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Dynamic expression and effect of TGF-β1 on extracellular matrix in premature rats with CLD

Jian-Hua FU, Xin-Dong XUE

Department of Pediatrics, Second Affiliated Hospital, China Medical University, Shenyang 110004, *China*

Abstract: Objective To investigate the effect of transforming growth factor-β1 (TGF-β1) on the extracellular matrix (ECM) of lungs in premature infants with chronic lung disease (CLD). **Methods** Sixty premature rats were randomly assigned into a Model group of CLD and a Control group (n = 30 each). CLD was induced by hyperoxia exposure. The distribution and expression of TGF-β1 and levels of type I collagen, fibronectin (FN) and hyaluronic acid (HA) from lung specimens were assayed by the immunohistochemical method and enzyme-linked immunoabsorbent technique on days 1, 3, 7, 14 and 21 of the experiment. **Results** In the Control group there was a slight expression of TGF-β1 in bronchial epithelial cells and vascular endothelial cells. In the Model group, the TGF-β1 expression was similar to that of the Control group on day 1 and day 3, while on day 7, the TGF-β1 expression in alveolar macrophages, alveolar epithelial cells and interstitial cells was found. Expression intensity and range were increased with the time of hyperoxia-inducement and reached a peak on the 21st day. On days 1, 3, and 7, there were no differences in the type I collagen level between the two groups. On day 14, however, it was higher in the Model group than that of the Control group ($P < 0.05$). This difference was more significant on day 21 ($P < 0.01$). Levels of FN and HA in the Model group did not differ from those of the Control group at any time point of the experiment. The level of TGF-β1 expression was postitively correlated with the type I collagen level on day 14 ($r = 0.709$, $P < 0.01$) and day 21 ($r = 0.711$, $P < 0.01$). **Conclusions** Over-expression of TGF-β1 may play an important role in ECM remodeling in rats with hyperoxia-induced CLD.

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Key words: Transforming growth factor-β1 (TGF-β1); Extracellular matrix (ECM); Chronic lung disease; Premature rat

CLD 早产鼠肺组织内 TGF-β1 的动态表达及其对细胞外基质的影响

富建华，薛辛东 中国医科大学附属第二医院儿科，辽宁 沈阳 110004

[摘 要] 目的 近年来发现慢性肺疾病(CLD)早产儿支气管肺泡灌洗液中转化生长因子 β1 (TGF-β1)及 I 型胶原等细胞外基质(ECM)水平增高，但由于临床研究的局限性，缺乏与肺组织 ECM 的对照研究。因此探讨 TGF-β1 在早产鼠 CLD 发生、发展中的变化规律及对 ECM 的影响对完善早产儿 CLD 的发生机制有重要意义。**方法** 将 60 例早产鼠随机分为模型组和对照组，于实验后 1,3,7,14 和 21 d，应用免疫组织化学和酶联免疫吸附法，分别观察及测定肺组织 TGF-β1 分布、表达强度及 I 型胶原、纤维连接蛋白(FN)及透明质酸(HA)的含量。**结果** 正常肺组织 TGF-β1 仅在支气管上皮细胞或血管内皮细胞有微弱表达；而模型组 1 d 和 3 d 时，TGF-β1 表达同正常对照组，7 d 时少量肺泡巨噬细胞、肺泡上皮细胞及肺间质细胞开始表达，其表达强度高于对照组，差异有显著性($P < 0.05$)，14 d 时明显增强，差异有极显著性意义($P < 0.01$)，21 d 达高峰；1,3 和 7 d 时，两组肺组织 I 型胶原含量无差异($P > 0.05$)，而 14 d 时，模型组高于对照组，差异有显著性($P < 0.05$)，21 d 时明显高于对照组，差异有极显著性意义($P < 0.01$)；不同吸氧时间，两组肺组织 FN 及 HA 含量均差异无显著性意义($P > 0.05$)；模型组在 14 d 和 21 d 时，肺组织 TGF-β1 表达与 I 型胶原含量呈显著正相关(r 分别为 0.709,0.711，均 $P < 0.01$)。**结论** 暴露高氧环境中的 CLD 早产鼠，肺组织 TGF-β1 过度表达与 ECM 重塑中 I 型胶原沉积密切相关。

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[Biography] Jian-Hua FU(1966-), Female, Doctor of Medicine, Lecturer, Specializing in Neonatology.
[Correspondence Author] Xin-Dong XUE, Department of Pediatrics, Second Affiliated Hospital of China Medical University, 36 Sanhao Street, Shenyang, Liaoning 110004, China (Email:fujianhua@163.com).

[关 键 词] 转化生长因子 β1;细胞外基质;慢性肺疾病;早产鼠
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Transforming growth factor-β1 (TGF-β1) is a kind of cytokine with multiple biological effects. In recent years, it has been shown that excessive synthesization of TGF-β1 and the mediated extracellular matrix play a critical role in adult idiopathic pulmonary fibrosis and bleomycin-induced rat pulmonary fibrosis model. Chronic lung disease (CLD) is the most common complication accompanying the long duration hyperoxia treatment in premature infants. Clinical studies suggest that the levels of TGF-β1 and type I collagen increase in bronchial alveolus lavage fluid (BALF) of premature infants with CLD. However, these clinical studies lacks of the control to lung tissue ECM, only based on their biological characters, in inferring the correlation between the TGF-β1 and type 1 collagen levels and the occurrence of CLD. So to study the rule of TGF-β1 in the occurrence and development of CLD and the effect on the synthesis of ECM, premature rats with hyperoxia-induced CLD were used to investigate the dynamic expression and distribution of TGF-β1 in lung specimens and to measure the contents of type I collagen, fibronectin (FN), and hyaluronic acid (HA) respectively, which are the main components of lung ECM.

Materials and methods

Subjects and grouping

Forty adult female SD rats(provided by Department of Laboratory Animal, China Medical University), weighing 220-250 g (mean 231 g), were placed with healthy male SD rats (female: male = 4:1) at night. The next day, vagina incretion were collected for smear. The first day of gestation was defined when sperms were found under the microscope. On the 21st day of gestation, infant rats were taken out by cesarean section. These were defined as premature rats[1]. The surviving premature rats after resuscitation (heat preservation, oxygen treatment and touch stimulation) were sent to to be raised by fungible mother rats. The premature rats were randomly assigned into a Model group and a Control group, and there were 30 rats in each group.

Establishment of animal model

Model group: Immediately after birth, premature SD rats were laid in oxygen boxes with $FiO_2 > 0.90$ (detected by oxygen-recorder), concentration of $CO_2 < 0.5\%$ (CO_2 absorbed by Sodalime), temperature 25℃-27℃, and humidity 50%-70%. The boxes were kept open for 1 hour every day, water and feed were added to them, and the cushions were changed. The ability of the mother rats to raise the premature rats may be decreased because of oxygen-poisoning and so they were replaced with mother rats from the Control group which were placed in oxygen boxes with $FiO_2 = 0.21$ (air)[1].

Samples collection and test methods

On days 1, 3, 7, 14 and 21 of the experiment, 6 rats from each group were selected randomly, and sacrificed after anesthesia. 5 μm wax slices from left lung specimens fixed by 4% poly-formaldehyde were prepared by routine methods. Right lung specimens were conserved in −80℃ refrigerator for later use.

Detection of the TGF-β1 expression in lung tissues

The SABC immunohistochemical technique was used. After the specimens were dewaxed according to the routine method, 3% H_2O_2 was used to block peroxidase. Citrate buffer was used for microwave repair and goat serum blocking. Subsequently, rabbit anti-rat TGF-β1 antibody (working concentration 1:100), biotin-labeled sheep anti-rabbit IgG, and HRP-labeled streptavidin were added and were stained with DAB (The above-mentioned antibody and reagent were bought from Wuhan Boshide Biotenique Inc). The procedures followed for the two groups were similar except that in the Negative control group PBS was used instead of resistant rat TGF-β1 antibody. The cells with yellow-brown particle deposition in cytoplasm were judged to be positive ones.

Semi-quantitative analysis of the TGF-β1 expression

Five clearly dyed slices at each time point were taken randomly. Then 5 random sight-shots for every slice were selected under microscope (×400), and the windows' square was fixed. Mean gray value,

representing the intensity of positive products, was measured using Meta Morph and computer image process softwares. It was found that the lower the mean gray value, the more intensive was the expression of the positive product and the higher the content of protein.

Contents of type I collagen, FN, and HA

The enzyme-linked immunoabsorbent technique was used. After the remaining blood of the lung specimens had been washed up by a pre-cooled saline solution and after the water had been sucked out, 1 g wet lung specimen was removed and was cut into fragments. These fragments were then smashed by ultrasonic waves in an ice-box and were then made into 10% tissue homogenate. Supernatants were collected after low temperature centrifugalization. The contents of type I collagen, FN, and HA were detected following the instructions of Kits (provided by Shanghai Sengxiong Biotechnique Inc). The results were expressed by the contents of type I collagen, FN, and HA per mg lung specimen (ng/mg).

Statistical analysis

SPSS version 10.0 was used to perform statistical analysis, with all data expressed as $\bar{x} \pm s$. The Dunnet t-Test was used for inter-group comparison, and the Spearman Analysis was used for correlation analysis.

Results

Morphological changes of lung tissues

On day 1 of the experiment, it was observed in both the Model and Control groups that the alveolar structure was irregular, the alveolar cavity was rather small, and the alveolar septum was thick. On day 3, the alveolar structure of the two groups was more regular, the size of alveolus was equal, and alveolar septum was thinner. The only difference between the two groups was that a few inflammatory cells exuded out from alveolar cavity in the Model group. On day 7, the alveolar size of the Control group was equal, while the alveolar cavity of the Model group became large, and alveolar septum became thin. On day 14, the alveolar cavity of the Model group grew significantly large, the quantity of alveolar reduced, with a few alveolus fusion, and interstitial cells partly increased. On day 21, the normal alveolar structure of the Model group disappeared, the quantity of alveolus decreased, the diameter of alveolar cavities extended, large quantities of alveolus fused, alveolar septum became very thin, and pulmonary interstitial fibroblasts partly increased.

Distribution of TGF-β1 expression in lung tissues

In normal lung tissues, TGF-β1 was slightly expressed in bronchial epithelial cells or vascular endothelial cells (Figure 1); in the Model group of hyperoxia-inducement, TGF-β1 expression was similar to the Control group on day 1 and day 3 of the experiment, while on day 7, TGF-β1 was found in alveolar macrophages, alveolar epithelial cell and interstitial cells (Figure 2). Expression intensity was increased and expression range was expanded with the time of hyperoxia-inducement (Figure 3). On day 21, TGF-β1 could be easily found in alveolus macrophages, alveolar epithelial cells and interstitial cells (Figure 4).

Intensity of TGF-β1 expression in lung tissues

On days 1 and 3 of the experiment, there were no differences in the gray values of TGF-β1 of the lung tissues between the two groups. On day 7, the gray values of the Model group were lower than those of the Control group ($P < 0.05$); On days 14 and 21, these differences were more significant ($P < 0.01$). See Table 1.

Levels of type I collagen, FN and HA in lung tissues

On days 1, 3, 7, 14 and 21 of the experiment, there were no significant differences in FN and HA levels between the Control and Model groups. On days 1, 3 and 7, there were also no distinct differences in the type I collagen level. However, on day 14, it was found that the type I collagen level in the Model group was higher than that in the Control group ($P < 0.05$). On day 21, this difference was more significant ($P < 0.01$). (Figures 2, 3 and 4)

The correlation between TGF-β1 expression and type I collagen level in lung tissues

There was a remarkable positive correlation between TGF-β1 expression and type I collagen level in lung tissues on day 14 ($r = 0.709$, $P < 0.01$) and day 21 ($r = 0.711$, $P < 0.01$).

Table 1 TGF-β1 expression in the Model and Control groups ($n=6, \bar{x} \pm s$)

Group	1 d	3 d	7 d	14 d	21 d
Control	85.24±5.65	86.17±3.99	84.56±2.14	84.56±2.56	84.16±2.16
Model	86.82±6.80	85.92±5.53	80.89±1.36[a]	73.83±5.21[b]	72.99±5.00[b]

Note: a vs the Control group $P<0.05$; b vs Control group $P<0.01$

Table 2 Type I collagen level in the Model and Control groups ($n=6, \bar{x} \pm s$, ng/mg)

Group	1 d	3 d	7 d	14 d	21 d
Control	406.73±20.32	491.25±15.16	501.50±18.17	496.01±11.02	511.86±49.55
Model	422.08±49.48	470.87±29.39	528.11±43.39	556.60±40.04[a]	874.89±81.25[b]

Note: a vs the Control group $P<0.05$; b vs the Control group $P<0.01$

Table 3 FN level in the Model and Control groups ($n=6, \bar{x} \pm s$, ng/mg)

Group	1 d	3 d	7 d	14 d	21 d
Control	309.18±28.64	302.05±14.03	341.83±28.08	362.22±38.45	366.33±14.30
Model	338.18±38.26	352.08±48.88	374.84±13.75	376.96±24.31	374.64±64.88

Table 4 HA level in the Model and Control groups ($n=6, \bar{x} \pm s$, ng/mg)

Group	1 d	3 d	7 d	14 d	21 d
Control	71.28±6.70	70.96±10.97	72.91±7.51	72.57±9.83	72.57±9.83
Model	69.56±18.45	75.58±7.51	76.43±4.85	76.43±4.85	72.04±9.96

Discussion

TGF-β1 is recently discovered type of cytokine with multiple biological effects, which plays an important role in the tissue repair. It has been found that a high level expression of TGF-β1 occurs in experimental pulmonary fibrosis, accompanied with an increased expression of type I and III collagen mRNA. It has also been found that the use of anti-TGF-β1 serum can reduce the content of hydroproline (an important index reflecting the level of fibrosis) in fibrotic lung tissues[2]. TGF-β1 has been proved to be a significant factor promoting lung fibrosis. Josson et al[3] have discovered that, within 1 week of life in infants with CLD, the TGF-β1 level in BALF is obviously higher than that in infants with RDS or in infants following operations other than lung disease operations, and increases continually until the 2nd week and even until the 4th week; TGF-β1 positive expression is also found in the corpses of infants with CLD. Therefore, TGF-β1 is thought to be closely correlated with the occurrence of premature infant CLD. However, the dynamic expression and distributed rule of TGF-β1 in lung tissues can not be clarified. This study showed that the expression of lung TGF-β1 in premature rats exposed to hyperoxia for 7 days was higher than that of the Control group and was centralized in a few alveolar macrophages, alveolar epithelial cells and interstitial cells. This suggested that CLD was developed on day 7 of hyperoxia-inducement. The expression of TGF-β1 strengthened significantly on day 14, and reached a peak on day 21, when it was scattered in groups of alveolar macrophages, large numbers of alveolar epithelial cells and interstitial cells. It has been suggested that the extent of TGF-β1 expression increases, and the range expands, with the lengthened time of oxygen inhalation. The variable trend of TGF-β1 in BALF of infants with CLD, that Josson et al[3] have reported, is consistent with this result of the level of TGF-β1 expression in lung tissues. So, detecting TGF-β1 level in BALF of premature infants may be of value in evaluating the possibility of the occurrence and prognosis of CLD.

ECM a is dynamic network among cells, composed by collagen, proteoglycans (eg. HA), and glycoproteins (eg. FN) etc. macromoleculars, of which collagen is the main component. In normal physiological condition, the synthesis and decomposition of ECM are in dynamic balance. Excessive synthesis of ECM can cause fibrosis. A great deal of ECM deposition is found in the lungs of adults with idiopathic pulmonary fibrosis and in the bleomycin-induced rat pulmonary fibrosis model, which has been proved in many studies. There are few reports about the effect of ECM on the occurrence of premature infant CLD. The expression of type I collagen has only been found in the lungs of few infants died from CLD[4]. However, some studies have also shown that the expression of type I collagen mRNA reduces in the lung specimens of newborn rats absorbing hyper-oxygen continually, and that retinoid acid could increase the synthesis of collagen in lungs, thereby helping the alveolus to mature[5,6]. While in this study, it was found that, though the contents of HA and FN in lungs had no difference, the content of type I collagen began to increase on day 14, and was significantly higher than that of the Control group on day 21. This suggested that there was the deposition of type I collagen in the lungs. It still requires thorough study to see whether this transformation is a kind of compensatory response by organism after hyperoxia-induced lung damage, or whether it causes further fibrosis.

Recently, the studies of pulmonary fibrosis have focused on the increased synthesis of ECM induced by TGF-β1 leading to the deposition of excessive collagen in lung tissues. The correlation between TGF-β1 and the occurrence of pulmonary disease of infants often involves the effect of TGF-β1 on the development of lungs. External experiments in vitro have confirmed that TGF-β1 can hamper the morphological development of pulmonary ramification[2]. Yet the results of this study have shown that TGF-β1 is expressed in lung tissues before the level of type I collagen increased, and that on days 14 and 21 after hyperoxia exposure, the TGF-β1 expression had a remarkable positive correlation with the level of type I collagen. Therefore, it can be inferred that TGF-β1 may cause ECM deposition in lung tissues of premature infants with hyperoxia-induced CLD, as well as hindering lung development.

(Figures are on the inside front cover)

[**References**]

[1] Xu F, Fok TF, Yin J. Hyperoxia-induced lung injury in premature rat: description of a suitable model for the study of lung disease in newborns [J]. Chin Med J, 1998, 111(7): 619-624.

[2] Fu J-H, Xue X-D. TGF-β1 and chronic lung disease of prematurity [J]. Chin J Pediatr (in Chinese), 2003, 41(4): 313-315.

[3] Jonsson B, Li YH, Noack G, Brauner A, Tullus K. Downregulatory cytokines in tracheobronchial aspirate fluid from infants with chronic lung disease of prematurity [J]. Acta Pediatr, 2000, 89(11): 1375-1380.

[4] Kotecha S. Cytokines in chronic lung disease of prematurity[J]. Eur J Pediatr, 1996, 155(21): 2814-2817.

[5] Cherif A, Marrakchi Z, Chaouachi S, Boukef S, Sfar R. Bronchopulmonary dysplasia and corticosteroid therapy [J]. Arch Pediatr, 2002, 9(2): 159-168.

[6] Rong Z-H, Chang L-W, Zhang Q-S. Protective effects of retionic acid on hyperoxia-induced lung injury in neonatal rats [J]. Chin J Pediatr (in Chinese), 2003, 41(4): 299-230.

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Podocin expression in rats with puromycin aminonucleoside nephropathy

(These figures refer to the paper on page 161)

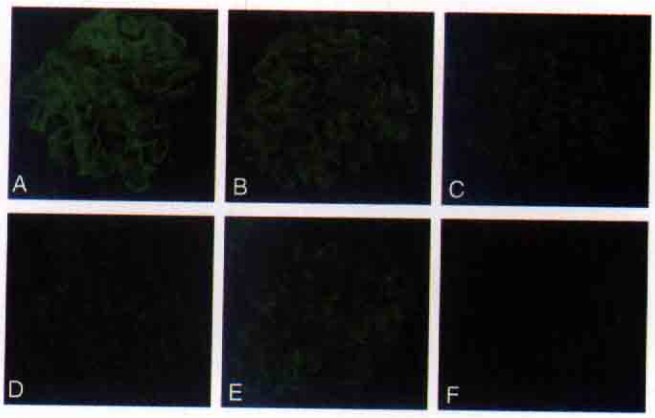


Figure 2 Immunofluorescence staining (magnification, × 200)

Figure 2A Linear-like pattern of the continuous fine granules in normal glomeruli

Figure 2B Partly discontinuous pattern on day 1

Figure 2C Diffusely granular pattern on day 3

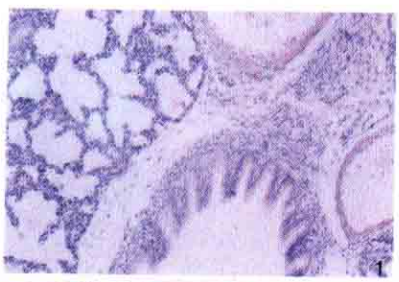
Figure 2D Coarse granular pattern on day 10

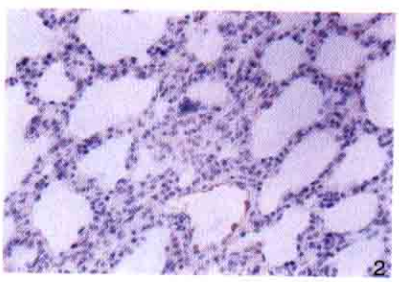
Figure 2E Partly retrieved expression on day 20

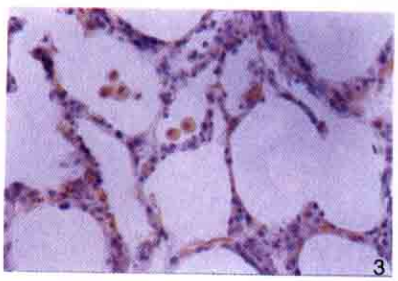
Figure 2F No staining on glomerular (negative control)

Dynamic expression and effect of TGF-β 1 on extracellular matrix in premature rats with CLD

(These figures refer to the paper on page 166)







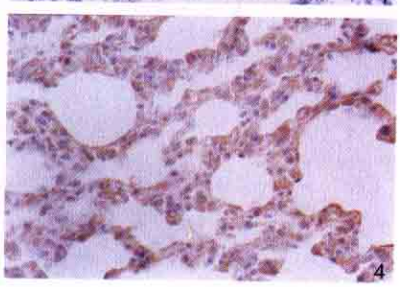


Figure 1 Expression of TGF- β 1 of the normal lung (SABC, × 200)

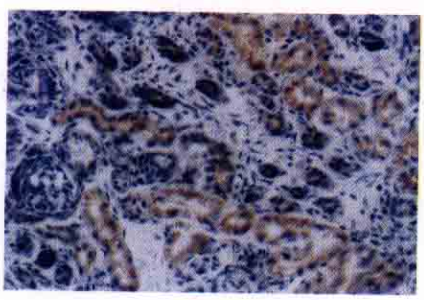
Figure 2 Expression of TGF- β 1 of the model lung on day 7 (SABC, × 200)

Figure 3 Expression of TGF- β 1 of the model lung on day 14 (SABC, × 400)

Figure 4 Expression of TGF- β 1 of the model lung on day 21 (SABC, × 400)

宫内窘迫后胎鼠肾脏细胞间粘附分子-1的表达及意义

（正文见第199页）



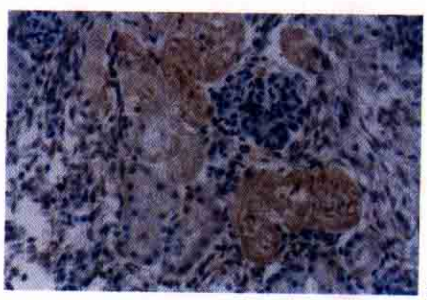


图1 ICAM-1在假手术组胎鼠肾组织即有少量表达，表达部位主要在肾小管上皮细胞胞浆中，近曲小管表达略强于远曲小管，肾小球区则未见表达。（免疫组化染色 × 360）

Figure 1 Small quantity expression of ICAM-1 in the cortical tubuli in the Sham-Operation group (HE × 360)

图2 缺血15 min再灌注15 h时，ICAM-1在近曲小管的表达达到高峰，在坏死和空泡变性明显的小管中其表达最强。（免疫组化染色 × 360）

Figure 2 ICAM-1 expression in the proximal tubuli reached a peak after a 15-minute ischemia and then a 15-hr reperfusion (HE × 360)