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Acute Inflammatory Response to Contrast Agent Aspiration and Its Mechanisms in the Rat Lung

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Objectives/Hypothesis: Contrast agent (CA) aspiration is an established complication of upper gastrointestinal and videofluoroscopic swallow studies. The underlying molecular biological mechanisms of acute response to CA aspiration in the respiratory organs remain unclear. The aims of this study were to elucidate the histological and biological influences of three kinds of CAs on the lung and to clarify the differences in acute responses.

Study Design: Animal model.

Methods: Eight-week-old male Sprague Dawley rats were divided into five groups (n = 6 in each group). Three groups underwent tracheal instillation of one of three different CAs: barium (Ba) sulfate, nonionic contrast agents (NICAs), and ionic contrast agents (ICAs). A control group was instilled with saline and a sham group was instilled with air. All animals were euthanized on day 2 after treatment and histological and gene analysis was performed.

Results: No animal died after CA or control/sham aspiration. Ba caused severe histopathologic changes and more prominent inflammatory cell infiltration in the lungs compared with the two other iodinated contrast agents. Increases in expressions of inflammatory cytokines (tumor necrosis factor [*Tnf*], interleukin-1β [*Il1b*], and interferon-γ [*Ifng*]) were observed in Ba aspiration rats, and upregulation of *Il1b* was seen in ICA aspiration rats. NICA did not cause obvious histologic changes or expressions of inflammatory cytokines and fibrosis-related genes in the lungs.

Conclusions: Ba caused significantly more acute lung inflammation in a rodent model than did ioinic and nonionic iodinated CAs. Nonionic contrast did not cause any discernible inflammatory response in the lungs, suggesting that it may be the safest contrast for videofluoroscopic swallow studies.

Key Words: Contrast agents, acute response, lung, inflammatory cells, inflammatory cytokines.

Level of Evidence: NA

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INTRODUCTION

Dynamic contrast videofluoroscopic swallow studies (VFSSs) are an essential part of the diagnostic workup of persons with dysphagia.[1] Radiographic contrast agents (CAs) are also essential in esophagography and more distal upper-gastrointestinal studies.[2] Barium (Ba) sulfate, nonionic contrast agents (NICAs: iohexol, iodixanol), and ionic contrast agents (ICAs: diatrizoic acid) are the most common CAs currently used for VFSS and upper-gastrointestinal investigations. These studies are frequently performed on persons at risk for aspiration, and the consequences of CA aspiration must be considered.

Clinical reports of pulmonary complications due to CA aspiration have emerged,[3,4] and previous studies have revealed that CA aspiration causes pulmonary complications such as inflammatory cell infiltration, pulmonary edema, pneumonitis, and fibrosis.[5,6] Since these initial reports, investigations regarding the pulmonary effects of CA aspiration have described conflicting findings. Aspiration of Ba sulfate, the most widely used CA, has been reported as having no negative effects on the respiratory system,[7,8] whereas other studies described its severe toxicity and adverse effects on the respiratory organs.[2,9–11] Moreover, only a few studies demonstrated the acute inflammation and histological changes in the lung following aspiration of NICAs and ICAs in animal studies,[5,12,13] but clinical effects have not been shown.

Although radiographic CAs are commonly utilized for radiographic testing, basic studies for the effects of aspirated CA on the respiratory system have been limited. To date, most studies have focused solely on morphological and histological examinations. We previously reported that large quantities of aspirated Ba, ICA, and NICA influenced survival and caused lung injury in a lagomorph model.[2] Further investigation is required to

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R.U. and T.S. developed the concept, designed and performed the experiments, and analyzed the data. T.G. developed the concept and designed and performed the experiments. N.Z., T.N, P.B., and T.Y. developed the concept and designed the experiments. All authors contributed to interpretation of the data and writing of the manuscript.

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evaluate the degree of pulmonary injury resulting from smaller doses of aspirated material that would more accurately represent aspiration during VFSS.

In this study, we aimed to explore the underlying molecular biological mechanisms of the acute response to CA aspiration on the respiratory organs. To this end, we performed immunohistological examinations of the lung at the acute phase following aspiration of small amounts of CAs (60 μL). In addition, we investigated the inflammatory and fibrotic responses by utilizing real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR).

MATERIALS AND METHODS

Rats

Eight-week-old male Sprague Dawley rats were purchased from CLEA Japan, Inc. (Tokyo, Japan). Rats were housed in a temperature-controlled environment under a 12-hour light–dark cycle with access to food and water ad libitum. All animal experiments were conducted in accordance with institutional guidelines and with the approval of the Animal Care and Use Committee of the University of Tokyo (no. P17-126).

Contrast Agents

In this study, we used three kinds of CAs: Ba suspension (Barytgen HD, 60% w/v; Fushimi Pharmaceutical, Kagawa, Japan), NICA (iohexol [Omnipaque] iodine, 300 mg/mL, osmolality 640 mOsm/kg H_2O, viscosity 6.1 mPa·s; Daiichi-Sankyo, Tokyo, Japan), and ICA (diatrizoate sodium solution [Gastrografin] iodine, 370 mg/mL, osmolality 1940 mOsm/kg H_2O, viscosity 8.9 mPa·s; Daiichi-Sankyo).

Rat Model of CA Aspiration

The rat model of CA aspiration was adapted from the rabbit model used in a previous study, in which CAs (3 mL/rabbit) were intratracheally administrated (Fig. 1A,B).[2] Rats were divided into five groups (n = 6). Group 1 received an air injection and served as a sham model. Group 2 received saline injection (60 μL) and served as a control model. Groups 3, 4, and 5 received administration of 60 μL of three kinds of CAs (Table I). CAs were administered via the larynx to the trachea under direct laryngoscopy under ketamine hydrochloride and xylazine hydrochloride anesthesia (50 μg/g body weight and 10 μg/g body weight, respectively, i.p.). Sham and control rats received air injection and saline administration according to the same schedule as CA-treated rats. All rats were sacrificed on day 2 after the intratracheal procedures. In addition, survival rate of rats was calculated in each group.

TABLE I.
Animal Groups and Administration Protocol.

Group	Agents Administered
1	Air, 60 μL
2	Saline, 60 μL
3	Ba sulfate suspension (60% w/v), 60 μL
4	ICA (diatrizoic acid [Gastrografin]), 60 μL
5	NICA (iohexol [Omnipaque]), 60 μL

Ba = barium; ICA = ionic contrast agent; NICA = nonionic contrast agent.

Tissue Preparation

The left and right lobes of the lung were harvested for histological and qRT-PCR analyses on day 2 after intratracheal administration of CA. Histological samples and qRT-PCR samples were obtained from the same rats. Immediately after sacrifice, the lungs were excised and tissue samples were fixed in 4% paraformaldehyde for a further 24 hours. Tissues were dehydrated in a series of graded ethanol solutions, then embedded in paraffin.

Histological Analyses

Histological analysis was performed on the inferior lobe of the right lung. Four-μm-thick serial paraffin sections were deparaffinized in xylene and dehydrated in ethanol before hematoxylin and eosin staining (for evaluation of whole tissue structure) or immunostaining.

Before immunostaining, antigen retrieval was performed using antigen retrieval solution (S1700; Dako, Tokyo, Japan) and sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were then incubated with Blocking One (Nacalai Tesque, Tokyo, Japan) for 30 minutes at room temperature to block nonspecific antibody binding. Primary antibodies against cluster of differentiation 3 (CD3; 1:300 dilution, rabbit monoclonal; Nichirei Corp., #413601, Tokyo, Japan) and myeloperoxidase (MPO; 1:300 dilution, rabbit polyclonal; ab9535; Abcam, Cambridge, United Kingdom) were detected with peroxidase conjugated appropriate secondary antibodies and a diaminobenzidine substrate. Images of the lungs were captured using a digital microscope camera (BZ-X700; Keyence, Itasca, IL) with 4× and 20× objective lenses.

qRT-PCR

Total RNA was isolated from the left robes of the lungs using TRIzol reagent (Life Technologies, Tokyo, Japan) on day 2, then reverse-transcribed into cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan), according to

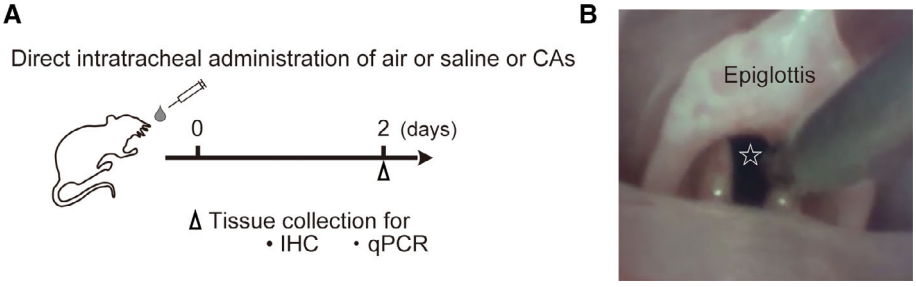

Fig. 1. (A) Experimental timeline. Rats were intratracheally administered CAs or air or saline. Subsequently, the lungs were collected for analyses by IHC and qRT-PCR on day 2. (B) The endoscopic view of intratracheal administration. A white star shows the airway lumen. CA = contrast agent; IHC = immunohistochemistry; qRT-PCR = real-time quantitative reverse transcription polymerase chain reaction. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

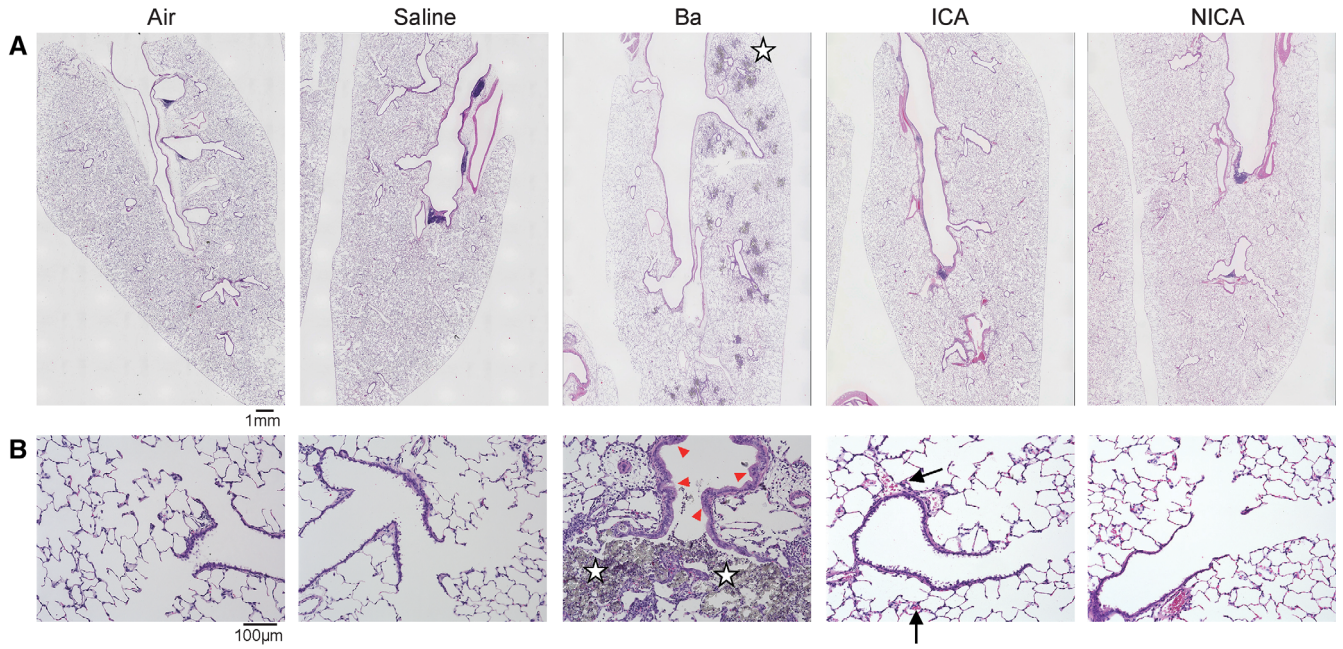

Fig. 2. Representative images of HE-stained sections of the lungs from all groups (A, 40× magnification; B, 200× magnification). Ba particles were diffusely found in alveolar cavities phagocytosed by inflammatory cells (white stars). Triangles indicate thickening of the bronchioles and alveolar ducts in the Ba-treated rats. In the ICA-treated group, only slight congestion of red blood cells in the alveoli was observed (black arrows). Ba = barium; HE = hematoxylin and eosin; ICA = ionic contrast agent; NICA = nonionic contrast agent. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

the manufacturer's instructions; analysis was performed in duplicate using Thunderbird Sybr. qPCR Mix (Toyobo) and an Applied Biosystems (ABI) 7500 sequence detection system (Life Technologies). Gene expressions of inflammatory cytokines (tumor necrosis factor [*Tnf*], interleukin-1β [*Il1b*], and interferon-γ [*Ifng*]) and fibrosis-related factors (transforming growth factor β1 [*Tgfb1*] and platelet-derived growth factor-α [*Pdgfa*]) were evaluated. The gene-specific primers and probes used were the following: rat *Actb* (actin, β) as endogenous control (forward, 5′-CTAAGGCCAACCGTGAAAAG-3′; reverse, 5′-ACCAGAGGCATACAGGGACA-3′); rat *Tnf* (forward, 5′-TGTGCCTCAGCCTCTTCTC-3′; reverse, 5′-GAGCCATTTGGGAACTTCT-3′); rat *Il1b* (forward, 5′-TGTGATGAAAGACGGCACAC-3′; reverse, 5′-CTTCTTCTTTGGGTATTGTTTGG-3′); rat *Ifng* (forward, 5′-TGAAAGCCTAGAAAGTCTGAAGAAC-3′; reverse, 5′-CGTGTTACCGTCCTTTTGC-3′); rat *Tgfb1* (forward, 5′-CATTGCTGTCCCGTGCAGA-3′; reverse, 5′-AGGTAACGCCAGGAATTGTTGCTA-3′); and rat *Pdgfa* (forward, 5′-CTGAGGATGCCTTGGAGACAAAC-3′; reverse, 5′-TCTTGCAAACTGCGGGAATG-3′). The expression levels of each gene were normalized to the level of *Actb* expression for each sample.

Statistical Analysis

Statistical comparisons between groups were performed by one-way analysis of variance with post-hoc Tukey tests using GraphPad Prism, version 6.0 (GraphPad Software, San Diego, CA). The qRT-PCR data were subjected to logarithmic transformation before analysis. A P value <.05 was considered to be statistically significant.

RESULTS

Mortality

There was no mortality in any group.

Ba Causes Severe Histopathologic Changes of the Lung Compared With ICA and NICA

First, we investigated pathological changes in the lungs of all groups. Histopathologic analyses by hematoxylin and eosin staining revealed that saline and NICA did not cause any detectable histologic changes in the lungs, as compared with the lung of the air-treated sham group. Pulmonary edema was not notable in the saline- and NICA-treated groups. In the Ba-treated group, however, Ba particles spread throughout the lungs, and significant inflammatory changes were detected on day 2 after Ba aspiration. Thickening of the bronchioles and alveolar ducts, inflammatory cell infiltration in the bronchiolar and alveolar walls, and yellowish-brown barium particles phagocytosed by inflammatory cells in alveolar cavities were all appreciated. In the ICA-treated group, only slight congestion of red blood cells in the alveoli was observed, but no pulmonary edema or other pathological changes were recognized (Fig. 2).

Ba Sulfate Induces Prominent Inflammatory Cell Infiltration in the Lung Compared With ICA and NICA

To discriminate the type of inflammatory cells, we next performed immunohistochemical staining. Myeloperoxidase $(MPO)^+$ granulocytes and macrophages and $CD3^+$ lymphocytes were infiltrated in the lungs of the Ba-treated group. MPO^+ and $CD3^+$ cells were rarely detected in the lungs of air-sham, saline-control, ICA-treated, and NICA-treated groups. In the Ba-treated group, MPO^+ granulocytes and macrophages were scattered and infiltrated in many

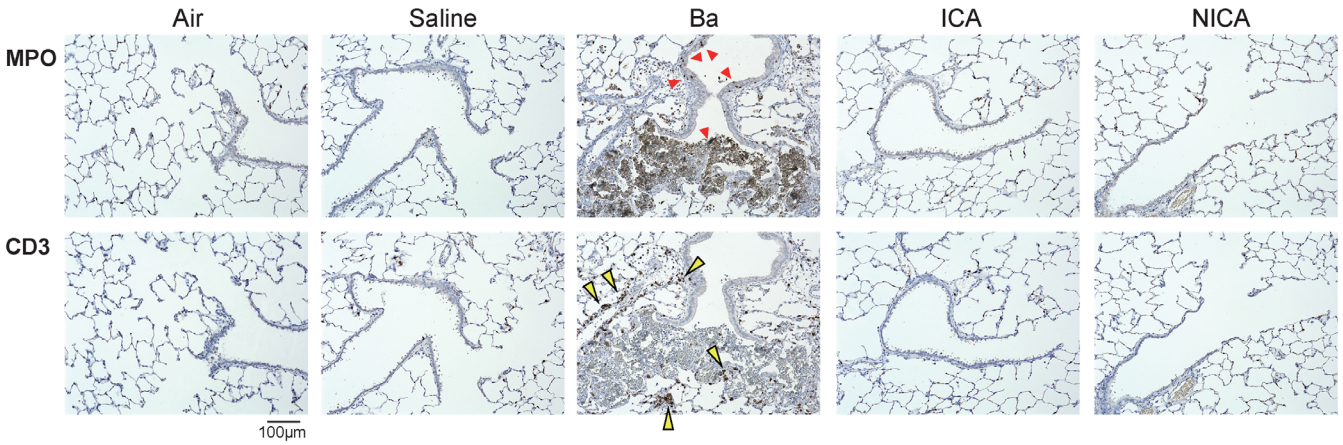

Fig. 3. Representative images (200× magnification) of tissues stained with antibodies against MPO (red arrowheads) and CD3 (yellow arrowheads) were shown. In the Ba-treated group, MPO^+ granulocytes or macrophages were scattered and infiltrated in many alveolar cavities, and $CD3^+$ lymphocytes were diffusely found and infiltrated in the walls of bronchioles. Ba = barium; CD3 = cluster of differentiation 3; ICA = ionic contrast agent; MPO = myeloperoxidase; NICA = nonionic contrast agent. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

alveolar cavities, and $CD3^+$ lymphocytes were diffusely found and infiltrated in the walls of bronchioles.

CA Aspiration Increases Expression of Inflammatory Cytokines and Fibrosis-Related Genes in the Lung

Finally, to determine the molecular background of respiratory inflammation by CA aspiration, we analyzed the expression of inflammatory cytokines and fibrosis-related genes in the lungs. The qRT-PCR analyses revealed that the expression levels of *Tnf*, *Il1b*, and *Ifng* in the Ba-treated group were significantly higher than in the air-treated (sham) and saline-treated (control) groups (*Tnf*: $P = .0003$ and $P = .0007$; *Il1b*: $P = .0007$ and $P = .0004$; *Ifng*: $P = .0005$ and $P = .001$, respectively). In addition, only an expression of *Il1b* in the ICA-treated group was significantly increased compared with those in

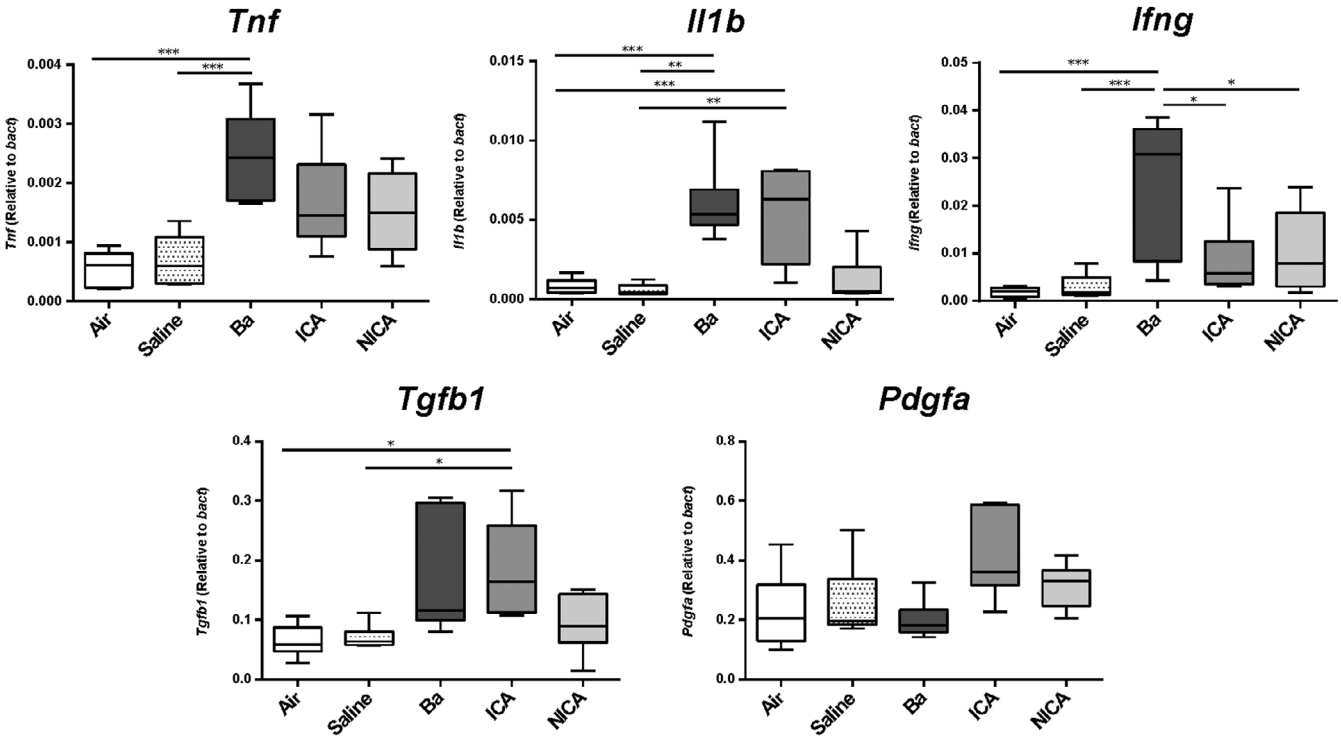

Fig. 4. *Tnf*, *Il1b*, *Ifng*, *Tgfb1*, and *Pdgfa* expression levels in the lungs were quantified by qRT-PCR and are expressed relative to the expression of the endogenous control gene *Actb.* Data represent the mean, minimum, and maximum (n = 6; $*P < .05$, $**P < .01$; $***P < .001$, one-way ANOVA). ANOVA = analysis of variance; Ba = barium; ICA = ionic contrast agent; *Ifng* = interferon-γ; *Il1b* = interleukin-1β; NICA = nonionic contrast agent; *Pdgfa* = platelet-derived growth factor-α; qRT-PCR = real-time quantitative reverse transcription polymerase chain reaction; *Tgfb1* = tumor growth factor-β1; *Tnf* = tumor necrosis factor.

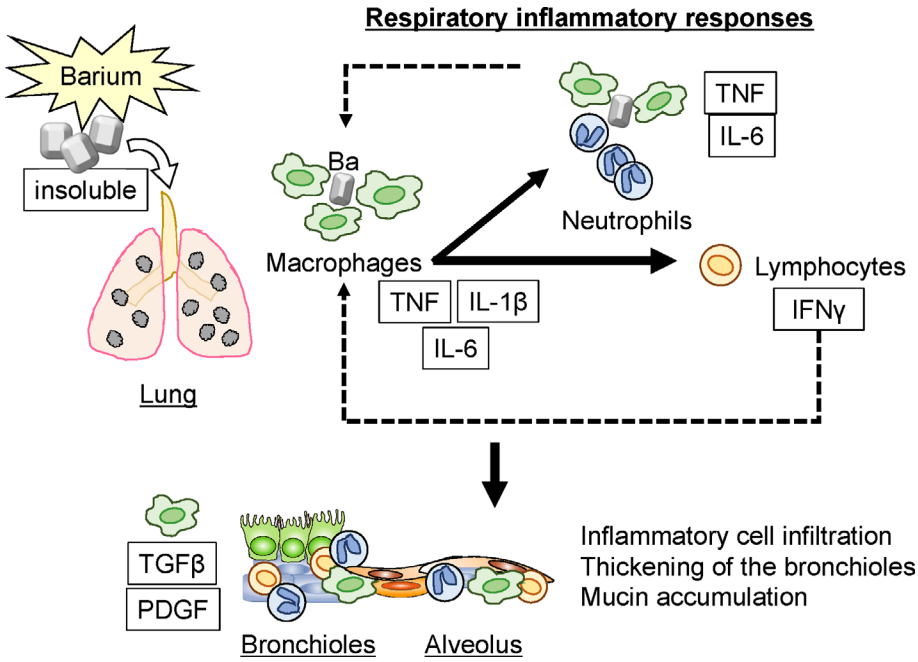

Fig. 5. Diagram of Ba-induced pulmonary inflammation. Ba = barium. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

the sham and control groups (P = .0027 and P = .0016, respectively). There were no significant differences in expression of inflammatory cytokines and fibrosis-related genes between the NICA group and the sham and control groups. These results were consistent with the results from histologic analyses and suggest that Ba aspiration induced more inflammatory responses and NICA caused virtually no inflammatory responses in the lungs.

DISCUSSION

Pulmonary complications including infiltration of inflammatory cells, pulmonary edema, pneumonitis, and fibrosis due to CA aspiration have been reported.[2–13] The underlying molecular biological mechanisms of acute responses to CA aspiration in the lungs have not been well examined. In this study, we performed histological and molecular-biological examinations of the lungs using more clinically representative animal models of small-dose contrast aspiration. Consequently, we obtained the following results: Ba induced thickening of the bronchioles, MPO^+ and $CD3^+$ inflammatory cell infiltration, and increases in expressions of inflammatory cytokines in the lungs. ICA induced congestion of red blood cells in the alveoli and increases in the expression of *Il1b* with no histological changes. NICA did not cause any histological changes and increases in gene expressions of inflammatory cytokines.

Ba particles are insoluble, but both ICA and NICA are water-soluble. Thus, the underlying mechanisms of pulmonary complications by aspiration can be supposed to be different depending on the type of aspirated chemicals. Therefore, we examined gene expressions of the lungs to elucidate the underlying molecular mechanisms. *Tnf*, an inflammatory cytokine, is produced by many different cell types, such as macrophages, lymphocytes, neutrophils, fibroblasts, and endothelial cells.[14] This cytokine induces inflammatory mediators, such as *Il1b* and interleukin-6,[15] which activate macrophages and neutrophils, potentially causing acute respiratory failure.[14,15] *Il1b* is released by macrophages, dendritic cells,[16] and stressed epithelial cells[17] in the lungs. Induction of *Il1b* expression in the lung of adult mice caused pulmonary inflammation characterized by neutrophil and macrophage infiltrates. In another study, *Il1b* increased the thickness of conducting airways, enhanced mucin production, and caused lymphocytic aggregates in the airways.[18] *Ifng* is synthesized mostly by T lymphocytes and natural killer cells after activation of these cells with antigen-specific immunity and inflammatory stimuli. The main function of *Ifng* is macrophage activation, rendering them able to exert its microbicidal functions.[19,20] *Pdgf* and *Tgfb* are representatives of fibrogenic-associated factors in the lung. *Pdgf* is produced by platelets and macrophages, and *Tgfb* is a product of alveolar macrophages and bronchiolar epithelial cells.[21,22]

In Ba-treated rats, gene expressions of inflammatory cytokines *Tnf*, *Il1b*, and *Ifng* were significantly increased after aspiration. This may result from immunological responses that identified insoluble Ba particles as foreign. We hypothesize that macrophages are recruited to the immunological reaction site and *Tnf* and *Il1b* are produced. These subsequently cause neutrophil and macrophage infiltrates and lymphocytic aggregates. Gathered T lymphocytes may produce *Ifng*, activating macrophages which induce respiratory inflammatory responses in a chain reaction, resulting in thickening of the bronchioles and mucin accumulation (Fig. 5).

ICA aspiration caused only slight congestion of red blood cells in the alveoli and increase in expression of *Il1b*, and it did not induce inflammatory cell infiltration in the lung. The high osmolality of ICA is considered to be associated with congestion.[2] Considering that the acute pulmonary edema caused by ICAs resolves and clears within 24 hours,[13,23] severe pulmonary congestion and edema may have possibly existed in the lung at an early stage after ICA administration and may have pathologically improved on day 2 after aspiration. In addition, NICA may induce little inflammation responses in the lung when aspirated in small quantities, as pathologic changes such as inflammatory cell infiltration and pulmonary edema were not recognized in the NICA-treated lungs and expressions of inflammatory cytokines did not increase on day 2 after aspiration.

Airway remodeling and pulmonary fibrosis can occur after acute inflammation in the lung.[18] High expression of *Tgfb1* on day 2 following ICA aspiration indicates that ICA may cause pulmonary fibrosis in the chronic stage. As the present study focused on acute effects of CA aspiration on the lung, further investigation is required to evaluate pulmonary inflammation and fibrosis in the chronic phase. In addition, detailed investigations of chronic stages following Ba aspiration are necessary to gain a further understanding of whether deposited Ba particles are cleared and to elucidate the possible influences of BA deposits on pulmonary tissue. Serious complications and several deaths have been reported due to Ba aspiration in humans.[10,11] The determination of the safest CA for use should be based on short- and long-term advantages with regards to radiographic opacity, water solubility and osmolality, viscosity, and pulmonary toxicity.

As for limitations of the present study, the evaluation timing was set on day 2 after CA aspiration, considering the possibility that CA aspiration could induce various inflammatory cells via the above mechanisms of cytokine production. Additional investigations in other timings would be desirable.

CONCLUSION

This study demonstrated that small amounts of Ba aspiration caused significantly more acute lung inflammation in a rodent model than ICA and NICA. NICA caused less inflammatory responses in the lungs, suggesting that NICA may be the safest contrast agent for VFSS. This study utilized both histopathologic and gene analyses to elucidate the underlying mechanisms of acute inflammatory responses to different contrast agents.

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