea Centers for Disease Control and Prevention (KCDC) disclosed that disinfectants nebulized from humidifiers might be the cause of this lethal lung injury.<sup>5-7</sup> This disinfectant, which was composed of [2-(2-ethoxy) ethoxyethyl] guanidium chloride (PGH), polyhexamethyleneguanidine (PHMG), 5-chloro-2-methylisothiazol-3(2H)-one (CMIT)/ 2-methylisothiazol-3-one (MIT), and didecyldimethylammonium chloride (DDAC), was reported to cause fatal lung injury and has been prohibited for any use that may lead to inhalation.<sup>6-9</sup> Recently, a case-control study and a multicenter intervention study in Korea provided further evidence suggesting that the cause of this fatal ILD appeared to be the humidifier disinfectant.<sup>10,11</sup>

The American Thoracic Society and European Respiratory Society have recently updated guidelines for the diagnosis, classification, and management of ILD and children's interstitial lung disease (chILD).<sup>12-14</sup> However, ILD is still a very diverse disease group, with a high mortality and morbidity and an increasing prevalence.<sup>15</sup> There have been continuing efforts to determine reliable prognostic factors and effective treatments through increased understanding of the pathophysiology of ILD.<sup>16,17</sup> Several possible prognostic markers have been proposed, including interleukin 8 (IL-8), matrix metalloproteinase (MMP), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF- $\beta$ ), Krebs von den Lungen-6 (KL-6; a representative inflammatory and fibrotic cytokine), and monocyte chemoattractant protein 1 (MCP-1). However, no biomarker has been established as a standard for the management of patients with ILD.<sup>18-22</sup>

The current paradigm for understanding the pathophysiology of ILD involves chronic inflammation by repetitive injury to lung epithelial cells, followed by aberrant repair processes leading to excessive irreversible lung fibrosis.<sup>23</sup> This process may progress gradually or rapidly, and it may cease when lung damage is mild or progress to severe lung dysfunction.<sup>16</sup> Despite current medical advances, we are still unable to predict the exact progression and ultimate prognosis of this disease. Existing studies, which have generally focused on investigating clinical characteristics and cytokines at a single time, have been unable to determine factors that reliably predict the progress and prognosis of ILD.<sup>21</sup>

There have been many efforts to determine the clinical prognostic factors of the humidifier disinfectant-associated children's interstitial lung disease (HD-chILD) because this ILD progressed rapidly and was refractory to all treatment in non-survivors, whereas the clinical course in survivors tended to be highly favorable.<sup>1,11</sup> Disappointingly, no initial clinical characteristics, such as radiologic, pathologic, and laboratory findings, were significantly associated with survival.<sup>1,3,11</sup> The recently reported nationwide intervention study indicated that humidifier disinfectants could be the possible cause, but it mentioned the limitations caused by our lack of knowledge about the differences in individual susceptibility.<sup>11</sup>

In this study, we compared serial changes in pulmonary inflammatory and fibrotic cytokines between survivors and non-survivors and evaluated whether patterns of change in these cytokines could be useful prognostic markers. We also examined potential correlations between changes in useful prognostic cytokines and clinical indices, such as radiologic, pathologic, and laboratory findings.

## METHODS

### Subjects

A total of 17 children diagnosed with HD-chILD who were included in the patients reported in the previous nationwide report<sup>11</sup> were enrolled in this study. All children were admitted to Severance Children's Hospital from February 2010 to May 2011, and satisfied the diagnostic criteria reported previously.<sup>10,11</sup> Briefly, the criteria were as follows: (1) age less than 18 years at the time of diagnosis; (2) rapid progression to acute respiratory distress; and (3) chest tomographic (CT) findings of diffuse or centrilobular ground-glass opacities and/or air leak syndrome, such as pneumothorax, pneumomediastinum, or subcutaneous emphysema.<sup>10,11</sup> All children were checked to have been exposed to humidifier disinfectants before diagnosis by interviewing with their parents and confirmed with HD-chILD thorough the previous nationwide report.<sup>11</sup> Serum samples were collected weekly from hospital admission until discharge or death.

To determine clinical prognostic factors, we collected information regarding the patients' demographic data, laboratory findings, treatment, CT results, and pathologic findings. This study was approved by the Institution Review Board of Severance Hospital (Seoul, Korea, IRB No. 4-2013-0670).

## Radiologic Review

All children underwent thin-section CT scans of the chest. The CT images were reviewed and scored by two radiology specialists. Details regarding the methodology used for the radiologic review and scoring are provided in an online data supplement.

## Pathologic Review

The pathologic diagnosis was made by lung biopsy obtained during video-assisted thoracoscopic surgery in 12 patients. These biopsies were performed within 1 week after admission. To evaluate the grade of pathologic findings, two pathologists reviewed the specimens. Details of the methods used for the pathologic review and scoring are provided in an online data supplement.

## Analysis of Serum Cytokines

Serum levels of the following were measured by enzyme-linked immunosorbent assay, according to the manufacturer's (R&D Systems, Minneapolis, MN) instructions: IL-8; IL-13; monocyte chemoattractant protein-1 (MCP-1); MMP-9; periostin; TGF- $\beta$ 1; vascular endothelial growth factor (VEGF); regulated on activation, normal T cell expressed and secreted (RANTES) cytokine; and granulocyte macrophage-colony stimulating factor (GM-CSF).

## Statistical Analysis

Patient characteristics, laboratory data, CT results, pathologic findings, and treatments were compared between the survivor and non-survivor groups.

The study protocol indicated that serum samples would be collected weekly during the hospital stay; however, in patients whose condition was extremely unstable or those who underwent concurrent blood sampling for monitoring of their treatment, not all of the serum samples for this study were collected at exact weekly intervals. Since the clinical course and length of hospital stay varied widely from child to child, a substantial quantity of serum cytokine data was missing. To account for missing data and irregular intervals between serial serum cytokine collections, we used a generalized linear mixed-effect model for comparing the slope of changes in the serial serum cytokines among the survivors and non-survivors.<sup>24</sup> We evaluated the slope of changes as estimate values with standard errors. Also, we used the slope comparison test for comparing the trend of TGF- $\beta$ 1 and the trends of the other cytokines according to survival. The differences of the trend of TGF- $\beta$ 1 and the trends of the other cytokines were significant in  $P < 0.05$  and the more close to zero the value of estimates were, the more significant the relationship with the trend of TGF- $\beta$ 1 and the trends of the other cytokines were.

A  $P$ -value  $< 0.05$  was considered statistically significant. Statistical software (SAS version 9.2, SAS Institute Inc., Cary, NC) was used for all analyses.

## RESULTS

### Clinical Characteristics

The clinical characteristics of children in this study are shown in Table 1. The mean age of all patients was 2.6 years, and 12 children (70.6%) were male. Twelve children had a weight below the 5th percentile for the same age and sex. On admission, the most common symptoms were cough, tachypnea, and fever. The ratio of arterial oxygen partial pressure to inspired oxygen fraction ( $\text{PaO}_2/\text{FiO}_2$ ) at admission of non-survivors was higher than that of survivors, although the difference between groups was not statistically significant. The admission peripheral blood leukocyte count and absolute neutrophil count of non-survivors were higher than those of survivors ( $P = 0.025$  and  $P = 0.010$ , respectively). Only one child had a respiratory virus detected by nasopharyngeal aspirate, and no patient had bacteria detected in sputum or blood cultures.

During the hospital stay, all patients required supplemental oxygen, and all non-survivors received mechanical ventilation. No survivors received extracorporeal membrane oxygenation (ECMO). Steroid therapy was used for all patients. Hydroxychloroquine, cyclophosphamide, or both were administered to 14 of the 17 children.

### Radiological Findings

The initial and follow-up high resolution CT scores for ground-glass attenuation, septal thickening, bronchiectasis, and sum of these three findings are shown in Table 2. The change in ground-glass attenuation, septal thickening, and sum scores from initial to follow-up was greater in non-survivors than in survivors ( $P = 0.002$ ,  $P = 0.002$ , and  $P = 0.005$ , respectively). Whereas air leak syndrome on the initial HRCT did not differ between groups, this finding was more common on the follow-up HRCT in non-survivors than survivors ( $P = 0.021$ ).

### Pathological Findings

The pathologic characteristics were bronchiolar destruction with peribronchiolar inflammation or fibrosis, accompanied by a predominantly centrilobular destructive lesion or diffuse alveolar damage, which were generally consistent with previous reports.<sup>3,11</sup> Additionally, type II pneumocyte hyperplasia was found frequently in our patients.

The only pathologic finding that differed significantly between survivors and non-survivors was the score for interstitial and intra-alveolar foamy macrophages. The

**TABLE 1—Clinical Characteristics and Laboratory Findings at Admission of Patients with Humidifier Disinfectant-Associated Children's Interstitial Lung Disease**

	Total (n = 17)	Survivors (n = 7)	Non-survivors (n = 10)
Age, years	2.6 ± 1.5	2.3 ± 2.1	2.7 ± 1.0
Gender, male	12	5	7
Weight < 5 percentile for the same age and sex	12	4	8
Height < 5 percentile for the same age and sex	1	0	1
Symptoms and signs at admission			
Cough	15	5	10
Tachypnea or dyspnea	8	5	3
Sputum	1	0	1
Chest wall retraction	0	0	0
Fever	8	2	6
Cyanosis	2	2	0
Weight loss	2	0	2
PaO <sub>2</sub> /FiO <sub>2</sub> at admission	280 ± 153	258 ± 107	296 ± 182
Peripheral blood leukocyte count, cells/μL	12,780 ± 5,572	9,493 ± 4,523*	15,081 ± 5,221
Absolute lymphocyte count	3,266 ± 3,015	3,663 ± 4,323	2,989 ± 1,868
Absolute neutrophil count	8,337 ± 5,039	4,846 ± 1,467*	10,781 ± 5,253
ESR, mm/hr	16.0 ± 12.6	17.3 ± 13.3	17.3 ± 13.3
CRP, mg/dL	1.3 (0.0–4.6)	1.1 (0.0–4.5)	1.5 (0.0–5.6)
Respiratory virus from nasopharyngeal aspirate			
Rhinovirus	0	0	0
Parainfluenza	0	0	0
Respiratory syncytial virus	0	0	0
Influenza	1	0	1
Adenovirus	0	0	0
Coronavirus	0	0	0
Metapneumovirus	0	0	0
No detection	16	7	9
Bacteria from sputum culture			
Positive	0	0	0
Blood culture			
Positive	0	0	0
Treatment for respiratory insufficiency/failure			
Oxygen supplementation	17	7	10
Ventilator care	12	2*	10
Extracorporeal membrane oxygenation	5	0*	5
Medication for treatment			
Steroid monotherapy	1	1	0
Steroid + IVIG	2	0	2
Steroid + IVIG + HC	7	3	4
Steroid + IVIG + HC + CPM	7	3	4

ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; IVIG, intravenous immunoglobulin; HC, hydroxychloroquine; CPM, cyclophosphamide.

Data are expressed as mean ± standard deviation or median (interquartile range) or number (%).

\* $P < 0.05$  versus Non-survivors.

interstitial and intra-alveolar foamy macrophages score was higher in non-survivors ( $P = 0.026$ ) (Table 3). The scores for bronchiolar and peribronchiolar destruction, type II pneumocyte hyperplasia, intraalveolar fibrinous exudates, alveolar inflammatory cell infiltrate, and alveolar fibrosis were not different between survivors and non-survivors. The frequency of centrilobular destruction also did not differ between groups.

### Serial Changes in Cytokines

The serial changes in cytokines (IL-8, IL-13, MCP-1, MMP-9, periostin, TGF-β1, VEGF, RANTES, and GM-

CSF) are shown according to survival in Figure 1 and Appendix 1. The TGF-β1 values of survivors were maintained at approximately 1,000–1,500 pg/ml from the admission to discharge, whereas TGF-β1 values in most non-survivors eventually decreased below 500 pg/ml. The estimate of the slope of TGF-β1 in non-survivors was  $-16.4469$ , which was significant different from the slope of  $-1.1223$  found in survivors ( $P = 0.0257$ ) (Table 4).

No differences between survivors and non-survivors were noted for the trends of the serial values of other cytokines (Appendix 1). The slopes for the other cytokines also did not differ significantly between groups (Table 4).

**TABLE 2—High Resolution Computed Tomographic Findings in Patients With Humidifier Disinfectant-Associated Children’s Interstitial Lung Disease**

	Survivors (n = 7)	Non-survivors (n = 10)
<b>Initial HRCT Findings</b>		
Scoring		
Ground glass opacity	10.0 (10.0–12.0)	10.0 (7.5/10.0)
Septal thickening	10.0 (9.0–10.0)	10.0 (8.3–10.0)
Bronchiectasis	4.0 (1.0–6.0)	2.0 (0.0–6.0)
GGO + septal thickening + bronchiectasis	24.0 (20.0–26.0)	21.0 (18.0–24.3)
Yes or no		
Consolidation	6	7
Airleak	3	5
<b>Follow-up HRCT Findings</b>		
Survivors (n = 7)		
Non-survivors (n = 6)		
Scoring		
Ground glass opacity	10.0 (8.0–12.0)	12.0 (10.8–12.0)
Septal thickening	10.0 (8.0–12.0)	12.0 (10.8–12.0)
Bronchiectasis	4.0 (1.0–6.0)	9.5 (0.0–12.0)
GGO + septal thickening + bronchiectasis	24.0 (19.0–29.0)	31.5 (23.5–36.0)
Yes or no		
Consolidation	4	5
Airleak	2*	6
<b>Changes in scores</b>		
Ground glass opacity	0.0 (–2.0–0.0)*	3.5 (1.8–4.5)
Septal thickening	0.0 (0.0–0.0)*	3.0 (1.8–6.3)
Bronchiectasis	0.0 (–1.0–0.0)	5.5 (0.0–7.0)
GGO + septal thickening + bronchiectasis	–1.0 (–3.0–0.0)*	12.5 (9.5–14.5)

HRCT, high resolution computed tomography; GGO, ground glass opacity.

Data are expressed as median (interquartile range) or number.

\**P* < 0.05 versus Non-survivors.

Comparing the trend of TGF-β1 and the trends of the other cytokines according to survival by the slope comparison analysis, the slopes of IL-8, MCP-1, periostin, and VEGF showed the similar trends with the slope of TGF-β1 while GM-CSF showed the opposite trend with the slope of TGF-β1 in non-survival group. In survival group, only the slope of MMP-9 had the similar trend with the slope of TGF-β1 in Supplementary Table S1.

**The Clinical Course of Non-Survivors Related to TGF-β1**

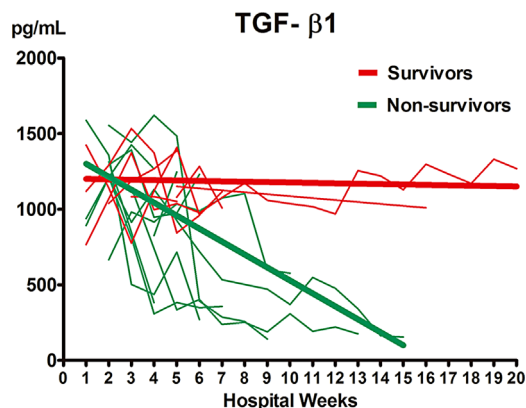
Since TGF-β1 in most non-survivors eventually fell below 500 pg/ml, we compared the serial changes in TGF-β1 with the clinical course in each non-survivor (Appendix 2). Except for three children, most non-survivors had a point at which the TGF-β1 abruptly

**TABLE 3—Pathologic Findings at Admission in Patients With Humidifier Disinfectant-Associated Children’s Interstitial Lung Disease**

	Survivors (n = 6)	Non-survivors (n = 6)
Scoring		
Bronchiolar and peribronchiolar destruction	0 = no 1.0 (0.0–2.0)	1.0 (1.0–2.0)
Type II pneumocyte hyperplasia	1 = focal 1.0 (1.0–1.3)	1.5 (1.0–2.0)
Intraalveolar fibrinous exudates	2 = diffuse 1.0 (0.8–1.3)	1.0 (0.8–1.0)
Alveolar inflammatory cell infiltrate	0 = no 2.0 (1.8–2.0)	1.5 (1.0–2.0)
Alveolar fibrosis	1 = mild 2.0 (0.8–2.0)	2.0 (1.0–2.0)
Foamy macrophages, interstitial and intraalveolar	2 = moderate 1.5 (1.0–2.0)* 3 = severe	2.5 (2.0–3.0)
Total	9.5 (5.8–10.3)	9.5 (7.0–11.3)
Yes or no		
Centrilobular destruction	2	2

Data are expressed as median (interquartile range) or number.

\**P* < 0.05 versus Non-survivors.



**Fig. 1. Serial changes in transforming growth factor beta 1 (TGF- $\beta$ 1) during hospitalization. TGF- $\beta$ 1 in survivors remained at approximately 1000 to 1500 pg/mL, whereas the TGF- $\beta$ 1 of most non-survivors eventually decreased below 500 pg/ml.**

decreased (shown as red lines in Appendix 2). Among the seven non-survivors with these abrupt decreases in TGF- $\beta$ 1, four were transferred to the intensive care unit (ICU) or managed with increasing the ventilator settings (e.g., by increasing the positive end-expiratory pressure [PEEP] or inspired oxygen fraction [ $\text{FiO}_2$ ]), and three other patients were initiated on ECMO at around the time that the TGF- $\beta$ 1 abruptly fell. In the three patients who did not demonstrate an abrupt decrease in TGF- $\beta$ 1 (shown as blue lines in Appendix 2), the last sampling was obtained prior to worsening of their respiratory failure, as exemplified by an increase in ventilator settings or initiation of ECMO.

As shown in Figure 2, the  $\text{PaO}_2/\text{FiO}_2$  ratios correlated well with TGF- $\beta$ 1 (pearson's correlation coefficient,  $r = 0.481$ ,  $P < 0.0001$ ). Since the values of  $\text{PaO}_2/\text{FiO}_2$  ratios and TGF- $\beta$ 1 were checked serially in all children, we adjusted the analysis for the serial values from each

patient using a generalized linear mixed model. In the adjusted model, the  $\text{PaO}_2/\text{FiO}_2$  ratios were not correlated with TGF- $\beta$ 1 (adjusted  $r = 0.375$ , adjusted  $P = 0.138$ ).

Since TGF- $\beta$ 1 has been reported to restore depleted VEGF secretion after acute lung injury,<sup>25</sup> we reviewed the serial changes in VEGF related to TGF- $\beta$ 1 in the seven non-survivors who showed the abrupt decrease in TGF- $\beta$ 1. As depicted in Figure 3, VEGF decreased just after or at the same time as the fall in TGF- $\beta$ 1 in these non-survivors.

## DISCUSSION

This study suggested that maintaining an elevated TGF- $\beta$ 1 predicted eventual improvement of excessive pulmonary inflammation and a favorable outcome in HD-chILD. By contrast, an abrupt decrease of the elevated TGF- $\beta$ 1 was associated with a decrease in VEGF and reflected worsening respiratory function. These findings suggest that TGF- $\beta$ 1 may play a key role in the repair of damaged lung and control of excessive inflammation in acute lung injury, rather functioning in promoting the progression of irreversible, fatal fibrosis.

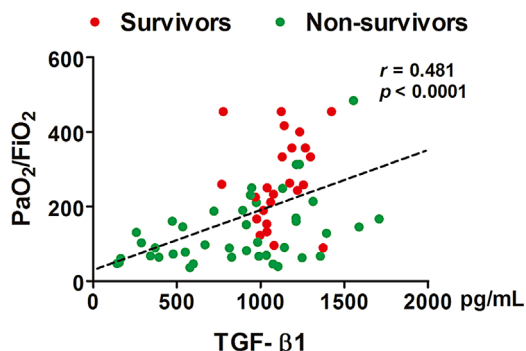
TGF- $\beta$ 1 is involved in lung development, inflammation, injury-related fibrosis, and repair.<sup>26</sup> In ILD, and especially idiopathic pulmonary fibrosis (IPF), TGF- $\beta$ 1 has usually been considered a poor prognostic factor, inducing pulmonary fibrosis through stimulation of fibrogenic cytokines and endothelial-mesenchymal transition. It has likewise been considered a potential therapeutic target.<sup>27</sup> Recently, TGF- $\beta$ 1 was reported to inhibit autophagosome activity, thus leading to pulmonary fibrosis in a lung injury model.<sup>28</sup> Conversely, TGF- $\beta$ 1 is also important in the development of the immature lung of neonates, and excessive TGF- $\beta$ 1 may induce bronchopulmonary dysplasia (BPD), in which it may inhibit alveolarization in extreme premature lung. The specific role of TGF- $\beta$ 1, therefore, appears to be tightly

**TABLE 4— Comparison of Dynamic Changes of Cytokines Between Survivors and Non-survivors by General Linear Mixed Model**

	Survivors (n = 7)		Non-survivors (n = 10)	
	Estimate	SE	Estimate	SE
Interleukin-8 (IL-8)	1.3397	1.2609	-0.3940	1.6046
Interleukin-13 (IL-13)	-0.2233	1.1915	-0.1763	1.1500
Monocyte chemoattractant protein-1 (MCP-1)	-1.6984	4.4156	8.0588	3.6864
Matrix metalloproteinase (MMP-9)	-4.1083	15.2734	-19.4410	11.7868
Periostin	-374.28	415.66	277.19	207.45
Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)*	-1.1223	4.5131	-16.4469	3.6996
Vascular endothelial growth factor (VEGF)	-1.6984	4.4156	8.0588	3.6864
Regulated on activation, normal T cell expressed and secreted (RANTES) cytokine	-4.3837	1.7465	-3.6605	1.3602
Granulocyte macrophage-colony stimulating factor (GM-CSF)	-1.0785	0.5048	-0.8951	0.3356

Data are expressed as estimate (standard error).

\* $P < 0.05$  Survivors versus Non-survivors.



**Fig. 2.** Correlation of transforming growth factor beta 1 (TGF- $\beta$ 1) with the ratio of arterial oxygen partial pressure and inspired oxygen fraction (PaO<sub>2</sub>/FiO<sub>2</sub>). PaO<sub>2</sub>/FiO<sub>2</sub> ratios were well correlated with TGF- $\beta$ 1 ( $r = 0.481$ ,  $P < 0.0001$ ). In the adjusted model considering the serial values in the same patient, the PaO<sub>2</sub>/FiO<sub>2</sub> ratios were not correlated with TGF- $\beta$ 1 (adjusted  $r = 0.375$ , adjusted  $P = 0.138$ ).

controlled, resulting in the appropriate amount of activity at the correct time and place.<sup>26</sup>

In an animal model of hyperoxic lung injury (a representative acute lung injury), TGF- $\beta$ 1 was reported to promote the repair of damaged lung and improve survival.<sup>25</sup> TGF- $\beta$ 1 also repaired damaged deoxyribonucleic acid and increased fibronectin secretion to accelerate closure of a monolayer of hyperoxic cells subjected to a scratch wound.<sup>25</sup> TGF- $\beta$ 1 has also been reported to play a key role in inhibiting excessive pulmonary inflammation.<sup>29,30</sup> In the HD-chILD of this study, the role of TGF- $\beta$ 1 seemed to be very complex in relating with repairing damaged lung, controlling severe inflammation, and promoting pulmonary fibrosis.

In non-survivors, the abrupt decrease in TGF- $\beta$ 1 correlated well with clinical deterioration in respiratory function, as exemplified by such events as increased ventilator settings (e.g., very high PEEP) or applying ECMO for support of impending severe respiratory failure. In addition, the PaO<sub>2</sub>/FiO<sub>2</sub>, which is the most widely recognized clinical index of lung injury, tended to correlate positively with TGF- $\beta$ 1.<sup>31</sup> However, this correlation was not significant statistically after adjustment for the several measurements in one subject. Considering the inevitably small number of subjects and irregular sampling times and intervals because of the fatal clinical course of this disease, it does not seem reasonable to consider only the strict statistical analysis results. A correlation between changes in TGF- $\beta$ 1 with the clinical severity of respiratory failure and lung oxygenation index may provide additional indirect evidence for the role of TGF- $\beta$ 1 in repairing damaged lung and controlling excessive inflammation.

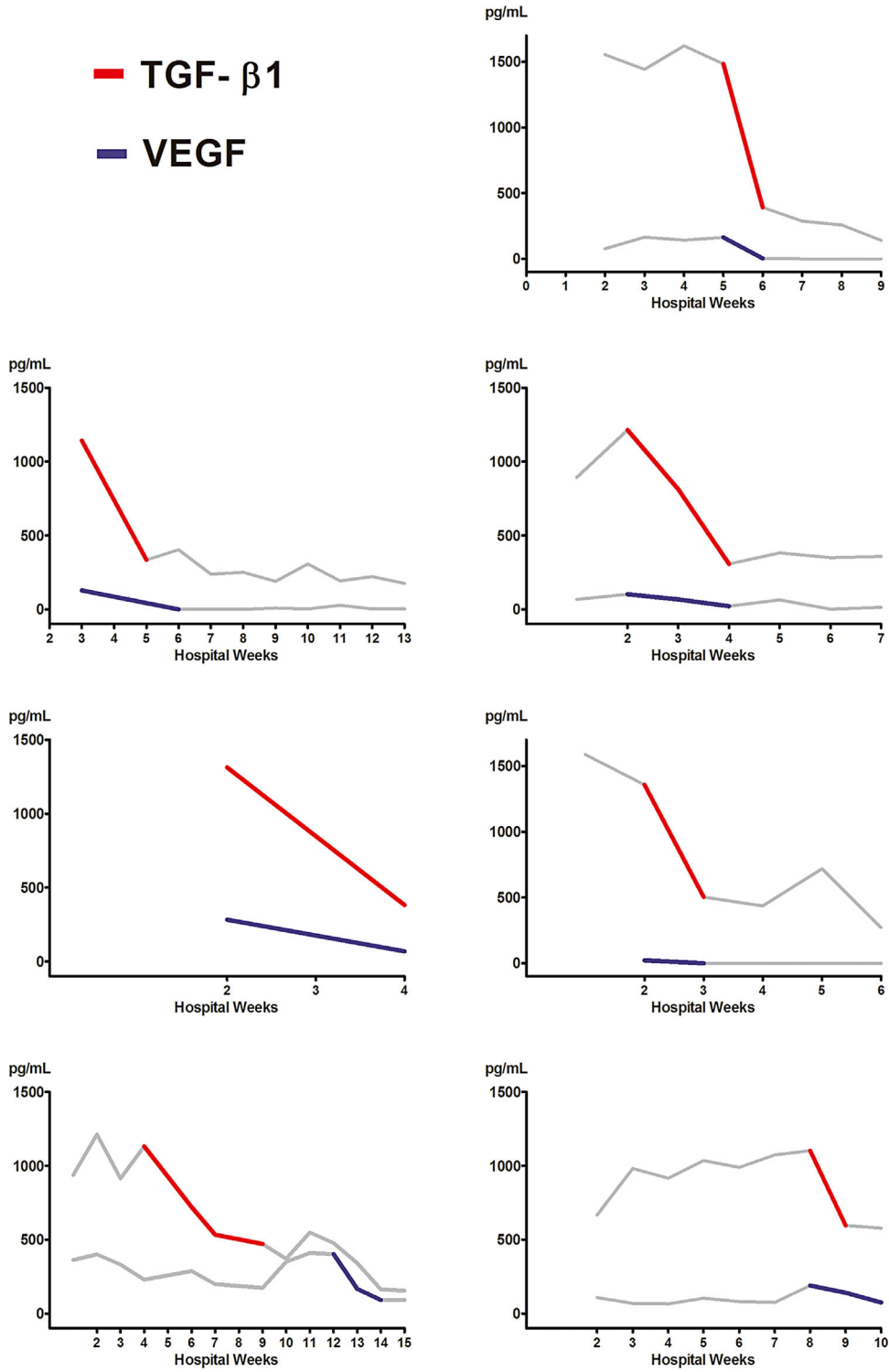
VEGF functions in promoting angiogenesis and increasing vascular permeability.<sup>32</sup> It has been identified as a reliable index in IPF, reflecting the degree of lung

fibrosis on radiologic imaging and the severity of the disease.<sup>19</sup> Paradoxically, VEGF has also been suggested as a possible therapeutic agent because of its role in stimulating growth and inhibiting apoptosis of alveolar epithelial cells.<sup>33</sup> In premature children, VEGF was reported to be elevated as a compensatory mechanism during severe respiratory distress proceeding to BPD.<sup>34</sup> VEGF, like TGF- $\beta$ 1, thereby exhibits paradoxical roles in lung injury, and in our patients, it seemed to function in repair of the damaged lungs.<sup>35</sup> Our study likewise provided evidence in humans supporting the results of an animal study in which TGF- $\beta$ 1 restored the depleted VEGF during repair of acute lung injury.<sup>25</sup>

IL-8 is a potent pro-inflammatory cytokine that is increased in patients with IPF.<sup>36</sup> IL-13 has been suggested as a possible therapeutic target, which was evaluated in a large-scale phase 2 study of anti IL-13 in IPF.<sup>37</sup> The inflammatory and fibrotic cytokines MCP-1 (CCL2) and RANTES were dramatically increased in a mouse model when DDAC, a component of humidifier disinfectants, was injected into the trachea and increased the susceptibility to infection by host immune modulation related to toll-like receptor 4 (TLR 4).<sup>7,9,22</sup> MMP-9, periostin, and GM-CSF have been reported to be important factors in pulmonary fibrosis.<sup>21,38,39</sup> These cytokines were increased in our patients with HD-chILD, but serial changes in these cytokines were not related to whether patients were survivors or non-survivors.

The recent nationwide intervention study of HD-chILD in Korea did not identify any useful clinical prognostic marker,<sup>11</sup> and clinical indices, such as radiologic, pathologic, and laboratory findings. In our current study, only small differences in clinical indices were noted between survivors and non-survivors. Non-survivors were more likely to have persistent air leak syndrome and more extensive ground-glass attenuation and septal thickening on follow-up CT, which showed a potential possibility of serial CT findings as a prognostic tool; a higher neutrophil count on laboratory tests; and a greater number of macrophages on pathologic findings. The ground-glass attenuation, increased neutrophil counts, and increased macrophages reflect inflammation more than fibrosis. The prognosis of most ILD is generally more closely associated with the severity of pulmonary fibrosis than the severity of inflammation because fibrosis is usually irreversible, whereas inflammation tends to be more reversible.<sup>15,16,21</sup> Considering all the clinical findings of our non-survivors, the prognosis of HD-chILD seemed to be more closely related to excessive inflammation than fibrosis. As well, the control of excessive inflammation seemed to be a key factor in determining survival in HD-chILD.

Children are more vulnerable to lung injury following exposure to toxic materials than adults, while at the same time, repair and regeneration of damaged lung may by



**Fig. 3.** Serial changes of transforming growth factor beta 1 (TGF-β1) and vascular endothelial growth factor (VEGF) during hospitalization in seven non-survivors. VEGF was decreased just after or at the same time as the decrease in TGF-β1 in all non-survivors with an abrupt fall in TGF-β1.



more prominent in children because alveolarization can persist up to puberty, although it is mostly complete by 18 months of age.<sup>40,41</sup> Since most children in this study were very young, with a mean age was 2.6 year old, it seems reasonable that excessive inflammation, followed by control of the inflammation and repair of the damaged lung, was more important than fibrosis in predicting their prognosis. Also, twelve children (70.6%) were an underweight in this study and it seemed to reflect on individual functional deterioration or increasing disease susceptibility by malnutrition.<sup>42</sup>

The strengths and limitations of this study might be considered. This study provides meaningful information about the role of TGF- $\beta$ 1 in controlling inflammation and repairing acute lung injury in humans, especially children. Furthermore, the subjects were young children, who are usually difficult to include in research studies. Our study is of particularly importance when one considers the increasing concerns in recent years about chemical terrorism. It is the only study of acute lung injury due to a toxic inhalant that provides information about the pathophysiology, related therapeutic considerations, and prognostic factors, as determined by analyzing serial changes in cytokines, instead of single data. The most important limitations are the very limited number of patients and the analyzing of the only blood samples without any lung tissue and exudates, but it was inevitable because the clinical course of HD-chILD was severe, aggressive, and unpredictable. The small number of subjects, plus the irregular sampling and substantial quantity of missing cytokine data, made the statistical analysis difficult, although a generalized mixed model called a spaghetti plot was used to help overcome this. Also, we could only rely on interviewing with the parents whether the humidifier disinfectant was exposed to the children. A further limitation was that this study could not define a clear cause and effect relationship; this should be re-evaluated in a well-designed animal model.

In conclusion, this study confirmed the possible role of TGF- $\beta$ 1 in the repair of damaged lung and control of vigorous inflammation, in acute lung injury associated with unexpected exposure to humidifier disinfectants in young children. It also suggested that such a repair mechanism could be more prominent in young children whose lungs are still actively growing and developing than adult. Further studies involving *in vitro* investigations and an *in vivo* animal model are required to directly confirm this mechanism.

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#### REFERENCES

- Cheon CK, Jin HS, Kang EK, Kim HB, Kim BJ, Yu J, Park SJ, Hong SJ, Park JD. Epidemic acute interstitial pneumonia in children occurred during the early 2000s. *Korean J Pediatr* 2008;51:383–390.
- Kim BJ, Kim HA, Song YH, Yu J, Kim S, Park SJ, Kim KW, Kim KE, Kim DS, Park JD, et al. Nationwide surveillance of acute interstitial pneumonia in Korea. *Korean J Pediatr* 2009;52:324–329.
- Lee E, Seo JH, Kim HY, Yu J, Jhang WK, Park SJ, Kwon JW, Kim BJ, Do KH, Cho YA, et al. Toxic inhalational injury-associated interstitial lung disease in children. *J Korean Med Sci* 2013;28:915–923.
- Lee E, Seo JH, Kim HY, Yu J, Song JW, Park YS, Jang SJ, Do KH, Kwon J, Park SW, et al. Two series of familial cases with unclassified interstitial pneumonia with fibrosis. *Allergy Asthma Immunol Res* 2012;4:240–244.
- Korea Centers for Disease Control and Prevention (KCDC). Interim report of epidemiological investigation on lung injury with unknown cause in Korea. *Public Health Weekly Report* 2011;4:817–825.
- Lee JH, Kim YH, Kwon JH. Fatal misuse of humidifier disinfectants in Korea: importance of screening risk assessment and implications for management of chemicals in consumer products. *Environ Sci Technol* 2012;46:2498–2500.
- Ohnuma A, Yoshida T, Tajima H, Fukuyama T, Hayashi K, Yamaguchi S, Ohtsuka R, Sasaki J, Fukumori J, Tomita M, et al. Didecyldimethylammonium chloride induces pulmonary inflammation and fibrosis in mice. *Exp Toxicol Pathol* 2010;62:643–651.
- Ohnishi H, Yokoyama A, Hamada H, Manabe S, Ito R, Watanabe A, Katayama H, Yasuhara Y, Ikezoe J, Higaki J. Humidifier lung: possible contribution of endotoxin-induced lung injury. *Intern Med* 2002;41:1179–1182.
- Ohnuma A, Yoshida T, Horiuchi H, Fukumori J, Tomita M, Kojima S, Takahashi N, Fukuyama T, Hayashi K, Yamaguchi S, et al. Altered pulmonary defense system in lung injury induced by didecyldimethylammonium chloride in mice. *Inhal Toxicol* 2011;23:476–485.
- Yang HJ, Kim HJ, Yu J, Lee E, Jung YH, Kim HY, Seo JH, Kwon GY, Park JH, Gwack J, et al. Inhalation toxicity of humidifier disinfectants as a risk factor of children's interstitial lung disease in Korea: a case-control study. *PLoS ONE* 2013;8:e64430.
- Kim KW, Ahn K, Yang HJ, Lee SY, Park JD, Kim WK, Kim JT, Kim HH, Rha YH, Park YM, et al. Humidifier disinfectant-associated children's interstitial lung disease. *Am J Respir Crit Care Med* 2013;189:48–56.
- Dishop MK. Paediatric interstitial lung disease: classification and definitions. *Paediatr Respir Rev* 2011;12:230–237.
- Kurland G, Detering RR, Hagood JS, Young LR, Brody AS, Castile RG, Dell S, Fan LL, Hamvas A, Hilman BC, et al. An official American thoracic society clinical practice guideline: classification, evaluation, and management of childhood interstitial lung disease in infancy. *Am J Respir Crit Care Med* 2013;188:376–394.
- Travis WD, Costabel U, Hansell DM, King TE, Jr., Lynch DA, Nicholson AG, Ryerson CJ, Ryu JH, Selman M, Wells AU, et al. An official American thoracic society/European respiratory society statement: update of the international multidisciplinary classification of the idiopathic interstitial pneumonias. *Am J Respir Crit Care Med* 2013;188:733–748.

15. Eves ND, Song Y, Piper A, Maher TM. Year in review 2012: acute lung injury, interstitial lung diseases, sleep and physiology. *Respirology* 2013;18:555–564.
16. Cottin V. Interstitial lung disease. *Eur Respir Rev* 2013;22:26–32.
17. Harari S, Caminati A. IPF: new insight on pathogenesis and treatment. *Allergy* 2010;65:537–553.
18. Richards TJ, Kaminski N, Baribaud F, Flavin S, Brodmerkel C, Horowitz D, Li K, Choi J, Vuga LJ, Lindell KO, et al. Peripheral blood proteins predict mortality in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2012;185:67–76.
19. Ando M, Miyazaki E, Ito T, Hiroshige S, Nureki SI, Ueno T, Takenaka R, Fukami T, Kumamoto T. Significance of serum vascular endothelial growth factor level in patients with idiopathic pulmonary fibrosis. *Lung* 2010;188:247–252.
20. Yokoyama A, Kohno N, Hamada H, Sakatani M, Ueda E, Kondo K, Hirasawa Y, Hiwada K. Circulating KL-6 predicts the outcome of rapidly progressive idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 1998;158:1680–1684.
21. Kelly M, Kolb M, Bonniaud P, Gauldie J. Re-evaluation of fibrogenic cytokines in lung fibrosis. *Curr Pharm Des* 2003;9:39–49.
22. Hartl D, Griese M, Nicolai T, Zissel G, Prell C, Reinhardt D, Schendel DJ, Krauss-Etschmann S. A role for MCP-1/CCR2 in interstitial lung disease in children. *Respir Res* 2005;6:93.
23. Homer RJ, Elias JA, Lee CG, Herzog E. Modern concepts on the role of inflammation in pulmonary fibrosis. *Arch Pathol Lab Med* 2011;135:780–788.
24. Cnaan A, Laird NM, Slasor P. Using the general linear mixed model to analyse unbalanced repeated measures and longitudinal data. *Stat Med* 1997;16:2349–2380.
25. Buckley S, Shi W, Barsky L, Warburton D. TGF-beta signaling promotes survival and repair in rat alveolar epithelial type 2 cells during recovery after hyperoxic injury. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L739–748.
26. Warburton D. Developmental responses to lung injury: repair or fibrosis. *Fibrogenesis Tissue Repair* 2012;5:S2.
27. Fernandez IE, Eickelberg O. The impact of TGF-beta on lung fibrosis: from targeting to biomarkers. *Proc Am Thorac Soc* 2012;9:111–116.
28. Patel AS, Lin L, Geyer A, Haspel JA, An CH, Cao J, Rosas IO, Morse D. Autophagy in idiopathic pulmonary fibrosis. *PLoS ONE* 2012;7:e41394.
29. Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, Pittet JF, Kaminski N, Garat C, Matthay MA, et al. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 1999;96:319–328.
30. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003;113:685–700.
31. Artigas A, Bernard GR, Carlet J, Dreyfuss D, Gattinoni L, Hudson L, Lamy M, Marini JJ, Matthay MA, Pinsky MR, et al. The American-European consensus conference on ARDS, part 2: Ventilatory, pharmacologic, supportive therapy, study design strategies, and issues related to recovery and remodeling. Acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1998;157:1332–1347.
32. Tzouveleki A, Anevlavis S, Bouros D. Angiogenesis in interstitial lung diseases: a pathogenetic hallmark or a bystander? *Respir Res* 2006;7:82.
33. Roberts JR, Perkins GD, Fujisawa T, Pettigrew KA, Gao F, Ahmed A, Thickett DR. Vascular endothelial growth factor promotes physical wound repair and is anti-apoptotic in primary distal lung epithelial and A549 cells. *Crit Care Med* 2007;35:2164–2170.
34. Lassus P, Turanlahti M, Heikkila P, Andersson LC, Nupponen I, Sarnesto A, Andersson S. Pulmonary vascular endothelial growth factor and Flt-1 in fetuses, in acute and chronic lung disease, and in persistent pulmonary hypertension of the newborn. *Am J Respir Crit Care Med* 2001;164:1981–1987.
35. Lindsay CD. Novel therapeutic strategies for acute lung injury induced by lung damaging agents: the potential role of growth factors as treatment options. *Hum Exp Toxicol* 2011;30:701–724.
36. Carre PC, Mortenson RL, King TE, Jr., Noble PW, Sable CL, Riches DW. Increased expression of the interleukin-8 gene by alveolar macrophages in idiopathic pulmonary fibrosis. A potential mechanism for the recruitment and activation of neutrophils in lung fibrosis. *J Clin Invest* 1991;88:1802–1810.
37. Baroke E, Gauldie J, Kolb M. New treatment and markers of prognosis for idiopathic pulmonary fibrosis: lessons learned from translational research. *Expert Rev Respir Med* 2013;7:465–478.
38. Dancer RC, Wood AM, Thickett DR. Metalloproteinases in idiopathic pulmonary fibrosis. *Eur Respir J* 2011;38:1461–1467.
39. Naik PK, Bozyk PD, Bentley JK, Popova AP, Birch CM, Wilke CA, Fry CD, White ES, Sisson TH, Tayob N, et al. Periostin promotes fibrosis and predicts progression in patients with idiopathic pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2012;303:L1046–1056.
40. Smith LS, Zimmerman JJ, Martin TR. Mechanisms of acute respiratory distress syndrome in children and adults: a review and suggestions for future research. *Pediatr Crit Care Med* 2013;14:631–643.
41. Wright RJ, Brunst KJ. Programming of respiratory health in childhood: influence of outdoor air pollution. *Curr Opin Pediatr* 2013;25:232–239.
42. Rodriguez L, Cervantes E, Ortiz R. Malnutrition and gastrointestinal and respiratory infections in children: a public health problem. *Int J Environ Res Public Health* 2011;8:1174–1205.

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