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Original Article

The role of efalizumab in protecting ventilator-induced lung injury in anesthesia rats and related mechanisms

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Abstract: Although important for advanced life support and critical care, mechanical ventilation frequently caused ventilator-induced lung injury (VILI), manifesting a severe impact on survival rate. As the new generation of immunosuppressant, efalizumab was studied on a VILI model in rats to elucidate the protective function against damage and possible mechanisms. SD rats were randomly enrolled into control, VILI model, IgG control, glucocorticoid and efalizumab treated groups (N=15 each). The VILI model was generated by mechanical ventilation. In the experimental group, 2.5 mg/kg efalizumab was applied before mechanical ventilation. The total number of nuclear cells and neutrophils in bronchoalveolar lavage fluid (BALF) were counted, and the expressions of tumor necrosis factor (TNF)-α and interleukin (IL)-8 were also determined. Real-time PCR and Western blotting were also employed to detect the expression levels of SP-A gene and protein. Both nuclear cell and neutrophil numbers were significantly increased in VILI model group ($P<0.05$). The intervention by efalizumab decreased inflammatory cell number, as well as impeding the levels of cytokines such as TNF-α and IL-8 ($P<0.05$ in all cases). In VILI and IgGgroups, mRNA levels of SP-A gene were significantly decreased ($P<0.05$) but were potentiated by the addition of efalizumab or glucocorticoid. SP-A proteins had consistent distribution patterns as those of mRNA did. Efalizumab protects lung tissues from VILI via decreasing the activation and infiltration of inflammatory cells, inhibiting inflammatory factor release and facilitating expression of surfactant proteins.

Keywords: Efalizumab, mechanical ventilation, ventilator-induced lung injury

Introduction

As the most important life-saving intervention in patients, mechanical ventilation (MV) mechanically assists or replaces spontaneous breathing, especially helping support people with acute pulmonary injury or acute respiratory distress syndrome (ARDS) [1, 2]. MV can improve functions of critical organs including heart, brain, liver and kidney, and maintain body homeostasis, thereby providing opportunities for functional recovery. It may, however, cause structural and functional injuries on the pulmonary tissue, leading to ventilator-induced lung injury (VILI). As one severe complication, VILI may aggravate patient's condition, or even cause death as the result of multi-organ failures, thus severely compromising patient's prognosis [3-5]. Currently the incidence of VILI in long-term MV patients is as high as 15%, with thousands of death even more than the death rate caused by hypoxic toxicity. Therefore the understandings of pathogenesis of VILI as well as development of potential drugs remain challenging and unrecognized [6, 7].

Efalizumab is a humanized monoclonal antibody (IgG1κ) produced by recombinant DNA technology, which belongs to human CD11a monoclonal antibody. With the approximate molecular size of ~150 KD, efalizumab is a novel immunosuppressive agent and has been certificated by FDA for treating chronic psoriasis (moderate or severe) [8-10]. During the usage of respiratory machine, on the other hand, VILI was mainly caused by the hyperextension of pulmonary tissues at the end of respiration cycle as a result of high pressure and/or high volume ventilation. The consequent periodic retension of pulmonary alveoli recruits pro-inflammatory cells and increases the production of inflammatory factors, resulting in damages on

Table 1. Number of nuclear cells and neutrophils in BALF

Group	N	Nuclear cell No. ($\times 10^5$/ml)	Neutrophil No. ($\times 10^5$/ml)
Control	15	2.7±0.51	0.73±0.06
VILI model	15	7.9±0.72*	3.8±0.71*
IgG control	15	8.6±0.34*	4.2±0.37*
Glucocorticoid	15	4.5±0.56*,#	1.88±0.76*,#
Efalizumab treated	15	4.6±0.65*,#	1.92±0.69*,#

Note: *P<0.05 compared to the control group; #P<0.05 compared to the model group.

the alveolar epithelial cells followed by the occurrence and progression of VILI [11, 12]. The inhibition of immune response and further down-regulation of pro-inflammatory cytokines may effectively suppress the activation and recruitment of neutrophils, thereby reversing the progression of VILI [13, 14]. This study aimed to evaluate the treatment efficacy of efalizumab in the therapy of VILI, via the functional study of efalizumab on VILI model of anesthesia rats.

Materials and methods

Animal model

A total of 75 healthy SD rats (SPF grade, female, 8 weeks old, body weight=250 g±30 g) were purchased from Laboratory Animal Unit of Weifang Medical University and were kept in a SPF-grade facility. The protocol has been pre-approved by the ethical committee of Weifang Medical University.

All animals were randomly divided into three groups (N=15 each): control group; VILI model group, IgG control, glucocorticoide and efalizumab treated group. Except control group, animals in other 4 groups were anaesthetized by 10% chloral hydrate (in-house prepared) followed by tracheal intubation. MV was then performed on an animal respiratory machine (Harvard, US) using the parameters (tidal volume: 20 mL/kg; oxygen concentration: 21%; respiratory rate: 70 per minute; H_2O PEEP: 0 cm). The MV lasted for four hours. In IgG, glucocorticoid andefalizumab groups, animals received drug injection of IgG, glucocorticoid andefalizumab (final concentrations were 2.5 mg/kg in PBS, Life technologies, US), respectively, 72 hours before the surgery as previously reported [15]. Control group received the same surgical procedure without connecting respiratory machine. Four hours after surgery, all animals were sacrificed for sample collection.

Analysis of BALF

1×PBS (2 mL per reaction) was added for rinsing left pulmonary tissues. The rinsing buffers were collected and centrifuged for 10 min at 4°C. The supernatant was frozen at -80°C. Cell pellets were re-suspended in 1×PBS (0.5 mL). Total cell number was deduced from a hematocytometer. Other suspensions were prepared using slide centrifugation for 15 min. A clear pattern of evenly distributed cell was stained for further classification.

Enzyme-linked immunosorbent assay (ELISA)

Both TNF-α and IL-8 levels in BALF were determined by ELISA using relevant test kits (Abcam, US) following manual instruction. In brief, serial diluted standard samples were added into 96-well plate. Test samples were also put into pre-designated wells, which were added with enzyme-linked reagents for 30-min incubation at 37°C. After gentle washing, chromogenic substrate A and B were sequentially added into each well, followed by 10-mn incubation at 37°C. The reaction was stopped by adding stopping buffer. A microplate reader was used to measure the optical density (OD) value at 450 nm in each well. A linear regression was performed to plot a standard curve, by which we could evaluate the sample concentration.

Real-time PCR

Animals were sacrificed for collecting pulmonary tissues, which were grinded by tissue homogenizer on ice. The lysis mixture was intensively rinsed by buffer, and was centrifuged for 2 min with its supernatants being transferred into a new tube. Total RNA was extracted by Trizol (Invitrogen, US) and RNA extraction kit (Invitrogen, US). cDNA was then synthesized using specific primers (SP-A-F: 5'-GATCG CTCGT AGCAC TAGGA C-3'; SP-A-R: 5'-TATCC CGGCC GGTCA GCCT-3'; GAPDH-F: 5'-CAGTA GTACC TGTTG CTGG-3'; GAPDH-R: 5'-TAATA GACCC GGTCT GGTAT G-3) with reverse transcription kit (Invitrogen, US). The real-time PCR was carried out using pre-mixture (Invitrogen, US) on a fluorescent PCR machine. PCR conditions were set as: 90°C denature for 30 sec, 58°C annealing

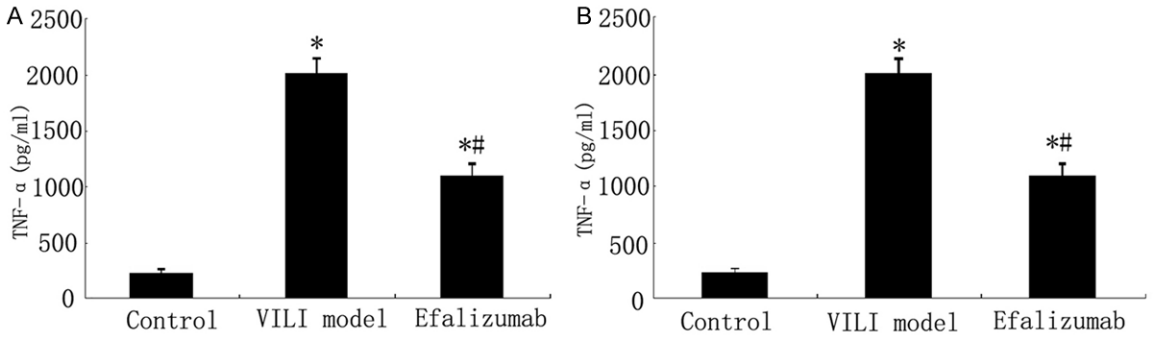

Figure 1. TNF-α and IL-8 levels in BALF. A. TNF-α concentrations; B. IL-8 concentrations. *P<0.05 compared to the control group; #P<0.05 compared to the model group.

for 50 sec, plus 72°C extension (35 cycles in total). The relative level of mRNA was determined using $2^{-\Delta Ct}$ method, and GAPDH gene was used as the reference gene.

Western blot

Pulmonary tissues were lysed in lysis buffer on ice for 15~30 min. After sonic rupture of cells for 5 sec (4 times), lysate was centrifuged at 10 000 g for 15 min, with the supernatant being transferred to a new tube. Proteins extracted were separated by 10% SDS-PAGE, and were transferred to PVDF membrane (Pall Life, US). Non-specific binding sites were blocked by blocking buffer contains 5% skimmed milk powder for 2 hours at room temperature. Rabbit anti-human SP-A monoclonal antibody (1:1000, Cell signaling, US) was used to incubate the membrane at 4°C overnight. After gentle washing in PBST, the membrane was further incubated in goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (1:2000, Cell signaling, US) for 30 minutes. The results were visualized by ECL reagents (AmershamBiosci., US) and were exposed under X-ray. Images were captured and analyzed for relative density by Quantity One software.

Statistical analysis

SPSS 20.0 software (SPSS, Chicago, IL, USA) was used for data analyses. Enumeration data were compared by using chi-square test, and measurement data were presented as mean ± standard deviation ($\bar{x}\pm s$). Multiple group comparison was finished by one-way analysis of variance (ANOVA). Differences P<0.05 indicated statistically significant.

Results

Effect of efalizumab on the cell count of nuclear cells and neutrophils

BALF collected from all groups were quantified for the numbers of nuclear cells and neutrophils. Results showed significantly elevated numbers of both cells in BALF of VILI model rats, compared to controlled animals (P<0.05). The intervention by efalizumabor glucocorticoid significantly decreased the number of inflammatory cells, although still higher than those in the control and IgGgroups (**Table 1**). These results suggest the up-regulation of inflammatory cells in VILI, and the protective role of efalizumab by decreasing inflammatory cell numbers.

TNF-α and IL-8 expression levels in BALF

The contents of TNF-α and IL-8 in BALF of all animals were determined by ELISA. Results illustrated significantly rise of TNF-α and IL-8 levels in BALF in VILI model rats in comparison to controlled animals (**Figure 1A** and **1B**, P< 0.05). The application of efalizumab significantly decreased levels of both cytokines in BALF, although still higher than those in the control and IgGgroup (**Figure 1A** and **1B**, P<0.05). The expressions of cytokines in glucocorticoid and efalizumab groups showed no significant difference.

SP-A gene expression in pulmonary tissues

We firstly used real-time PCR to detect mRNA levels of SP-A gene in rat pulmonary tissues. It has been demonstrated that there were significantly depressed level of SP-A mRNA in VILI

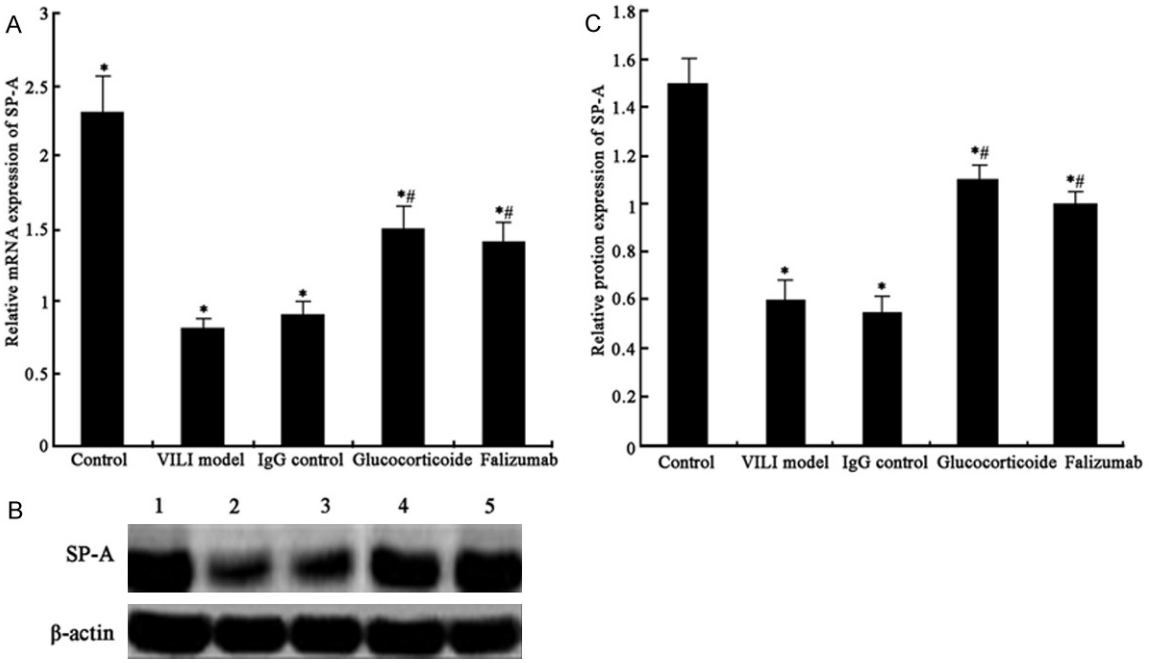

Figure 2. SP-A expression level in pulmonary tissues. A. SP-A mRNA relative level (against GADPH); B. Western blotting bands of SP-A protein in pulmonary tissues from all rats (1. Control; 2. VILI model; 3. IgG control; 4. Glucocorticoid; 5. efalizumab); C. Quantitative results of SP-A protein levels against β-actin levels. $^{*}P<0.05$ compared to the control group; $^{\#}P<0.05$ compared to the model group.

model rats, compared to the control group (**Figure 2A**, P<0.05). The application of efalizumab restored SP-A mRNA levels, although still lower than the control and IgGgroups. These results indicated that the potential injury of MV on pulmonary tissues was via decreasing SP-A expression. Efalizumab, on the other hand, may provide protection of pulmonary tissues via facilitating SP-A gene expression.

Western blotting assay consistently confirmed that VILI rats had significantly suppressed SP-A protein expression compared to those in the control group (**Figure 2B**, **2C**, *P*<0.05). Similar to the result in the glucocorticoid group, the intervention by efalizumab elevated SP-A protein levels but still lower than the control and IgGgroups. These results further suggest the role of efalizumab in protecting pulmonary tissues via potentiating SP-A protein expression levels.

Discussion

MV plus primary pulmonary disease may aggravate the biological injury of the lung tissue, leading to other organ failure or even multiple organ dysfunction syndromes. It has been shown that the hyperextension of lung tissue by MV may elicit biochemical signals transducing into the cell, thus activating local inflammatory cells and inducing release of large amounts of inflammatory cytokines or factors [16, 17]. Our study demonstrated the aggravated pulmonary damages during VILI by the increased secretion of TNF-α and IL-8. Efalizumab, as a humanized monoclonal antibody (IgG1κ), a novel immunosuppressive agent, inhibits the release of inflammatory cytokines. Our data demonstrated thatefalizumab decreased expressions of TNF-α and IL-8by interfering the function of IgG1κ. Nonetheless, the presence of IgG showed elevated expressions of TNF-α and IL-8 during VILI that even caused severer injury, suggesting that efalizumabexerts an alleviated effecton VILI treatment.

Mainly targeting T cell for immune suppression, efalizumab interferes with primary T cell activation, thus mediating the release of inflammatory cytokines in addition to the regulation of memory T cell production. It also inhibits body's immune system via impeding the migration of T cells, and hindering the binding between lymphocyte adhesion molecule LFA-1 and endothelial ICAM-1 in a rate-limit step of early migration phase [18, 19]. Efalizumab therefore exerts its anti-inflammatory roles in inhibiting the release

of pro-inflammatory cytokines. During the occurrence and progression of VILI, inflammatory cells including neutrophils and polymorphic nuclear cells may lead to inflammatory response. The mechanical stretch may also facilitate the recruitment of neutrophils in pulmonary alveoli and release of inflammatory factors. For examples, TNF induces endothelial cells in micro vessels to expression E-selectin, initiating an interaction with its partners to activate adhesion, migration, penetration and chemotactic aggregation of neutrophils into the inflammatory sites, further aggravating pulmonary damage [20, 21]. Our study showed significantly elevated numbers of both nuclear cells and neutrophils in BALF of VILI model rats. The intervention of efalizumab suppressed the total number of those two types of cells, suggesting the potentiation of inflammatory cells in VILI and protection by efalizumab by decreasing inflammatory cell number.

Pulmonary surfactant (PS) is a mixture containing lipoproteins and sugars, and is synthesized and secreted by type II epithelial cells of pulmonary alveoli. It plays an important role in maintaining normal cell structure and function via decreasing surface tension of pulmonary alveoli, keeping alveoli dry, preventing pulmonary edema, relaxing smooth muscle, participating local defense and improving mucosa properties. SP-A belongs to one of specific markers of PS [22] and was shown to be down regulated in VILI pulmonary tissues via both mRNA and protein assays. The application of efalizumab, besides the abovementioned functions including inhibiting chemotactic recruitment of inflammatory cells and suppressing inflammatory factors, also facilitate the expression of mRNA and protein of SP-A in rat lung tissues. Glucocorticoid, on the other hand,exerts anti-inflammatory function that reduces alveolar tension and also plays a favorable role in the treatment of VILI. However, long-term use of glucocorticoids causesa variety of side effects including the aggravation of infection, induction of cardiovascular disease [23]. Therefore in this study, we compared theefficacy of damage alleviation between the treatment of glucocorticoid and efalizumab. The results unraveleda similar role of both treatments in reducingon the cell count of nuclear cells and neutrophils, inhibiting the secretion of inflammatory factors, and preventing further injuries in lung tissue.

Our study thus suggests the contribution of efalizumabin facilitating the therapy of VILI without potential side effects of glucocorticoid. In summary, efalizumab may provide protections against the damage of VILI, via multiple pathways including inhibiting recruitment of inflammatory cells, suppressing release of pro-inflammatory cytokines, and potentiating SP-A expression in lung tissues. This study elucidates the protection of efalizumab on VILI and possible mechanisms, and provides novel drug targets for future clinical treatment of VILI.

Disclosure of conflict of interest

None.

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