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Lung Injury Indices Depending on Tumor Necrosis Factor-α Level and Novel 35 kDa Protein Synthesis in Lipopolysaccharide-Treated Rat†

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= 초 록 =

내독소처치 흰쥐에서 Tumor Necrosis Factor-α치 상승에 따른 폐손상 악화 및 35 kDa 단백질 합성†

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연구배경:

급성폐손상의 병태생리학적 기전에는 염증세포들이 분비하는 다양한 염증성 매개물질들이 매우 중요한 역할을 한다. 이중 특히 tumor necrosis factor-α (TNF-α)는 다른 염증세포들의 화학주성 및 각종 염증성 매개물질 분비에 영향을 미치는 proin-flammatory cytokine으로 작용하는 한편, 직접적으로 세포손상을 야기시키는 세포독성 cytokine으로도 작용하는데, 급성폐손상에서 TNF-α와 폐조직 손상과의 직접적인 관련성에 대해서는 아직 구체적으로 확인된 바가 많지 않다.

또한, 최근에 생체내 방어기전으로 스트레스 단백질에 대한 관심이 높아지면서, 단핵구에 내독소를 처치하거나, 동물에 내독소를 투여하기 전에 미리 스트레스 단백질을 합성시킨 경우, 내독소에 의한 손상을 감소시켜 준다는 연구가 보고되었지만, 내독소 자극 자체만으로 스트레스 단백질 합성이 유도되는지는 아직 분명하지 않다.

이에 저자들은 내독소 유도성 급성 폐손상에서 TNF-α 분비와 폐조직 손상을 포함한 일련의 염증반응과의 관계를 분석하고, 생체내 내독소 자극에 대하여 폐포대식세포에서 스트레스 단백질을 포함한 새로운 단백질 합성이 유도되는지 여부를 분석하고자 하였다.

연구방법:

흰쥐의 기관내로 내독소를 투여한 후 시간별로 기관지폐포세척액내 TNF-α농도, 염증세포 백분율 변화, 병리조직

† 본 논문은 1997년도 가톨릭 중앙의료원 및 가톨릭대학교 성바오로병원 임상의학 연구비의 지원으로 이루어졌음.

학적 소견을 관찰하고, 또한 각 시간대의 폐포대식세포에서 sodium dodesyl sulfate-polyacrylamide gel electrophoresis와 inducible heat stress protein72에 대한 면역화학염색을 시행하여 단백질 합성양상을 분석하는 한편, 폐포대식세포에 다양한 농도의 내독소 자극과 열처리를 가한 후, 배양상층액에서 tumor necrosis factor-α 농도를 측정하고, 폐포대식세포의 단백질 합성양상을 분석하였다.

연구결과:

내독소 투여 후 tumor necrosis factor-α는 첫 1시간째부터 현저하게 증가하여 ($p<0.0001$) 3시간째 최고치에 이르렀고 6시간째는 감소하기 시작하여 12시간째는 정상 대조군 수준으로 감소하였다.

내독소 투여 후 염증세포 백분율의 변화는 2시간째부터 시작하여 6시간째 최고에 이르러 12시간째까지 지속하였으며, 24시간째에 정상 대조군 수준으로 회복하였다.

병리조직학적 소견상 폐손상 지표 점수는 내독소 투여후 6시간째 최고치에 이르러 24시간째까지 지속하였다.

내독소 투여 후 분리한 폐포대식세포에서 첫 1시간째부터 24시간째까지 정상 대조군에서는 관찰할 수 없던 35 kDa의 새로운 단백질 띠가 관찰되었으며, 면역화학염색상 inducible heat stress protein72는 관찰되지 않았다.

내독소 자극을 가하지 않은 정상 대조세포군에 비해 내독소 자극을 가한 세포군의 배양상층액에서 tumor necrosis factor-α 농도가 유의하게 높았으며 ($p<0.001$), 내독소 자극만 가한 세포군에 비해 열충격 전처치후 내독소 자극을 가한 세포군의 배양상층액에서 tumor necrosis factor-α 농도가 10 μg/ml 내독소 자극군만 제외하고 모두 유의하게 감소하였다 ($p<0.05$).

내독소 자극만 가한 세포군은 10 μg/ml의 고농도에서만 35 kDa의 단백질 띠가 합성되었고 inducible heat stress protein72는 관찰되지 않았다. 열충격 전처치후 내독소 자극을 가한 세포군은 모두 inducible heat stress protein72가 관찰되었다.

결 론:

기관내 내독소 투여에 의한 급성 폐손상에서 tumor necrosis factor-α는 폐손상 정도와 밀접한 관련이 있다. 또한 내독소 자극에 의해서는 폐포대식세포에서 inducible heat stress protein72 합성이 유도되지 않으며, 35 kDa의 새로운 단백질 합성이 유도되었는데, tumor necrosis factor-α 농도 및 병리조직소견과의 관계를 볼 때, 급성 폐손상에 있어 35 kDa 단백질이 방어적인 역할을 담당하지는 않을 것으로 보이며, 이에 대해서는 향후 더 연구가 필요할 것으로 생각된다. (Tuberculosis and Respiratory Diseases 1998, 45 : 1236-1251)

중심단어 : 내독소, 급성폐손상, Tumor Necrosis Factor-α, 스트레스 단백질

Introduction

The acute respiratory distress syndrome (ARDS) is an acute, severe alteration in lung structure and function characterized by hypoxemia, stiff lungs with low functional residual capacity, and diffuse radiographic infiltrates due to increased lung microvascular permeability[1,2]. Acute lung injury (ALI) is considered a less severe form of ARDS, and often progresses to ARDS. Many investigators have studied the early pathogenetic mechanisms of ALI and ARDS in order develop new treatment modalities. Initial pathogenetic mechanisms are different, depending on the etiology (sepsis versus trauma). ALI caused by sepsis shows high mortality rate. Specifically, sepsis due to diffuse infectious processes and pneumonias results in

the highest mortality rate[3,4]. Therefore, in this study, we investigated the initial pathogenetic mechanisms of ALI using direct lung injury model of rat induced by intratracheal LPS instillation.

Tumor necrosis factor-α (TNF-α) appears to be a central mediator of the host response to sepsis[5-8]. While TNF-α is mainly considered a proinflammatory cytokine, it can also act as a direct cytotoxic cytokine. However, there are not so many studies about the relationship between TNF-α level and lung injury severity in ALI, particularly regarding the case of ALI caused by direct lung injury such as diffuse pulmonary infection.

Recently, a natural defense mechanism, known as the stress response or the heat shock response, has been reported in cellular or tissue injury reaction[9]. Heat shock proteins (HSP) or stress proteins are produced by a broad spectrum of pathogens and have been shown to be among the dominant target antigens recognized in immune responses to pathogens. The stress exerted by phagocytes may increase HSP induction in microbial pathogens in order to protect themselves from the host effector mechanism. On the other hand, host cells also need to protect themselves from the noxious molecules which pathogens produce. This may be achieved in part by HSP induction in host cells. In fact there are a number of reports about HSP response of host cells after in vitro infection with a variety of pathogens. However, there are a few reports about the in vivo HSP response of host cells. Zhang et al reported the in vivo production of HSP in mouse peritoneal macrophages by administration of lipopolysaccharide (LPS), one of the noxious products of gram-negative organisms[10]. LPS is also frequently used in ALI animal model because gram-negative infection is one of most common causes of ALI or ARDS.

On the other hand there are a number of reports examining the protective role of pre-induced heat stress proteins on subsequent LPS-induced TNF-α release from monocyte or macrophage and also on subsequent LPS-induced ALI in animals[11-15].

However it is not well established whether the stress protein synthesis such as HSP can be induced from rat alveolar macrophages by in vitro or in vivo LPS stimulation. We hypothesized the HSP synthesis may be induced from rat alveolar macrophages by LPS itself and it will contribute to the time course of alveolar fluid TNF-α level in LPS-induced ALI rat model.

Our purpose in this article is that, in first to investigate the relationship of TNF-α level and lung injury severity, in second to investigate the stress protein response of alveolar macrophages to in vitro and in vivo LPS stimulation in LPS-induced ALI rat model.

Methods

Experimental animals

Male Sprague-Dawley rats weighing 175-250g were used in these experiments. Rats were housed in standard wire-topped cages. Food and water were supplied ad libitum.

Lipopolysaccharide

Escherichia coli LPS B (strain 026 : B6 ; Difco

laboratories, Detroit) was prepared for in vitro and in vivo experiments by resuspension in pyrogen-free saline. Control rats received intratracheal instillations of an equal volume of saline. The LPS concentration for intratracheal challege was 5mg/kg, a sublethal dose which did not kill any animals within 24 hours in preliminary experiments.

Characterization of pulmonary cell population and isolation of macrophages

Bronchoalveolar lavage (BAL) was performed with five 3-ml aliquots of sterile saline. BAL fluid was centrifuged at 400 g for 10min at 4℃, and cell-free supernatant was stored at 70℃ until TNF-α assay. The cell pellet was washed twice and resuspended in minimal essential medium (MEM, from Gibco BRL Laboratories, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS). Total cell counts were determined in duplicate in a hemocytometer, and differential cell counts were determined after cytocentrifuge (Cytospin 2, Shandon, Pittsburg, PA, USA) and Diff-Quick stain. BAL cells suspended in MEM containing 10% FBS were plated at 4×10^6 cells/well in 6-well culture plate, and allowed to adhere for 2 h at 37℃ in 5% CO2-95% air atmosphere. To confirm whether the adhered cells were macrophages, adhered cells were scraped with a rubber policeman, cytocentrifuged, and stained with Diff-Quick stain. More than 95% of the adhered cells were macrophages.

Experiment protocol

In vivo experiment.

Rats were anesthetized with intraperitoneal injection of mixture of ketamine hydrochloride 50 mg/kg and xylazine 2mg/kg, and an incision was made in the soft tissue overlying the trachea. LPS was injected via a 25-gauge needle into the visualized trachea. Control rats received intratracheal sterile saline. BAL was done in control rats and at 1 h, 2 h, 3 h, 4 h, 6 h, 12 h, and 24 h after intratracheal LPS instillation. Histopathologic examination of the lung was also done in other rats which did not receive BAL and at 1 h, 2 h, 3 h, 4 h, 6 h, 12 h, and 24 h after intratracheal LPS instillation and at same points in saline treated rats, because BAL procedure itself can affect histopathologic findings.

In vitro experiment.

Isolated non-stimulated macrophages were incubated for 2 h with different concentration of LPS (0, 1, 10, 100ng/ml, 1, or 10 μg/ml). Other non-stimulated macrophages were exposed at 43℃ for 15 min, then returned to at 37℃ in 5 % CO2-95 % air for 1 hour, and then incubated for 2 h with LPS (0, 1, 10, 100ng/ml, 1, or 10 μg/ml).

Analysis of TNF-α concentration

TNF-α levels of cell-free supernatant of BAL fluid (in vivo experiment) and culture supernatant (in vitro experiment) were measured using specific ELISA (mouse TNF-α ELISA kit ; Genzyme, Cambridge, MA) according to the suppliers instructions.

Analysis of stress proteins in macrophages

After washing with methionine-free MEM (from Gibco BRL Laboratories, NY, USA), adhered

cells were further cultured for 1 h in methionine -free MEM containing 10 Ci [^{35}S]methionine. The labelling was terminated by aspirating the medium and washing three times with ice-cold PBS. The adhered macrophages were lysed in 100 l aliquot of sample buffer (0.125 M Tris/HCl (pH 6.8), 4% sodium dodesyl sulfate, 20% glycerol and 10% 2-mercaptoethanol), and then sonicated with sonic dismembrator (Fisher Co., NY, USA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrphoresis, transferred to nitrocellulose (NC) membrane. The NC membrane was stained with Coomassie staining solution (0.025% Coomassie Blue R-250, 40% methanol, 7% acetic acid) and destained with destaining solution (30% methanol, 10% acetic acid). The NC membrane was dried and exposed to X-ray film (Kodak Co.).

The NC membrane was immunostained with a primary monoclonal antibody, mouse antihuman IgG, specific against the inducible form of HSP72 (StressGen, Victoria, BC, Canada). After secondary labeling with biotinylated goat anti-mouse IgG and incubating with ABC reagent solution (VECTASTAIN ABC kit ; Vector Laboratories, USA), protein was visualized using peroxidase substrate solution.

Histopathologic examination and lung injury index scoring

Experimental animals were killed by cervical dislocation before and at 1, 2, 3, 4, 6, 12, 24 hours after intratracheal LPS instillation, and their lungs and heart were excised en bloc. The pulmonary vessels were divided and the heart was dissected from the lung specimen. The lungs were then gently inflated with a fixative (10% formalin in PBS) and maintained in the fixing solution for 7 days. All lung specimens were examined by a expert pathologist, who was blind to the treatment of the animals. Tansverse, 2-cm-thick sections from the upper, middle, and lower part of each lung were then obtained by the pathologist. From those sections the most severely injured section was selected. The slides were prepared from this severely injured tissue section and stained with hematoxylin-eosin. The slides were then examined by the pathologist to establish the pattern and extent of histologic changes. Five indices were established as lung injury indices : (1) degree of exudation of inflammatory cells such as alveolar macrophages and neutrophils, hemorrhage, edema, and fibrin deposition within alveolus ; (2) degree of exudation of inflammatory cells such as alveolar macrophages and neutrophils, hemorrhage, edema, and fibrin deposition within interstitium ; (3) degree of inflammatory exudate within terminal bronchioles ; (4) degree of atelectasis ; (5) degree of thrombus formation. After examination of the slide under low power field of light microscope, the most severely injured lobule was selected and then was examined under high power field. Each of the five indices were calculated as follows : 5, if each index occupied 80% to 100% of the lesion observed under one high power field ; 4, if 60% to 80% ; 3, if 40% to 60% ; 2, if 20 % to 40% ; 1, if not more than 20% ; 0, if 0%.

Saline-treated control rats also received the same histopathologic examination and lung injury index scoring.

Statistical analysis

All data were expressed as means±SEM. Data were analyzed using analysis of variance and

Table 1. Differential cell counts and tumor necrosis factor-α level in bronchoalveolar lavage fluid after intratracheal lipopolysaccharide instillation in rat

Time (hour)	Tumor necrosis factor-α level of bronchoalveolar lavage fluid (pg/ml)	Percentage of Alveolar macrophages	Percentage of Neutrophils
0(Control)	55.5±11.1	85.8±6.1	6.1±3.9
1	307.2±40.0‡	86.2±1.3	8.5±3.2
2	1002.7±315.5‡	51.7±7.4*	35.7±6.2*
3	5904.7±123.1‡	12.7±3.1†	78.5±2.3†
4	5882.9±9.1‡	10.4±4.2†	88.9±4.2†
6	3891.3±207.2‡	4.2±0.5†	92.8±1.0†
12	50.5±6.0	30.5±9.2†	68.5±9.6†
24	53.2±10.2	44.5±16.6	54.5±17.1†

*$p<0.05$, †$p<0.01$, ‡$p<0.001$ vs 0 hour (Control)

Students independent t-test where indicated.. Differences were regarded as significant at $p<0.05$.

Results

In Vivo Experiment

TNF-α level and the percentage of inflammatory cells in BAL fluid before and after intratracheal LPS instillation

TNF-α levels began to increase significantly at 1 h, reached peak at 3 h, began to decrease at 6 h, and reached the level of normal control at 12 h after LPS instillation (Table 1) (Fig. 1).

The percentage of inflammatory cells (neutrophils and alveolar macrophages) began to change significantly at 2 h, showed maximal change at 6 hours, began to recover but still showed significant change at 12 h, and showed

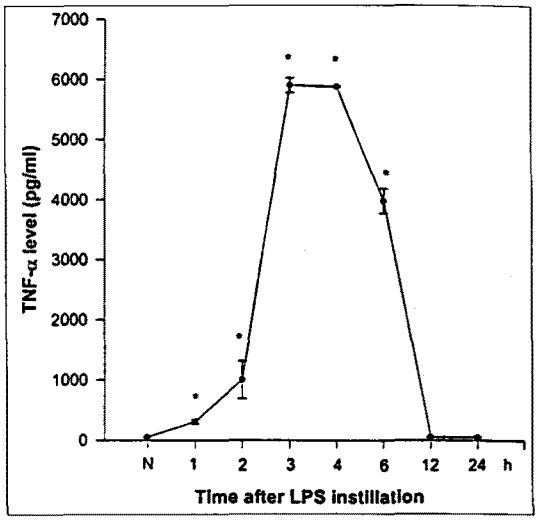

Fig. 1. Time-course of TNF-α level in bronchoalveolar lavage (BAL) fluid after intratracheal LPS (5 mg/kg of E. coli LPS) instillation in rat
Data are means±SEM (bars).
*$p<0.0001$ vs. N. N means normal control.

Table 2. Histologic lung injury score in the course of time after intratracheal lipopolysaccharide instillation in rat

Time (hour)	Alveolus					Interstitium					Exudate Within TB[5]	Atelect -asis	Thro -mbus	Total
	N[0]	M[1]	H[2]	E[3]	F[4]	N[0]	M[1]	H[2]	E[3]	F[4]				
1	1	0	0	0	0	0	0	0	0	0	0	0	0	1
2	1	0	0	0	0	1	1	0	0	0	0	0	0	3
3	2	0	2	0	1	3	2	2	2	0	3	0	0	17
4	4	2	4	1	1	3	1	2	1	0	2	5	0	26
6	5	2	3	3	2	3	2	4	3	2	4	0	0	35
12	4	5	2	2	2	4	5	4	3	0	5	0	0	37
24	4	5		0	2	4	5	3	2	0	5	0	0	32

N[0] (neutrophil), M[1] (macrophage), H[2] (hemorrhage), E[3] (edema), F[4] (fibrin), TB[5] (terminal bronchiole)

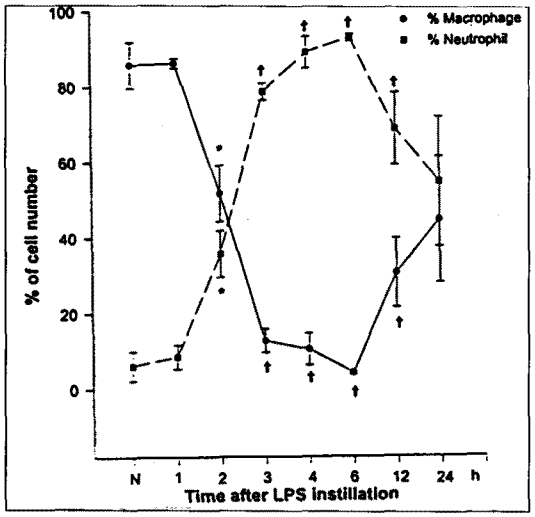

Fig. 2. Time-course of the percentage change of macrophage or neutrophil per 500 cell in bronchoalveolar lavage (BAL) fluid after intratracheal LPS (5mg/kg of E. coli LPS) instillation in rat.
Date are means±SEM (bars). *$p<0.05$, †$p<0.01$ vs. N.
N means normal control.

insignificant change at 24 h after LPS instillation compared with the normal control (Table 1, Fig. 2).

Histologic finding and lung injury index score

After intratracheal LPS instillation, histologic changes were not prominent at 1 and 2 h, but neutrophils began to infiltrate along acini and alveolar walls at 3 h. On histologic examination, neutrophils increased the most during the initial several hours of lung injury, while after 12 hours macrophages as well as neutrophils began to increase in the interstitium and alveoli (Fig. 3).

The histologic lung injury index reached a maximum value at 6 h and remained at this level for 24 hours (Table 2).

Any significant histopathologic change was not found in saline-treated control rats, and their lung injury index score was zero.

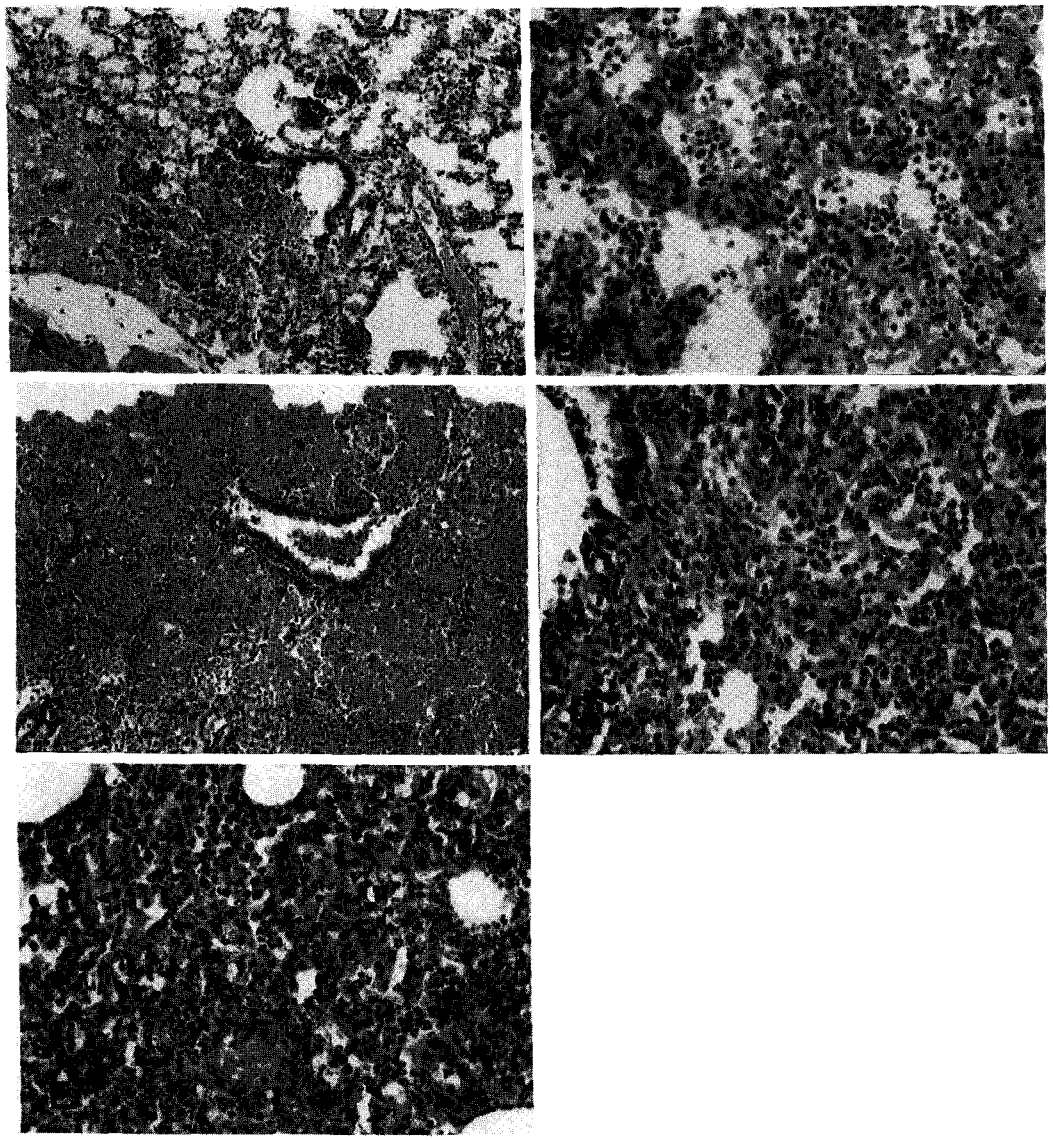

Fig. 3. Photomicrograph of a rat lung after intratracheal LPS instillation, (A) at 3 hours after intratracheal LPS instillation, (B) at 4 hours after intratracheal LPS instillation, (C) at 6 hours after intratracheal LPS instillation, (D) at 12 hours after intratracheal LPS instillation, (E) at 24 hours after intratracheal LPS instillation.
Hematoxylin & eosin stain, original magnification : ×200 (A,C), ×400 (B,D,E).

The pattern of stress protein synthesis in macrophages

We observed a 35 kDa protein band in alveolar macrophages from 1 hour to 24 hours after intratracheal LPS instillation (Fig. 4). Inducible HSP72 was not found by electrophoresis of homogenates obtained from any macrophage before and after intratracheal LPS instillation(Fig. 5).

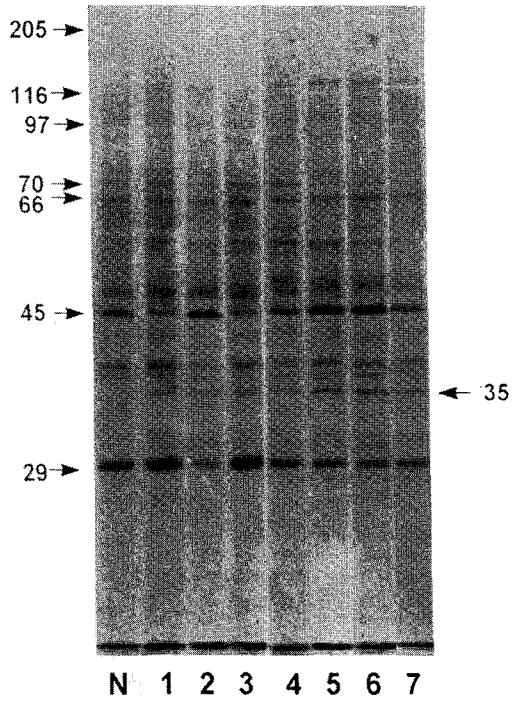

Fig. 4. Autoradiography after SDS-PAGE of homogenates obtained from alveolar macrophages (AM). Alveolar macrophages were labeled for 1 hour and harvested at the indicated time after intratracheal LPS instillation.
Lane N, normal control AM ; Lane 1, AM at 1 hour ; Lane 2, AM at 2 hours ; Lane 3, AM at 3 hours ; Lane 4, AM at 4 hours ; Lane 5, AM at 6 hours ; Lane 6, AM at 12 hours ; Lane 7, AM at 24 hours after intratracheal LPS instillation.
Long arrows indicate the molecular markers (kDa).

In Vitro Experiment

There was a dose related increase in TNF-α levels from the culture supernatants of LPS-stimulated macrophages such that at a dose of 10μg/ml there was a 150-fold increase in TNF-α (Fig. 6). TNF-α levels in culture supernatants of LPS -stimulated cells after heat treatment were significantly lower than in those of LPS-stimualted cells without heat pretreatment($p<0.05$) (Fig. 6). TNF-α levels in culture supernatants of LPS of 10μg/ml stimulated cells with heat pretreatment also exhibited a tendency to lower levels than in cells without heat pretreatment ($p=0.085$) (Fig. 6).

In cells stimulated with 10mg/ml of LPS, a 35 kDa protein band was found : this band was not found with stimulation at other concentrations of LPS (Fig. 7). In cells with heat pretreatment, stress protein of 72 kDa was found (Fig. 7). We confirmed this stress protein of 72 kDa to be the inducible HSP72 (Fig. 8). In cells with LPS stimulation only, inducible HSP72 was not found at any concentrations of LPS (Fig. 8).

Discussion

Alveolar macrophages are one of the initial effector cells of ALI and are important in the regulation of the inflammatory response. This cell produces many inflammatory mediators such as TNF-α, IL-1, IL-6, IL-8, MIPs, MCP, and oxygen free radicals[16-19]. The major source of TNF-α is the alveolar macrophage. While a number of studies examining TNF-α secretion has been reported, there are very few studies examining the relationship between the severity of lung injury and TNF-α levels over time in a direct acute lung injury animal model, doing intratracheal endotoxin administration.

In this study, TNF-α levels were increased before the onset of histologic changes and major

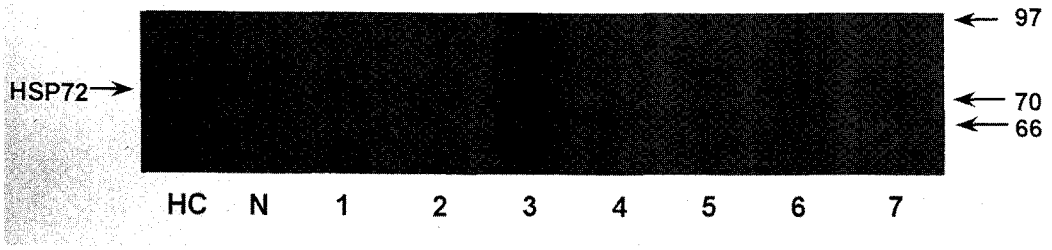

Fig. 5. Immunochemical staining after electrophoresis of HSP 72 induced by in vivo LPS stimulation in alveolar macrophages (AM). Alveolar macrophages were harvested at the indicated time after intratracheal LPS instillation.
Lane HC, heat-treated AM ; Lane N, normal control AM ; Lane 1, AM at 1 hour ; Lane 2, AM at 2 hours ; Lane 3, AM at 3 hours ; Lane 4, AM at 4 hours ; Lane 5, AM at 6 hours ; Lane 6, AM at 12 hours ; Lane 7, AM at 24 hours after intratracheal LPS instillation.
Long arrows indicate the molecular markers (kDa).

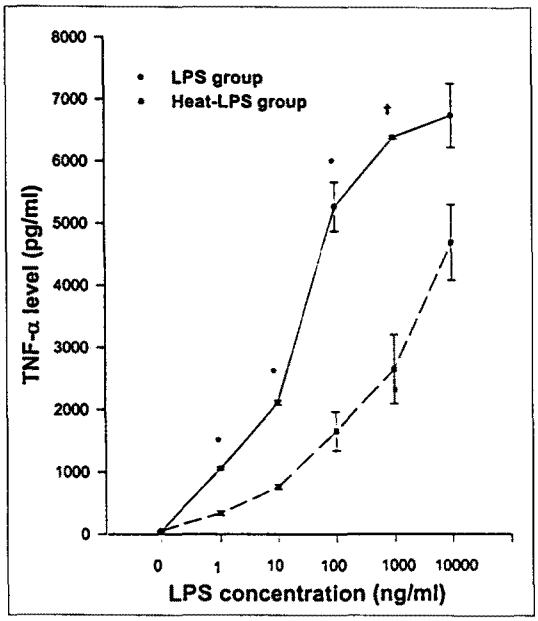

Fig. 6. The change of TNF-α levels in cell-free culture supernatants of alveolar macrophages after incubation at 37℃ for 2 hours with various concentrations of LPS (LPS group) or at 43℃ for 15 minutes and then with various concentrations of LPS (Heat-LPS group).
Date are means ± SEM (bars).
*$p<0.01$, †$p<0.05$ (LPS group vs. Heat-LPS group).

changes in inflammatory cell composition, and the histologic lung injury score and the percentage change of differential inflammatory cell count remained steady despite the fact that TNF-α levels decreased to their basal levels. This suggests that TNF-α acts mainly as a proinflammatory cytokine and has a direct or indirect close relationship with lung injury severity in ALI or ARDS.

There are a number of possibilities for the relatively early peak and decline in TNF-α level. First, TNF-α secretion may have decreased because the administered LPS was metabolized and inactivated. Second, TNF-α secretion may have been suppressed by other inhibitory inflammatory mediators such as interleukin-10 (IL-10) and IL-13[20-24].

Nelsons study was similar to our study[25]. In his study, TNF-α levels peaked at 3 h and returned to basal level at 4 h after LPS administration in rats. In our study, the time of peak TNF-α secretion was similar, but the time needed for TNF-α to return to its basal level was more delayed. This may related to the dif-

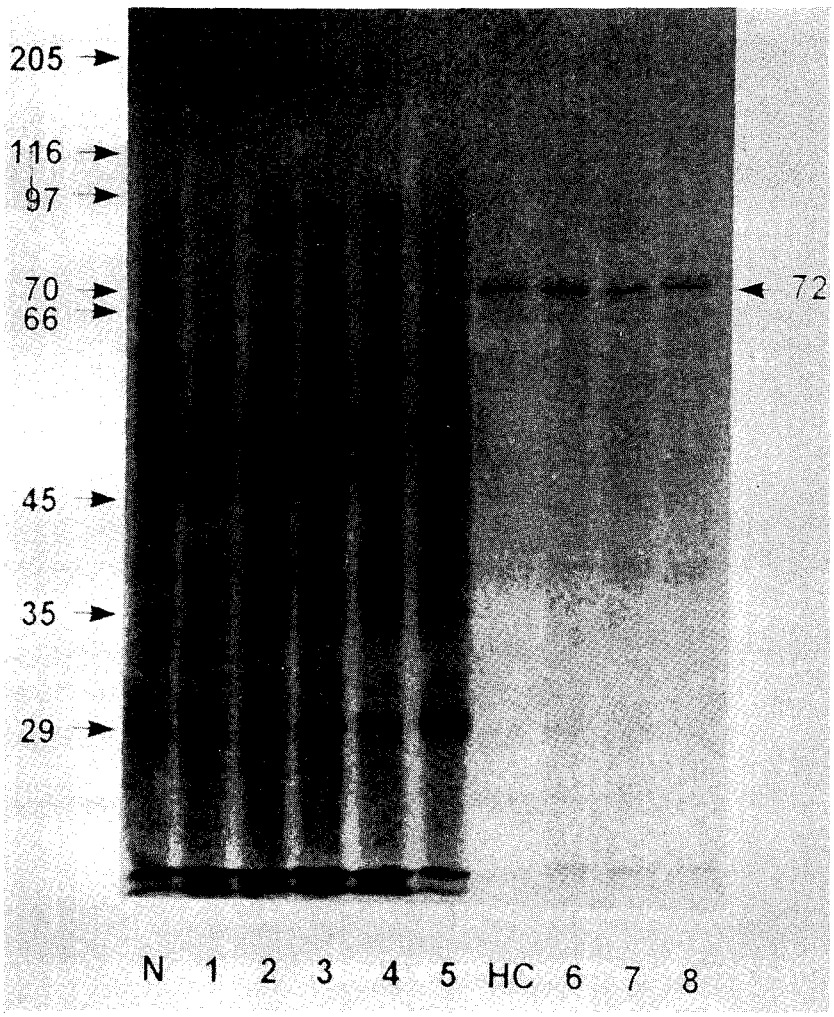

Fig. 7. Autoradiographic patterns after SDS-PAGE of homogenates obtained from alveolar macrophages (AM). Alveolar macrophages were incubated at 37℃ for 2 hours with various concentrations of LPS or at 43℃ for 15 minutes and then at 37℃ for 2 hours with various concentrations of LPS.
Lane N, normal control AM ; Lane 1, AM incubated with LPS 1 ng/ml ; Lane 2, AM incubated with LPS 10ng/ml ; Lane 3, AM incubated with LPS 100ng/ml ; Lane 4, AM incubated with LPS 1 μg /ml ; Lane 5, AM incubated with LPS 10 μg /ml ; Lane HC, heat-treated normal control AM ; Lane 6, AM incubated with LPS 1 ng/ml after heat treatment ; Lane 7, AM incubated with LPS 100ng/ml after heat treatment ; Lane 8, AM incubated with LPS 10 μg /ml after heat treatment. Long arrows indicate the molecular markers (kDa).

ferent doses of LPS and in part from not having used the specific pathogen-free state rats. Although the rats used in this study were not pathogen-free, we bred the rats in clean environment, and all lungs used in our experiments were examined by an expert pathologist. Furthermore, any

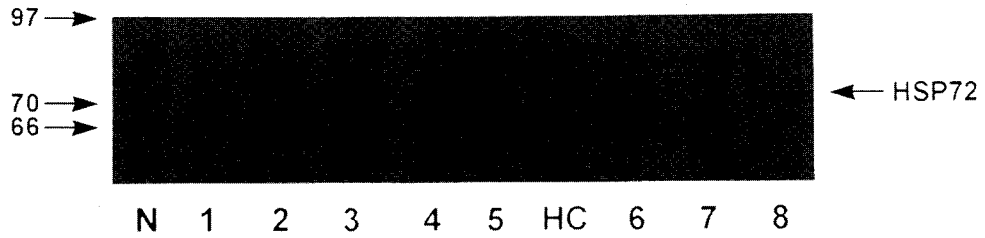

Fig. 8. Immunochemical staining after electrophoresis of HSP72 induced by LPS or heat in alveolar macrophages (AM). Alveolar macrophages were incubated at 37℃ for w hours with various concentrations of LPS or at 43℃ for 15 minutes and then at 37℃ for 2 hours with various concentrations of LPS. Specific monoclonal antibody to HSP72 and polyclonal antibody to HSP32 were used to detect the inducible HSPs. Lane N, normal control AM ; Lane 1, AM incubated with LPS 1 ng/ml;Lane 2, AM incubated with LPS 10ng/ml;Lane 3, AM incubated with LPS 100ng/ml;Lane 4, AM incubated with LPS 1 μg /ml;Lane 5, AM incubated with LPS 10 μg /ml;Lane HC, heat-treated normal control AM;Lane 6, AM incubated with LPS 1 ng/ml after heat treatment; Lane 7, AM incubated with LPS 100ng/ml after heat treatment;Lane 8, AM incubated with LPS 10 μg /ml after heat treatment.

control rats bred in the same environment did not show abnormal finding in their lungs.

It is widely accepted that neutrophils increase in BAL fluid and lung tissue as the injury progresses in ALI. Neutrophils are considered to be the major effector cells in the progressive stage of ALI while alveolar macrophages are considered the major effector cells in the early stage of ALI. However, there are reports showing that ALI can occur in neutropenic patients[26,27]. This implies neutrophil-independent mechanisms such as direct endothelial injury by endotoxin or other inflammatory cell-mediated mechanism. From this point of view, we focused on the role of alveolar macrophages in ALI. In inflammatory disease, these alveolar macrophages and neutrophils usually participate in a defense mechanism through phagocytosis. However, excessive inflammatory response by these inflammatory cells induces self-induced tissue injury, which is the fundamental pathophysiological event in ALI. For example, oxidative stress by reactive oxygen species (ROS) also causes self-induced tissue injury. In eukaryotes, both nonenzymatic and enzymatic antioxidant defenses have evolved to protect cells against the deleterious effects of ROS. As an antioxidant mechanism, the host synthesizes antioxidant enzymes or stress proteins such as HSP, metallothionein (MT), and heme oxygenase-1 (HO-1) to protect tissues from excessive inflammatory reaction in ALI. Stress proteins are produced in most prokaryotic and eukaryotic cells in response not only to an oxidative injury but also to thermal stress, viral infections, heavy metals, ethanol, amino acid analogs, inhibitors of energy metabolism or, in vivo, ischemia and reperfusion injury[28-31]. There are a number of reports examining the protective role of heat-induced HSP72 in subsequent endotoxin stimulation in monocytes or peritoneal macrophages and in subsequent endotoxin-induced ALI in rodents. Recently HSP32 (heme

oxygenase-1) induction has also been reported to have an important role in the defense against endotoxic shock[32,33]. However, the stress protein response of alveolar macrophages to in vitro and in vivo endotoxin stimulation is still unclear.

LPS stimulation induced HSP72 synthesis in human monocytes in the study of Fincato et al[34], while it induced HO-1 mRNA expression and HO-1 protein synthesis in RAW 264.7 murine macrophage cells in the study of Camhi et al[32]. In the studies of Fincato et al and Camhi et al, the strain of LPS (Salmonella enteritidis) and the serotype of LPS (E. coli serotype 055 : B5) used were different from those (E.coli serotype 026 : B6) used in our study. It is possible that this difference in strain and serotype of LPS have produced different results. In our study, we did not examine the mRNA level. We examined only the protein level through SDS-PAGE and immunochemical stain.

In this study, a new 35 kDa protein was induced in alveolar macrophages. This protein is considered an acute phase reactant, because it is induced very early in the course of the inflammatory response by LPS stimulation. It is likely that 35 kDa protein has no specific relationship with the increase in TNF-α release, because it is still observed even after TNF-α has decreased. 35 kDa protein also does not appear to have a defense role in ALI from the fact that histologic lung injury progressed despite the presence of the protein. Maybe it would have been worse without induction of this protein. Further studies for the nature and role of 35 kDa protein should be performed.

In conclusion, TNF-α has a direct or indirect close relationship with lung injury severity in acute lung injury or acute respiratory distress syndrome. In vivo and in vitro LPS stimulation neither induce heat stress protein 72 nor heat stress protein 32 synthesis in alveolar macrophages. It is likely that 35 kDa protein, newly synthesized by alveolar macrophages does not have a defense role in ALI after in vivo and in vitro LPS stimulation.

Summary

Background : TNF-α appears to be a central mediator of the host response to sepsis. While TNF-α is mainly considered a proinflammatory cytokine, it can also act as a direct cytotoxic cytokine. However, there are not so many studies about the relationship between TNF-α level and lung injury severity in ALI, particularly regarding the case of ALI caused by direct lung injury such as diffuse pulmonary infection.

Recently, a natural defense mechanism, known as the stress response or the heat shock response, has been reported in cellular or tissue injury reaction. There are a number of reports examining the protective role of pre-induced heat stress proteins on subsequent LPS-induced TNF-α release from monocyte or macrophage and also on subsequent LPS-induced ALI in animals. However it is not well established whether the stress protein synthesis such as HSP can be induced from rat alveolar macrophages by in vitro or in vivo LPS stimulation.

Methods : We measured the level of TNF-α, the percentage of inflammatory cells in bronchoalveolar lavage fluid, protein synthesis in alveolar macrophages isolated from rats at 1, 2, 3, 4, 6, 12, and 24 hours after intratracheal LPS instillation. We performed histologic examination and also obtained histologic lung injury index score in lungs from other rats at 1, 2, 3, 4, 6, 12, 24 h after

intratracheal LPS instillation. Isolated non-stimulated macrophages were incubated for 2 h with different concentration of LPS (0, 1, 10, 100 ng/ml, 1, or 10 μg/ml). Other non-stimulated macrophages were exposed at 43℃ for 15 min, then returned to at 37℃ in 5% CO2-95% for 1 hour, and then incubated for 2 h with LPS (0, 1, 10, 100ng/ml, 1, or 10 μg/ml).

Results : TNF-α levels began to increase significantly at 1 h, reached a peak at 3 h ($P<0.0001$), began to decrease at 6 h, and returned to control level at 12 h after LPS instillation. The percentage of inflammatory cells (neutrophils and alveolar macrophages) began to change significantly at 2 h, reached a peak at 6 h, began to recover but still showed significant change at 12 h, and showed insignificant change at 24 h after LPS instillation compared with the normal control. After LPS instillation, the score of histologic lung injury index reached a maximum value at 6 h and remained steady for 24 hours. 35 kDa protein band was newly synthesized in alveolar macrophage from 1 hour on for 24 hours after LPS instillation. Inducible heat stress protein 72 was not found in any alveolar macrophages obtained from rats after LPS instillation. TNF-α levels in supernatants of LPS-stimulated macrophages were significantly higher than those of non-stimulated macrophages ($p<0.05$). Following LPS stimulation, TNF-α levels in supernatants were significantly lower after heat treatment than in those without heat treatment ($p<0.05$). The inducible heat stress protein 72 was not found at any concentrations of LPS stimulation. Whereas the 35 kDa protein band was exclusively found at dose of LPS of 10 μg/ml.

Conclusion : TNF-α has a direct or indirect close relationship with lung injury severity in acute lung injury or acute respiratory distress syndrome. In vivo and in vitro LPS stimulation dose not induce heat stress protein 72 in alveolar macrophages. It is likely that 35 kDa protein, synthesized by alveolar macrophage after LPS instillation, does not have a defense role in acute lung injury.

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