Int J Clin Exp Med 2016;9(5):8421-8425
www.ijcem.com /ISSN:1940-5901/IJCEM0016575

Original Article

Expression of RAGE and sRAGE in adenoidal hypertrophy with secretory otitis media in children

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Received September 21, 2015; Accepted March 29, 2016; Epub May 15, 2016; Published May 30, 2016

Abstract: Objective: To investigate the expression of receptor for advanced glycation endproducts (RAGE) in adenoid hypertrophy in children and the change in the level of soluble receptor for advanced glycation endproducts (sRAGE) in tympanic effusion. Methods: Fifteen adenoidal specimens were collected from patients with adenoidal hypertrophy and secretory otitis media and patients with simple adenoidal hypertrophy without secretory otitis media who underwent surgical treatment at our hospital from April 2010 to December 2014. In addition, fifteen tympanic effusion specimens were collected from patients with simple secretory otitis media and from patients with adenoidal hypertrophy and secretory otitis media. Western blot analysis and immunohistochemistry were used to detect RAGE expression in adenoidal tissue, and an enzyme-linked immunosorbent assay (ELISA) was performed to determine the sRAGE levels in tympanic effusion. Results: Immunohistochemical staining showed that RAGE was expressed in each hypertrophic adenoidal tissue specimen and that RAGE protein expression levels were significantly higher in hypertrophic adenoidal tissue from patients with secretory otitis media than from those without secretory otitis media ($P<0.05$). Moreover, the sRAGE levels were significantly higher in tympanic effusion from patients with secretory otitis media and adenoidal hypertrophy than from patients with simple secretory otitis media ($P<0.05$). Conclusion: RAGE and sRAGE may play an important role in the pathogenesis of adenoidal hypertrophy with secretory otitis media.

Keywords: RAGE, sRAGE, adenoidal hypertrophy with secretory otitis media in children

Introduction

Secretory otitis media is a common disease in children, and persistent chronic secretory otitis media can cause severe hearing impairment, which leads to learning and behavioral problems. Therefore, early detection and prompt treatment are important for treating secretory otitis media [1, 2]. Previous studies show that adenoidal hypertrophy and the immune response are important causes of secretory otitis media [3]. Receptor for advanced glycation endproducts (RAGE) is a transmembrane protein with more than 400 amino acids, and recent studies show that RAGE is an important transmembrane protein for initiating inflammatory and immune responses [4, 5]. In this study, we used immunohistochemical staining to detect RAGE expression levels in adenoidal tissue from children with adenoidal hypertrophy and secretory otitis media and in children with simple adenoidal hypertrophy. The soluble receptor for advanced glycation endproducts (sRAGE) levels were determined in tympanic effusions from children with simple secretory otitis media (with no significant adenoidal hypertrophy) and from children with adenoidal hypertrophy and secretory otitis media to generate novel ideas and methods for preventing and treating secretory otitis media in children.

Materials and methods

General data

In this study, we investigated 30 patients with adenoidal hypertrophy who underwent surgical treatment at our hospital from April 2010 to December 2014, including 21 males and 9 females from 3-13 years old (median: 7 years old). Fifteen patients had adenoidal hypertrophy with secretory otitis media, and 15 patients had simple adenoid hypertrophy without secretory otitis media. We also collected tympanic

effusions from 15 patients with adenoid hypertrophy and secretory otitis media and from 15 patients with simple secretory otitis media (with no significant adenoidal hypertrophy) who were in the same age group and received inpatient or outpatient treatment during the same period. The specimens were processed as follows: a small piece of adenoidal tissue was cut from the resected specimen, placed and fixed in paraformaldehyde, and embedded in paraffin. A second piece of adenoidal tissue and a tympanic effusion specimen were also stored in a freezer at -80°C. The inclusion criterion was no previous history of surgery. Patients who received antibiotics or glucocorticoid therapy three weeks before surgery were excluded from this study.

Western blot analysis of RAGE protein expression

We collected 100 mg of resected adenoidal tissue, and the tonsil tissue was homogenized using protein lysate (Biyuntian Biotech Co., Ltd., Nantong, China) followed by centrifugation at 12,000 rpm for 10 minutes. Next, the supernatant was collected, and we determined the protein quantity using the bicinchoninic acid (BCA) method. The protein sample was then mixed (at a certain ratio) with protein loading buffer (Biyuntian Biotech Co., Ltd., Nantong, China) and boiled in water for 10 minutes. Next, 35 μg of the protein sample was used for electrophoresis and then transferred to a membrane. The membrane was blocked in 5% skim milk followed by adding β-actin and RAGE antibodies (Santa Cruz Biotechnology, Inc., USA, 1:200 diluted with 5% milk) for incubation at 4°C overnight. After a TBST wash, the secondary horseradish peroxidase-labeled goat anti-rabbit antibody (diluted with 5% milk) was added, the sample was incubated followed by a TBST wash, and 1:1 enhanced chemiluminescence (ECL) agent was added (according to the instructions) for gel imaging. Image J software was used for gray-scale analysis.

Immunohistochemical staining

The resected tissue specimen was fixed in 10% formalin and embedded in paraffin, and 4-μm sections were prepared. The slides were baked at 60°C for 8 hours for later use. After soaking with distilled water, antigens were prepared in EDTA buffer at 100°C for 20 minutes and then cooled to room temperature. After washing with distilled water and PBS, a peroxidase-blocking solution was added followed by incubation at room temperature for 10 minutes. After a PBS wash, non-immune serum of animal source was added and then discarded after incubation at room temperature for 10 minutes. The primary anti-RAGE antibody (1:50 dilution; Santa Cruz Biotechnology, Inc., USA) was added followed by incubation at room temperature for 60 minutes. PBS was used as a negative control. After a PBS wash, a biotin-labeled secondary antibody (goat anti-rabbit IgG, Zhongshan Jinqiao Biotech Co., Ltd., Beijing, China) was added followed by incubation at room temperature for 10 minutes. After a PBS wash, a streptavidin-anti-biotin-peroxidase solution was added followed by incubation at room temperature for 10 minutes. After a PBS wash, DAB chromogenic reagents were added. Next, the slides were washed with tap water, re-stained with hematoxylin, washed with tap water until the color turned blue, dehydrated and dried with ethanol, cleared in xylene, and mounted with neutral resin. Two experienced and blinded pathologists independently interpreted the results; they counted 15 fields on each slide at a high magnification (× 400), and the mean number of RAGE-positive cells was used as the final result for each patient.

Enzyme-linked immunosorbent assay (ELISA)

The human sRAGE ELISA kit was purchased from UNOCI Biological Technology Co., Ltd. (Hangzhou, China), and the procedure was performed strictly in accordance with the kit instructions. Before the assay, the kit and specimens were returned to room temperature. Next, 50 μl of standard protein dilutions and the testing specimens were added to specific reaction wells, and 50 μl of a biotin-labeled antibody was then immediately added. After the plate was sealed with a seal membrane, it was shaken gently to mix the components and then placed in an incubator at 37°C for 1 hour. Next, the solution in the wells was discarded, washing buffer was added, and the plate was shaken for 30 seconds. The washing buffer was then removed, and the plate was pat dry using a piece of absorbent paper. Next, 80 μl of biotin streptavidin-HRP was added to each well, and the plate was gently shaken to mix the components and placed in an incubator at 37°C for 30 minutes. The solution in the wells

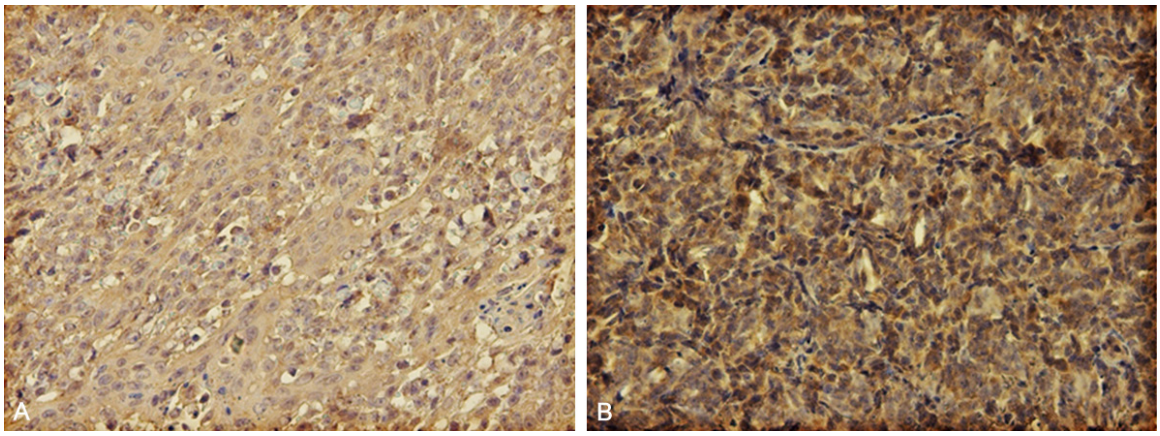

Figure 1. Immunohistochemical staining (× 400, upper panel: simple adenoidal hypertrophy without secretory otitis media; lower panel: adenoidal hypertrophy with secretory otitis media).

Table 1. Immunohistochemical RAGE staining

Group	n	RAGE (positive cell number)	RAGE/β-actin
Simple adenoidal hypertrophy without secretory otitis media	15	32 ± 9	0.23 ± 0.09
Adenoidal hypertrophy with secretory otitis media	15	195 ± 14#	1.52 ± 0.98
t value		37.93	5.08
P value		<0.001	<0.001

#t=37.93, P<0.001, compared with the simple adenoidal hypertrophy without secretory otitis media group.

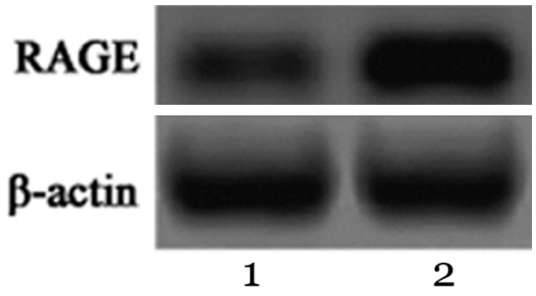

Figure 2. Western blot analysis (1. simple adenoidal hypertrophy without secretory otitis media; 2. adenoidal hypertrophy with secretory otitis media).

was then discarded, washing buffer was added, and the plate was shaken for 30 seconds. Next, the washing buffer was removed, and 50 μl each of substrate A and B was added to each well. The plate was then gently shaken to mix the components and placed in the dark in an incubator at 37°C for 10 minutes. Next, the microtiter plate was removed, 50 μl of stop solution was added quickly, and the results were determined immediately. The optical density (OD) at 450 nm was measured in each well. The OD value was then used for the y-coordinate, and the standard solution concentration was used for the x-coordinate to produce a standard curve; the protein concentration of each plasma sample was determined using the standard curve based on the OD value.

Statistical analysis

SPSS17.0 software was used for statistical analyses. A t-test was performed for inter-group comparison. P<0.05 was considered statistically significant.

Results

RAGE protein expression in adenoidal tissue

RAGE protein immunohistochemical staining is shown in **Figure 1**. Positive staining for RAGE protein is shown as yellow and brown particles. The results show that RAGE protein was expressed in the specimens from each patient with adenoidal hypertrophy with or without secretory otitis media, and the statistical analysis (**Table 1**) shows significantly more RAGE-positive cells in patients with adenoidal hypertrophy and secretory otitis media than with simple adenoidal hypertrophy without secretory otitis media (P<0.05). Western blot

Table 2. The sRAGE expression level in tympanic effusions from patients

Group	n	sRAGE (pg/ml)
Adenoidal hypertrophy with secretory otitis media	15	6105 ± 547.1
Simple secretory otitis media	15	3003 ± 412.3
t value		17.54
P value		<0.001

analysis shows that the RAGE protein expression level was significantly higher in patients with adenoidal hypertrophy and secretory otitis media than in those with simple adenoidal hypertrophy without secretory otitis media (**Figure 2**; **Table 1**, P<0.05).

The sRAGE expression level in tympanic effusion

The ELISA (**Table 2**) shows that the sRAGE expression level was significantly higher in tympanic effusions from patients with adenoidal hypertrophy and secretory otitis media than from patients with simple secretory otitis media (with no significant adenoidal hypertrophy) (P<0.05).

Discussion

RAGE is a member of the immunoglobulin superfamily and is expressed in membranes of monocytes, macrophages, vascular endothelial cells, smooth muscle cells, and fibroblasts. The RAGE protein consists of a large extracellular fragment, a transmembrane fragment, and a short intracellular fragment. The extracellular fragment has an immunoglobulin-like structure with a V-shaped fragment linking two C-shaped fragments, and each fragment contains a pair of conserved cysteine residues. The V-shaped fragment also contains two N-coupling glycosylation sites. These features are important for the RAGE molecular structure stability and specific ligand recognition. The main function of the extracellular fragment is to bind specific ligands. The intracellular fragment is richly charged and binds intracellular signaling molecules to produce biological effects. Ligands that bind RAGE include advanced glycation end-products (AGEs), amphotericin, and calgranulin, which bind RAGE to activate it; activated RAGE then exerts its biological effects mainly through activating nuclear factor (NF)-κB. NF-κB binds target gene sequences to regulate expression for many inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6; thus, it is involved in the body's inflammatory and immune responses [6]. Previous studies show that adenoidal abnormalities and immune response are two key factors in the development of adenoidal hypertrophy with secretory otitis media [7]. This study shows that the RAGE protein is mainly expressed in the cell membrane and in the adenoidal tissue specimens from patients with adenoid hypertrophy with or without secretory otitis media. Statistical analyses showed that the RAGE protein expression level was significantly higher in adenoidal tissue from patients with adenoidal hypertrophy and secretory otitis media than from patients with simple adenoidal hypertrophy. The results herein suggest that high RAGE expression levels may play an important role in the pathogenesis of adenoidal hypertrophy with secretory otitis media by regulating the immune response.

Previous studies demonstrate a complex local cytokine network in the ear cavities of patients with secretory otitis media. Further, a variety of cytokines interact to induce migration and activation of inflammatory cells, which leads to changes in mucosal enzyme activity and a delay in healing or persistent secretory otitis media, further aggravating middle ear effusion, mucosal lesions, bone destruction, fibrosis, and, in severe cases, hearing impairment [11, 12]. Currently, cytokines isolated and detected in middle ear effusions include interleukins (including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, and IL-10), interferons, TNF, granulocyte-macrophage colony stimulating factor, and transforming growth factor β [8-10]. sRAGE is an endogenous secretory RAGE and does not include the RAGE transmembrane fragment; it has become a reliable inflammatory biomarker in many immune disorders and inflammation-related diseases [13, 14]. This study shows that the sRAGE expression level was significantly higher in patients with adenoidal hypertrophy and secretory otitis media than in patients with simple secretory otitis media, which further suggests that RGAE may play an important role in the pathogenesis of adenoidal hypertrophy with secretory otitis media.

In summary, the results suggest that RAGE may play an important role in the pathogenesis of adenoidal hypertrophy with secretory otitis

media and that animal experiments are necessary to further confirm the specific mechanisms.

Disclosure of conflict of interest

None.

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References

[1] Wallace IF, Berkman ND, Lohr KN, Harrison MF, Kimple AJ, Steiner MJ. Surgical treatments for otitis media with effusion: a systematic review. Pediatrics 2014; 133: 296-311.

[2] Elicora SS, Ozturk M, Sevinc R, Derin S, Dinç AE, Erdem D. Risk factors for otitis media effusion in children who have adenoid hypertrophia. Int J Pediatr Otorhinolaryngol 2015; 79: 374-7.

[3] Wang B, Tang X, Xu J, Yao H. Differential expression of Immunoglobulin A in the adenoids of children with and without exudative otitis media. Int J Pediatr Otorhinolaryngol 2012; 76: 728-30.

[4] Ott C, Jacobs K, Haucke E, Navarrete Santos A, Grune T, Simm A. Role of advanced glycation end products in cellular signaling. Redox Biol 2014; 2: 411-29.

[5] Song J, Lee WT, Park KA, Lee JE. Receptor for advanced glycation end products (RAGE) and its ligands: focus on spinal cord injury. Int J Mol Sci 2014; 15: 13172-91.

[6] Zhao J, Randive R, Stewart JA. Molecular mechanisms of AGE/RAGE-mediated fibrosis in the diabetic heart. World J Diabetes 2014; 5: 860-7.

[7] Somuk BT, Soyalic H, Koc S, Gürbüzler L, Doğru S, Eyibilen A. Mean platelet volume as an inflammatory marker of chronic otitis media with effusion. Int J Pediatr Otorhinolaryngol 2014; 78: 1958-60.

[8] Lee HY, Chung JH, Lee SK, Byun JY, Kim YI, Yeo SG. Toll-like receptors, cytokines & nitric oxide synthase in patients with otitis media with effusion. Indian J Med Res 2013; 138: 523-30.

[9] Zhang Y, Li S, Wang G, Han D, Xie X, Wu Y, Xu J, Lu J, Li F, Li M. Changes of HMGB1 and sRAGE during the recovery of COPD exacerbation. J Thorac Dis 2014; 6: 734-41.

[10] Kim SH, Cha SH, Kim YI, Byun JY, Park MS, Yeo SG. Age-dependent changes in pattern recognition receptor and cytokine mRNA expression in children with otitis media with effusion. Int J Pediatr Otorhinolaryngol 2015; 79: 229-34.

[11] Lee SY, Ryu EW, Kim JB, Yeo SG. Clinical approaches for understanding the expression levels of pattern recognition receptors in otitis media with effusion. Clin Exp Otorhinolaryngol 2011; 4: 163-7.

[12] Thomas K, Boeger D, Buentzel J, Esser D, Hoffmann K, Jecker P, Mueller A, Radtke G, Geißler K, Finkensieper M, Guntinas-Lichius O. Pediatric adenoidectomy: a population-based regional study on epidemiology and outcome. Int J Pediatr Otorhinolaryngol 2013; 77: 1716-20.

[13] El-Seify MY, Fouda EM, Nabih ES. Serum level of soluble receptor for advanced glycation end products in asthmatic children and its correlation to severity and pulmonary functions. Clin Lab 2014; 60: 957-62.

[14] Al RM, Schneider AL, Alonso A, Maruthur N, Parrinello CM, Astor BC, Hoogeveen RC, Soliman EZ, Chen LY, Ballantyne CM, Halushka MK, Selvin E. sRAGE, inflammation, and risk of atrial fibrillation: results from the Atherosclerosis Risk in Communities (ARIC) Study. J Diabetes Complications 2015; 29: 180-5.