Upregulation of Galectin-3 by *Corynebacterium kutscheri* Infection in the Rat Lung

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Abstract: *Corynebacterium* (C) *kutscheri* and *Staphylococcus aureus* were isolated from two Sprague-Dawley (SD) rats with a hemisected spinal cord. Grossly, gray-white bulging foci and abscesses were distributed throughout the parenchyma of the lung. Pathologically, severe necrotizing lobar pneumonia with abscesses and fibrinous pleuritis were observed. Immunohistochemical analysis found accumulation of galectin-3 in alveolar macrophages and the alveolar interstitial region. No other viral or bacterial pathogens were detected in these animals. In addition, similar pathogenic changes and accumulation of galectin-3 were observed in the lungs of SD rats experimentally infected with *C. kutscheri*. Using northern blot analysis, the relative galectin-3 and GAPDH mRNA levels were 4.6 to 9.3 times higher in *C. kutscheri*-infected lung than in uninfected controls. These results demonstrate that a single *C. kutscheri* infection can induce the upregulation of galectin-3 in the lung and that this molecule may have an important pathogenic role in *C. kutscheri* infections in rats.

Key words: *Corynebacterium kutscheri*, galectin-3, immunohistochemical, *Staphylococcus aureus*

Introduction

Corynebacteria are Gram-positive, aerobic, non-motile, rod-shaped bacteria that form irregularly shaped, club-shaped or V-shaped arrangements during normal growth [3]. Infection with *Corynebacterium* (C) *kutscheri* in laboratory mice and rats is usually subclinical but stresses such as nutritional deficiencies, immunosuppressive drugs, irradiation, experimental manipulation, increased ammonia concentrations and concurrent infections can induce clinical diseases [1, 15]. *C. kutscheri* infection in mice and rats has been

reported previously but the pathogenic mechanism of this bacterium has not been well described.

Galectin-3, a type of mammalian lectin, has β -galactoside-binding ability. Galectin-3 is expressed and secreted by a wide variety of cells, including monocytes, macrophages, granulocytes, eosinophils, basophils and mast cells, and has been shown to have roles in cell differentiation, proliferation, adhesion and spreading [4].

Recently, using a murine *Streptococcus pneumoniae* model, extracellular galectin-3 that was released by alveolar macrophages or pulmonary parenchymal cells was shown to act as an adhesion molecule for neutro-

(Received 25 July 2006 / Accepted 27 October 2006)

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phil extravasation. Expression of galectin-3 was dependent on the presence of some bacteria, such as *Streptococcus pneumoniae*, but not others, such as *Escherichia coli*, although both bacteria induced migration of neutrophils into the alveoli and the release of a proinflammatory factor [13].

Here we describe a case of co-infection with *C. kutscheri* and *Staphylococcus aureus* in immunosuppressed Sprague-Dawley (SD) rats and the role of galectin-3 in *C. kutscheri* infection.

Materials and Methods

History

Eight male SD rats, 6 weeks of age, were subjected to spinal cord hemisection at a non-barrier facility of Yonsei University (Seoul, Korea) and injected with cyclosporine A (CsA). The SD rats were bought from Samtaco (Gyeonggi, Korea). Six of these rats died at 4 weeks after the hemisection, and two of the remaining rats showed weight loss and dyspnea. These latter two rats were transferred to the Korea Research Institute of Biotechnology and Biology (KRIBB) for health monitoring as reported previously [17]. Here we present our findings regarding these two rats.

Gross findings and bacterial isolation

In the two rats showing weight loss and dyspnea, grossly, numerous gray-white bulging foci and abscesses were distributed throughout the parenchyma of the lung, and fibrinous adhesions to the thoracic wall were observed (Fig. 1). In addition, gray-white pus was found on the hemisected spinal cord. Bacteria were collected from the trachea and abscesses of each animal using a sterile cotton swab and streaked onto trypticase soy agar plates supplemented with 5% sheep blood, and the plates were incubated for 2 days at 37°C. Cultures from both rats exhibited substantial growth of bacteria, which, based on morphological characteristics after Gram staining, were assumed to be *Corynebacterium sp.* and *Staphylococcus sp.* Eight strains of *Staphylococcus sp.* were selected from two rats and characterized biochemically by API Staph V.4.0 (BioMerieux, Marcy l'Etoile, France) and a coagulase test with EDTA-rabbit plasma (Difco Laboratories, Detroit, MI, USA). In addition, eight strains of *Corynebacterium sp.* were selected from two rats and characterized biochemically

Fig. 1. Gray-white abscesses in the lung and fibrinous adhesions in the thorax.

using API Coryne V. 2.0. To detect antibody of the pathogen-related respiratory diseases such as cilia-associated respiratory (CAR) bacillus, Sendai virus and rat parvovirus, we used the enzyme linked immunosorbent assay (ELISA) and the indirect immunofluorescent antibody (IFA) method as reported previously [17]. We also used PCR to detect *Pneumocystis carinii* in pulmonary tissues as described previously [8].

16S rRNA gene sequencing

The extraction of genomic DNA for 16S rRNA gene-sequence determination, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described previously [9, 16]. Briefly, each *Corynebacterium* strain was grown on blood agar plates at 37°C for 48 h. The cells were harvested, washed and suspended in 150 µl of sterile phosphate-buffered saline solution. Genomic DNA was prepared from the suspensions using a DNeasy Tissue kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer's instructions, and the 16S rRNA gene amplicon of 1498 bp was amplified and sequenced using a BigDye terminator cycle sequencing kit (Perkin-Elmer Biosystems, Foster City, CA, USA) and an ABI PRISM 377 sequencer. For sequencing reactions, we used 16S rRNA universal primers, and primers specific to *Corynebacterium*, which were designed using the 16S rRNA gene sequences of the genus *Corynebacterium* (Cory1, CCG TGA GGG ATT GAA

GG; and Cory2, ATT TGA CGT CAT CCC CAC CTT CC). The following conditions were used for amplification: denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and elongation at 72°C for 45 s, with 5 s added for each elongation step. A total of 25 cycles were performed, followed by a final elongation step at 72°C for 15 min. Sequence analysis was performed using the DNASTar program (DNASTAR Inc., Madison, WI, USA).

Histopathological and immunohistochemical findings

For histopathological examination, the kidneys, livers, lungs, heart, and spleen of each rat were fixed in phosphate-buffered 10% formalin and processed routinely for paraffin sectioning. The specimens were stained with hematoxylin and eosin (HE) or silver stain. For immunohistochemical analysis, mouse monoclonal antibody against human galectin-3 (NCL-GAL3, Novocastra Laboratories, Newcastle, UK) was used. Standard avidin-biotinylated peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA) immunostaining was performed according to the manufacturer's instructions [2]. The stained cells were visualized by incubating the sections for 5 min with 3,3'-diaminobenzidine tetrahydrochloride.

Experimental infection with C. kutscheri

Ten specific pathogen-free (SPF) male SD rats, 6 weeks of age, were purchased from Samtaco (Gyeonggi, Korea) and maintained under the SPF conditions. The rats were housed in autoclaved polycarbonate cages with wire lids. All feed, water and bedding were sterilized by steam autoclaving. They were housed in a temperature (22°C ± 2°C) and humidity (50%–60%) controlled room with air changes (15 times/day) and a 12-h light and dark cycle. Each rat was inoculated with 10⁷ *C. kutscheri* isolated in this study, as measured by viable cell count. The bacteria were suspended in 50 µl of PBS, and inoculated by intranasal injection. At the same time, the rats were injected with CsA (2.5 mg/kg per 12 h). These rats were euthanized after 2 weeks, and the lung was collected for histological studies and RNA isolation. All experimental procedures were carried out according to a protocol approved by the Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology.

Northern blotting

We performed northern blotting using a galectin-3-specific ³²P-labeled probe according to a previously reported method [10], with some modifications. Briefly, total RNA was isolated from infected rat lungs using TRIZOL (Invitrogen, CA, USA) reagent, according to the manufacturer's instructions. RNA electrophoresis and transfer were performed according to standard procedures, and 20 µg of total RNA were loaded in each lane. Hybridization was carried out using Express Hyb solution. Transcript levels were quantified by autoradiography and densitometric analysis was performed using Photocapt version 97-04 for Windows and Bioprofile Bio1D Windows application version 99-04. Hybridization with glyceraldehyde phosphate dehydrogenase (GAPDH) probe [10] was used as a normalizing control. Relative galectin-3 and GAPDH mRNA levels are expressed as galectin-3/GAPDH mRNA ratios.

Results

Bacterial isolation

The eight isolated *Staphylococcus sp.* were all shown to be Gram-positive cocci, positive for coagulase agglutination, and all strains were identified as *Staphylococcus aureus* by API Staph V.4.0. The isolated *Corynebacterium sp.* were all shown to be Gram-positive irregular rod-shaped bacteria, and were identified as *C. kutscheri* by API Coryne V. 2.0. Several new species of the genus *Corynebacterium* have recently been isolated from human and animal sources [14]. We confirmed the identification of our eight *C. kutscheri* by 16S rRNA sequencing. Each of the 16S rRNA sequences was 100% identical to that of *C. kutscheri* type strain CIP 103423 (ATCC 15677). We did not detect any pathogens except *Staphylococcus aureus* and *Corynebacterium kutscheri*.

Pathological and immunohistochemical findings

In the lungs of the two rats subjected to spinal cord hemisection that subsequently showed weight loss and dyspnea, we observed severe necrotizing lobar pneumonia and abscesses of variable size. Alveoli were filled with neutrophils, an admixture of various cell debris, proteinaceous fluids, fibrin and macrophages (Fig. 2A). The centers of these lesions were necro-

Fig. 2. Severe lobar pneumonia with abscess formation in the lung. (A) Lobar pneumonia due to co-infection with *Corynebacterium kutscheri* and *Staphylococcus aureus*. Hematoxylin and eosin (HE) staining. Bar=100 μ m. (B) *Staphylococcus aureus* colony (arrowhead) in the left ventricle of a heart. HE staining. Bar=100 μ m. (C) Irregular form of bacteria (arrows) in the infected lung. Silver staining. Bar=20 μ m. Insert: high magnification of an irregular form of bacteria. Bar=44 μ m. (D & E) Absence of labeling with galectin-3-specific antibodies in normal lung (D) and a neutrophil-infiltrated region (E). Avidin-biotinylated peroxidase complex (ABC) staining. Bar=100 μ m. (F) Strong galectin-3 immunolabeling induced by *C. kutscheri*. ABC staining. Bar=100 μ m. Insert: high magnification of galectin-3 positive macrophage. Bar=283 μ m.

tized. Alveolar walls were thickened with infiltrating mononuclear cells. Occasionally, *Staphylococcus* colonies and *Corynebacterium*-specific irregular rod-shaped bacteria were found in lesions in the heart and lung (Fig. 2B and 2C). We observed only one abscess with *Staphylococcus* colonies in the left ventricle of heart. This abscess had originated from direct bacterial invasion of epicardium from empyema in the thoracic cavity.

Immunohistochemical results showed that alveolar macrophages and mononuclear cells at the lung lesions were strongly galectin-3-positive. In addition, diffuse accumulation of galectin-3 was observed in the alveolar space with lobar pneumonia induced by co-infection with *C. kutscheri* and *Staphylococcus aureus*. We did not observe galectin-3-positive cells in normal parts or abscesses of the lung (Fig. 2D and 2E). In addition, mucin accumulation was found around the alveolar macrophages using periodic acid-Schiff (PAS) stain (data not shown). We did not detect any other significant lesions or bacteria in any of the other rat tissues examined.

Experimental infection with *C. kutscheri*

To investigate whether galectin-3 expression can be induced in the lung by a single *C. kutscheri* infection, we experimentally inoculated 10 CsA-treated SD rats with the *C. kutscheri* isolated in this study. The typical lobar pneumonia and abscesses caused by *C. kutscheri* infection were founded in 4 of the 10 rats. As we observed in the cases of natural infection, galectin-3-positive cells were observed in the alveolar space and alveolar interstitium using immunohistochemical analysis (Fig. 2F), a finding similar to that observed in a *Streptococcus pneumonia* model [13]. The intensity of galectin-3 immunolabeling was the greatest in alveolar macrophages (Fig. 2F).

To confirm upregulation of galectin-3 in infected lung tissue, we performed northern blotting. Like the ICH results, galectin-3 expression was significantly increased in infected lungs compared with uninfected controls. Relative galectin-3 and GAPDH mRNA levels in *C. kutscheri*-infected lungs were 4.6 to 9.3 times higher than those in uninfected controls (Fig. 3).

Discussion

In this present study, we isolated *C. kutscheri* and

Fig. 3. Northern blot analysis. Galectin-3 RNA expression was upregulated in *C. kutscheri*-infected lungs in SD rats. Lanes 1 and 2: uninfected control; lanes 3-6: *C. kutscheri*-infected lung.

Staphylococcus aureus from two SD rats with a hemisected spinal cord. Severe necrotizing lobar pneumonia with abscesses and fibrinous pleuritis were also observed in these two rats. *Staphylococcus* and *Corynebacterium* infections are among the most common opportunistic post-surgery infections in humans and animals [5]. During organ transplantation and some other types of surgery, immunosuppressive agents are used to prevent graft rejection and to suppress autoimmune responses. However, immune suppression increases the risk of opportunistic infection by subclinical pathogens. Immunosuppressive agents have been reported to increase the prevalence of bacterial isolates, as well as to change the spectrum of bacterial flora [7, 11]. Thus, spinal cord hemisection and treatment with the immunosuppressive agent, CsA may have been important factors in the cases of pneumonia induced by co-infection with *Corynebacterium* and *Staphylococcus*.

Using immunohistochemistry, we observed the accumulation of galectin-3 in alveolar macrophages and the alveolar interstitial region in the lungs of the two rats from which *C. kutscheri* and *Staphylococcus aureus* were isolated. So we investigated whether galectin-3 expression can be induced in the lung by a single *C. kutscheri* infection. In this study, similar pathogenic changes and accumulation of galectin-3 were observed

in the lungs of SD rats experimentally infected with *C. kutscheri*. Also galectin-3 expression was significantly increased in the infected lungs compared with uninfected controls using northern blot analysis. We used an antibody against human galectin-3 to detect rat galectin-3. Gebhardt *et al.* [6] reported that the normal rat pancreas is stained with two antisera against human galectin-3 (Gal169 and Gal170), and that the alignment of human and rat galectin-3 amino acid sequences revealed an 80% homology of these proteins.

Galectin-3 is a powerful proinflammatory signal in immune responses. When secreted and externalized, galectin-3 may affect inflammatory cells by an autocrine or paracrine mechanism. This molecule can induce respiratory burst in neutrophils and monocytes, and induce mediator release by mast cells. Galectin-3 also promotes adhesion of human neutrophils to laminin and endothelial cells, and acts as a chemoattractant for monocytes and macrophages [4, 12]. However, *in vivo*, galectin-3 expression and function in lung infections have not been well characterized. In a mouse asthma model, galectin-3 is one of the main pathogenic factors involved in disease progression and the immune response [18]. In addition, in a murine *Streptococcus pneumonia* model, extracellular galectin-3 released by alveolar macrophages or pulmonary parenchymal cells was shown to act as an adhesion molecule for neutrophil extravasation [13]. In our study, we observed upregulation of galectin-3 in alveolar macrophages and the alveolar interstitial region in *C. kutscheri*-infected lungs. Our results indicate that upregulation of galectin-3 plays an important role in the pathogenic mechanism of *C. kutscheri*. Thus, the *C. kutscheri* infection model may be a useful *in vivo* model for understanding galectin-3-related immunological responses in lung diseases caused by bacterial infection.

Acknowledgments

This work was supported by the faculty research fund of Konkuk University in 2005.

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