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Different Patterns of Acetylation and Dimethylation of Histone H3 between Young and Aged Cases with Chronic Tonsillitis: Influences of Inflammation and Aging

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Introduction: Epigenetics is now considered to be crucially involved in normal genetics and differentiation and in pathological conditions, such as cancer, aging, and inflammation. Epigenetic mechanisms involve DNA methylation and histone modifications. The purpose of this study was to investigate the effects of inflammation on epigenetics in young subjects and the effect of aging.

Materials and Methods: The palatine tonsils were extracted from child and adult patients with chronic tonsillitis. Hematoxylin-eosin staining was performed to examine the morphology of the palatine tonsils. A fluorescence immunological examination was also performed to detect acetyl-histone H3 or dimethyl-histone H3. Confocal scanning microscopy was used for observations.

Results: Acetylated histone H3 was detected in tonsils from child patients but not from adult patients. Dimethylated histone H3 was not detected in tonsils from either group of patients. Degeneration of the tonsillar structures was apparent in tonsils from adult patients.

Discussion: The differential expression of acetylated histone H3 Lys9 may reflect immunological differences between young and aged tonsils. The decrease observed in the activity of histone methyltransferase induced the down-regulated expression of methylated histone H3.

Conclusion: Our results suggest that epigenetic changes participate in chronic inflammation and aging in the palatine tonsils. Although the results do not lead to a direct treatment, the epigenetic pathogenesis of chronic inflammation, such as immunoglobulin A nephropathy, by focal infections will be described in greater detail in future studies, which will lead to new treatments being developed.

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Key words: epigenetics, histone modification, chronic tonsillitis, acetylation, dimethylation

Introduction

Epigenetics is now considered to be critically involved in normal genetics and differentiation and in pathological conditions, such as cancer, aging, inflammation, and immunological disorders[1–4]. Epigenetic mechanisms are known to involve DNA methylation and histone modifications that alter gene expression without changing original DNA sequences[1]. The modifications of DNA methylation and histone are closely related to each other. Epigenetic modifications have been detected in inflammatory diseases, such as[5] chronic kidney disease[6], and rheumatoid arthritis[7]. Aging has also been shown to involve DNA methylation and histone modifications[2,8].

Chronic tonsillitis is a common disorder in otorhinolaryngology. Patients with chronic tonsillitis have recurrent high fever, a sore throat, and, sometimes, peritonsillar and parapharyngeal abscesses[9]. A radical treatment for chronic tonsillitis is tonsillectomy. The incidence of tonsillectomy is reportedly highest among patients 4 to 16 years old, with 81% of patients with chronic tonsillitis who undergo tonsillectomy being younger than 20 years, after which the incidence of tonsillectomy decreases[10]. We

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Table 1 Patient information

Patient	Age, years	Sex	Chief complain	Clinical course
1	2	female	fever sore throat	chief complaint recurs
2	5	female	fever, sore throat	chief complaint recurs
3	6	male	fever	chief complaint recurs
4	4	male	fever	chief complaint recurs
5	40	male	fever, sore throat, dysphagia	chief complaint recurs
6	46	male	fever, sore throat	chief complaint recurs
7	37	male	fever, sore throat	chief complaint recurs
8	37	male	fever, sore throat	chief complaint recurs

The palatine tonsils of 8 patients (6 male and 2 female) with chronic tonsillitis were extracted. The main symptom was fever. None of our patients had focal infections, such as immunoglobulin A nephropathy and palmoplantar cystic disease.

performed tonsillectomy for patients with chronic tonsillitis. The etiology of age varied from young to aged patients.

The purpose of the present study was to investigate histone modification patterns under inflammation, particularly in young patients, and to determine the effects of aging on these patterns.

Materials and Methods

Palatine tonsils were extracted from 8 patients (6 male and 2 female) with chronic tonsillitis. All patients had previously had 6 or more episodes of tonsillitis per year or 3 or more episodes in the 2 years preceding this study and underwent tonsillectomy in our hospital in 2012 or 2013. Tonsillectomy was performed with the patient under general anesthesia. The tonsils were grasped with a clamp, the tonsillar capsule was separated from the tonsillar bed, and the tonsils were then extracted.

Patients (**Table 1**) were divided into 2 groups: a child group (n=4) aged 2 to 6 years (mean age ± SD, 3 ± 2 years) and an adult group (n=4) aged 37 to 46 years (mean age, 40 ± 4.2 years). Patients did not have focal infections, such as immunoglobulin A nephropathy, and palmoplantar cystic disease.

The protocol used was in accordance with the Declaration of Helsinki and the guidelines for research and was approved by the ethics committee of our institution (No. 339). All patients or their parents gave their informed written consent to be included in this study. The ethics committee approved only the examination of chronic tonsillitis. The excision of normal tonsils was not allowed for ethical reasons, and the collection of other tissues was not permitted.

Therefore, as a positive control the staining results of an aged mouse model from our previous study were added[11]. Eight female mice (C57BL/6) were divided into a group of 4 young mice aged 4 weeks and a group of 4 old miced aged 132 weeks. The temporal bones were extracted and embedded in paraffin. All staining procedures used were the same as those described for the tonsils. These staining results shown have not been published in previous studies.

Immunohistochemical Study for CD4

After paraffin had been removed, the sections were heated for 7 minutes at 105℃ with Target retrieval solution (pH 9.0; Dako, Glostrup, Denmark). Sections were then incubated with the primary antibody to CD4 at a 1 : 100 dilution (anti-CD4, NCL-CD4-IF6, Leica Biosystems, Newcastle, UK) overnight at 4℃. The tissues were then incubated with 0.1% H_2O_2 for 30 minutes, followed by a second antibody labeled by biotin. After being reacted with avidin-biotin complex reagents, tissues were developed with diaminobenzidine. Tissues were washed with distilled water and then stained with hematoxylin.

Fluorescence Immunological Examination

The extracted palatine tonsils were immediately cut into small pieces and fixed with 4% (w/v) paraformaldehyde. These tissues were immersed in the same fixative overnight. The specimens were embedded in paraffin and sectioned into 6-μm-thick slices with a microtome (RM2165, Leica, Bartels and Stout, Inc., Issaquah, WA, USA). Hematoxylin and eosin staining was performed in order to examine the morphology of these tissues under a light microscope (BX41, Olympus Optical Co., Ltd., Tokyo, Japan). After paraffin had been removed, the sections were immersed in 3% H_2O_2 for 20 minutes and then in Triton-X for 10 minutes. Sections were incubated with the primary antibody to acetyl-histone H3 Lys9 at a

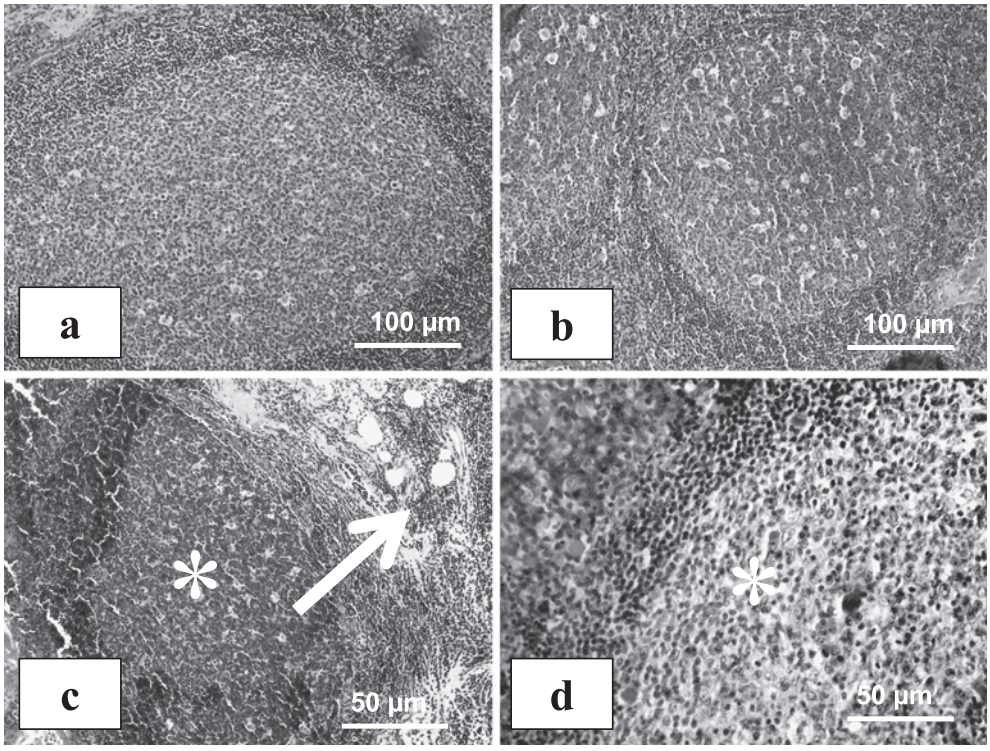

Fig. 1 Six-micrometer-thick paraffin sections of the palatine tonsils. Hematoxylin and eosin staining.
(a) The tonsils of patient 2 presented a mature structure, in which the germinal center and a large number of lymphocytes were detected, ×20.
(b) The same mature structure of the tonsils was observed in patient 3, ×20.
(c) The degeneration of lymphoid tissues (*) and fatty changes (**arrow**) were observed in case 5, ×20.
(d) The degeneration of the germinal center (*) was also detected in patient 6, ×20.

1 : 1,000 dilution (rabbit polyclonal antibody, 9671, Cell Signaling Technology, Danvers, MA, USA) or dimethyl-histone H3 Lys9 at a 1 : 1,000 dilution (rabbit polyclonal antibody, 6847, Cell Signaling Technology) overnight at 4℃. The tissues were then incubated with the second antibody at a 1 : 500 dilution (anti-rabbit, Cy3, Dako, Glostrup, Denmark) and were immersed in 4′,6-diamidino-2-phenylindole at a 1 : 50,000 dilution and a fluorescent dye (DRAQ5, BOS-889-001-R200, Biostatus Ltd., Shepshed, Leicestershire, UK) at a 1 : 2,000 dilution. Confocal scanning microscopy was performed with a Zeiss microscope (Laser model LS710, Carl Zeiss Microimaging, Jena, Germany). A laser was adjusted at wavelengths of 405 and 561 nm. Fluorescent images were captured with a software program (Zen 2011 ver. 700285, Carl Zeiss Microimaging).

Results

Morphological Changes Revealed by Hematoxylin and Eosin Staining in Human Patients

The tonsils of patient 2, a child, presented a mature structure, in which the germinal center and a large number of lymphocytes were detected (**Fig. 1a**). The same mature structure was observed in the tonsils from patient 3 (**Fig. 1b**). The structure of the tonsils presented the same features in all patients who were children.

The degeneration of lymphoid tissues and fatty changes were observed in the tonsils of patient 5, an adult (**Fig. 1c**). The degeneration of the germinal center was also detected in patient 6 (**Fig. 1d**). Degenerative changes in the germinal center were a common feature in the tonsils from all patients who were adults.

Immunohistochemical Study for CD4

Palatine tonsil tissues in the young group showed positive stainings for CD4. CD-positive cells (T-cells) were scattered in the germinal center and surrounding areas (**Fig. 2a, b**). In the older group, T-cells were also

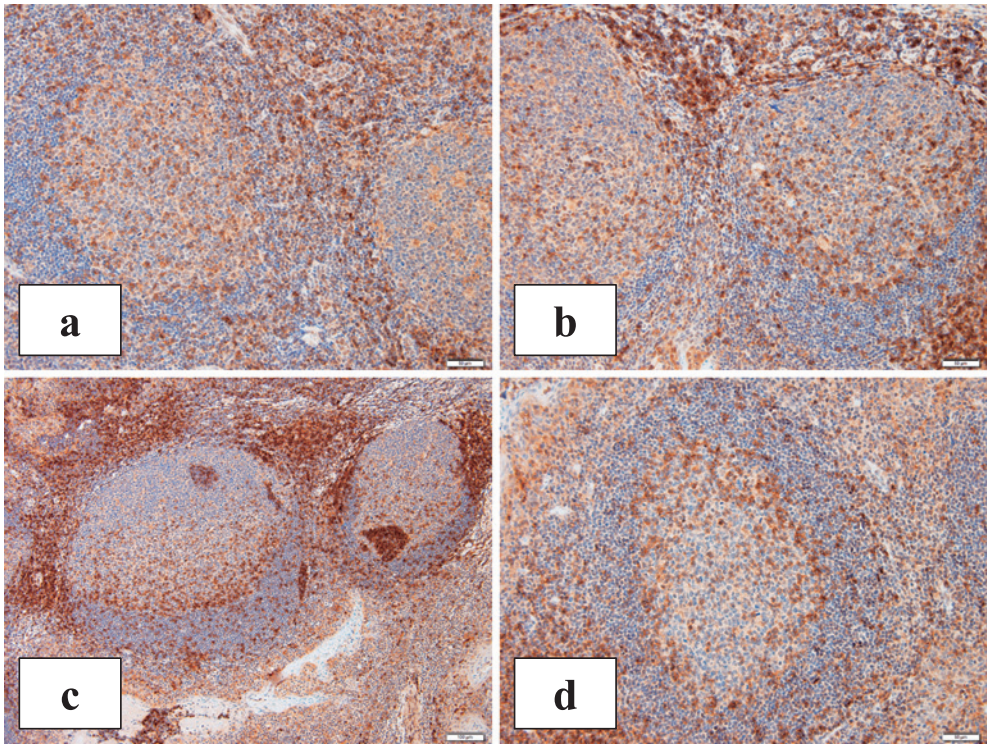

Fig. 2 Six-micrometer-thick paraffin sections of the palatine tonsils. Immunohistological results of staining of T-cells using an anti-CD4 antibody (brown) and hematoxylin (blue).
(a) Palatine tonsil tissues in the young group.
CD-positive cells (T-cells) were scattered in the germinal center and surrounding areas.
(b) The tonsils of the young group.
T-cells were also observed in the tonsils.
(c) The tonsils of the older group.
T-cells were detected, particularly in the area surrounding the germinal center. Some atrophic changes were observed around the germinal center.
(d) The tonsils of the older group;
T-cells were detected in the palatine tissues.

detected, particularly in the area surrounding the germinal center. Some atrophic changes were observed around the germinal center (**Fig. 2c, d**).

Fluorescence Immunohistochemical Expression of Acetylated and Dimethylated Histones

Acetylated histone H3 lysine 9 (Lys9) was detected in the nuclei of the tonsillar germinal center in young group (**Fig. 3a**), whereas dimethylated histone H3 Lys9 was not (**Fig. 3b**). These immunoreactivities were not observed in the area surrounding the germinal center or epithelium.

Acetylated histone H3 Lys9 and dimethylated histone H3 Lys9 were not detected in the nuclei of the germinal centers in the old group (**Fig. 3c, d**).

The organ of Corti in young mice showed positive staining for acetylated histone H3 Lys9 (**Fig. 3e**), whereas that in old mice showed positive staining for dimethylated histone H3 Lys9 (**Fig. 3f**). Negative staining was observed in the young group for dimethylated histone H3 Lys9 (**Fig. 3g**) and in old mice for acetylated histone H3 Lys9 (**Fig. 3h**).

Discussion

In the present study, observations with a light microscope revealed that the structure of the palatine tonsils was mature in child patients, whereas that in adult patients had degenerated. A previous study has demonstrated that the structure of the palatine tonsils is fully developed in children younger than 10 years[12]. Active nodules and the infiltration of dense lymphocytes have previously been reported. Furthermore, degeneration of the tonsils and the appearance of adipose tissues occurred after the age of 21 years. Our morphological results are consistent with previous findings.

The palatine tonsils are located on either side of the

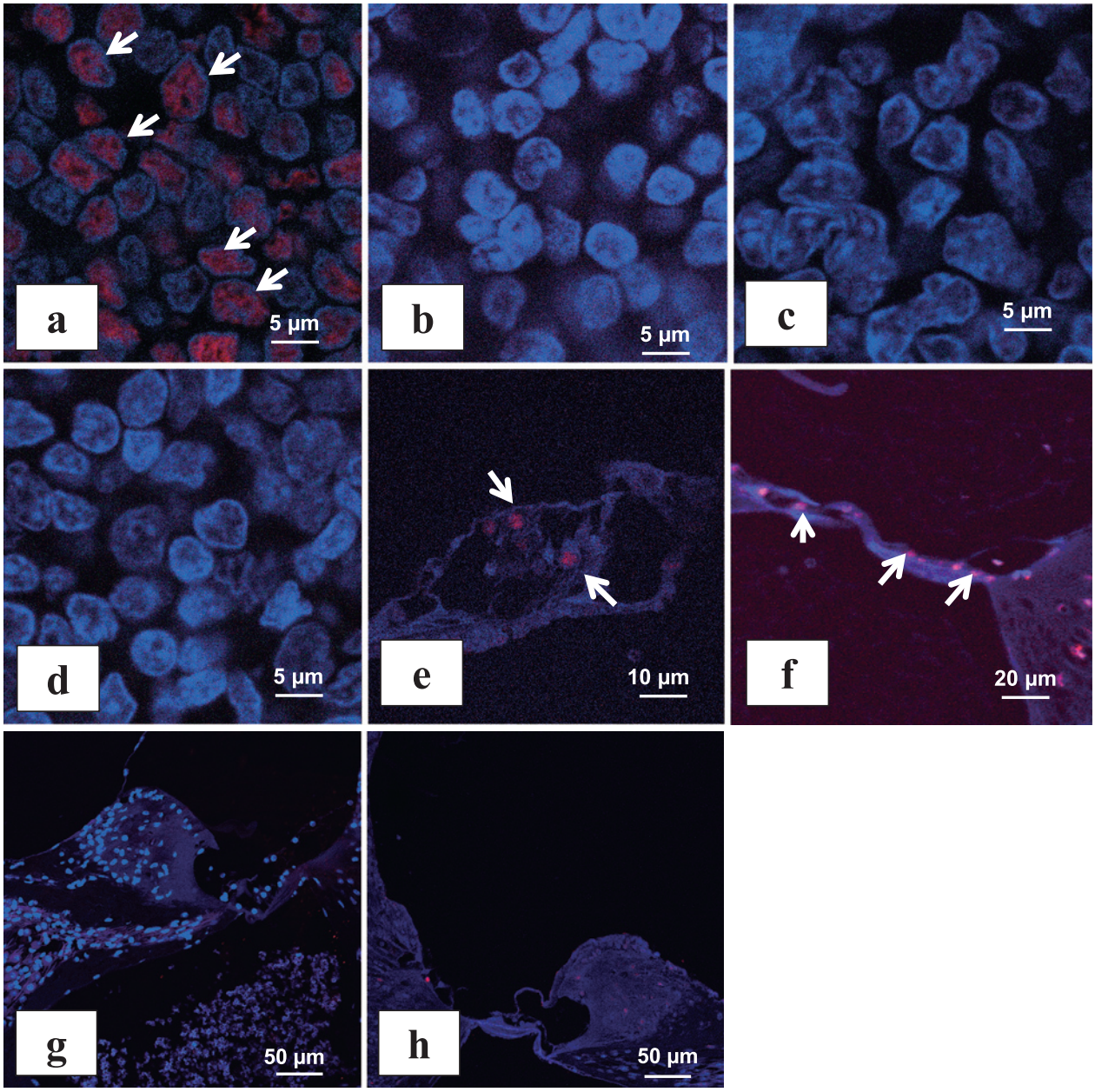

Fig. 3 Six-micrometer-thick paraffin sections of palatine tonsils. The distribution of acetylated H3 Lys9 or dimethylated H3 Lys9, DNA stained with DAPI and DRAQ5 (blue), acetylated H3 Lys9 and dimethylated H3 Lys9 stained by immunofluorescence using a Cy3-conjugated antibody (red).
(a) Palatine tonsil tissues in the young group.
Acetylated H3 Lys9 was detected in the nuclei of germinal center cells (**arrow**).
(b) Palatine tonsil tissues in the young group.
Dimethyl H3 Lys9 was not detected.
(c) Palatine tonsil tissues in the old group.
Acetylated H3 Lys9 was not detected.
(d) Palatine tonsil tissues in the old group.
Dimethyl H3 Lys9 was not detected.
(e) The organ of Corti in young mice (C57BL/6) at the age of 8 weeks.
Positive staining for acetylated histone H3 Lys9 was observed (**arrow**).
(f) The organ of Corti in old mice (C57BL/6) at the age of 132 weeks.
Positive staining for dimethylated histone H3 Lys9 was observed (**arrow**).
(g) The organ of Corti in young mice
No staining for dimethylated histone H3 Lys9 was observed.
(h) The organ of Corti in old mice
No staining for acetylated histone H3 Lys9 was observed.

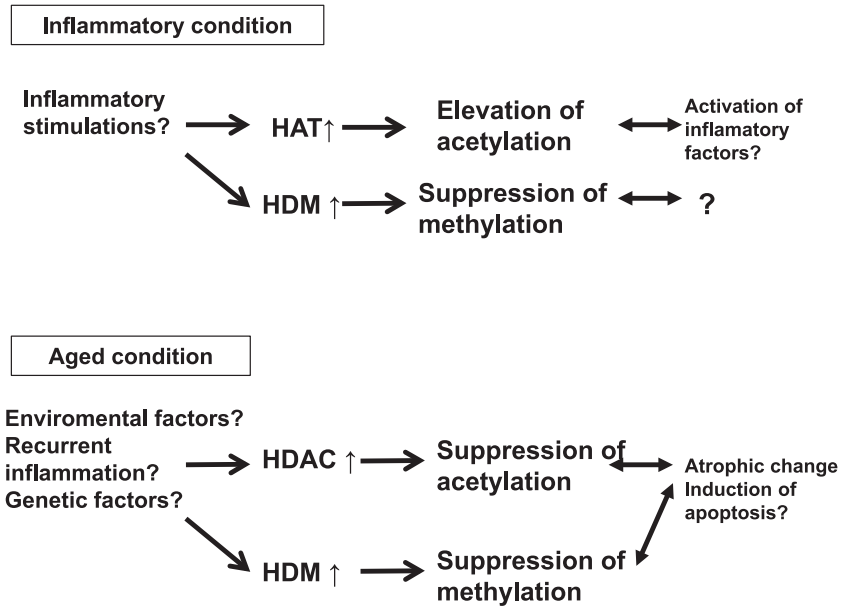

Fig. 4 Schematic drawing of histone modifications under inflammatory and aged conditions.
Inflammatory factors trigger the activation of histone acetyl transferase and histone demethylase.
Aging reflects many factors, such as environmental factors, recurrent inflammation, and genetic factors. These influences increase the activity of histone diacetyl transferase and histone demethylase. The suppression of acetylated histones induces apoptosis.

pharynx, whereas lingual tonsil adenoids are located at the entrance to the oropharynx, which is called Waldeyer's ring. The surface of the tonsils is covered by a squamous epithelium. Crypts enter the tonsils. Many nodules display the infiltration of lymphocytes; these nodules contain the germinal centers[13]. The palatine tonsils function as an immune barrier against unwanted organisms and antigens[14] and are immunologically active between the ages of 4 and 10 years[12]. The palatine tonsils contain T cells, macrophages, dendritic cells, and B cells, which account for 50% to 65% of all tonsillar lymphocytes in the germinal centers. Information regarding the infiltration of an antigen is relayed to mature T cells and B cells to start the production of antibodies against it. The B cell population decreases after puberty, when the ratio of T to B cells increases[9]. Immunological activity appears to decrease after the age of 21 years[12].

In the present study, acetylated histone H3 Lys9 was detected in the germinal centers of the tonsillar tissues in young mice, whereas dimethylated histone H3 Lys9 was not. In contrast, acetylated histone H3 Lys9 and dimethylated histone H3 Lys9 were not detected in old mice.

Chronic tonsillitis is characterized by the permanent inflammation of the palatine tonsils as a result of recurrent acute or subclinical infections[15]. The hypermethylation of DNA has been detected with chronic inflammation, such as atherosclerosis[16]. Epigenetic mechanisms have been shown to modulate the expression of the proinflammatory cytokine, tumor necrosis factor-α, interleukins, and tumor suppression genes under chronic inflammatory conditions[17]. Chronic obstructive pulmonary disease is a chronic inflammatory disease of the lung, and in patients with this disease the expression of histone deacetyltransferase is down-regulated. Levels of histone acetylation are high in proinflammatory genes[5]. Immune responses are enhanced by Jumonji D3 domain-containing protein 3, which is a histone demethylase[8]. Histone methyltransferase regulates chromatin and suppresses its expression in pulmonary diseases[18]. This is a reason that methylation is suppressed. Elevated levels of acetylated histones are known to contribute to inflammation[19]. The immunological activity of the palatine tonsils was previously reported to be the highest in young children. Taken together, these findings support the expression of acetylated histones and suppression of dimethylated histones in the palatine tonsils in the young subjects of our study.

A previous study has demonstrated that the immu-

nological responses of the palatine tonsils decrease after the age of 21 years[12]. The differential expression of acetylated histone H3 may reflect the immunological differences observed between the younger and older subjects. On the other hand, monozygotic twins show an altered pattern of epigenetic modification, that is, various factors are involved in the DNA methylation and histone modification[2]. Histone deacetyltransferase plays key roles in cellular aging[20]. Aging also leads to a decrease in histone acetylation, which results in reduced memory activity[21] and induced apoptosis[22]. Histone demethyltransferase has been shown to trigger the aging process[8]. In the present study, we did not detect acetylated or dimethylated histone H3 in the older subjects. Therefore, aged tonsillar tissue may be influenced by inflammation and aging. The acetylation of histones gradually decreases during the process of aging. We summarize the relationships of inflammation, aging, and histone modifications in **Figure 4**.

Complications associated with chronic tonsillitis in young and aged patients include peritonsillar and parapharyngeal abscesses, recurrent high fever, and glomerulonephritis[9]. The radical treatment currently employed for chronic tonsillitis is tonsillectomy. Abnormalities have been detected in DNA methylation and histone modifications in cancer cells. A histone diacetyl transferase inhibitor has recently been clinically applied to the treatment of leukemia[23]. For ethical reasons, we did not compare the tissue examined with normal tonsils. Although the results of the present study do not lead to a direct treatment, the epigenetic pathogenesis of chronic inflammation, such as immunoglobulin A nephropathy, by focal infections will be described in greater detail in future studies, which will lead to new treatments being developed.

In conclusion, acetylated histone H3 was detected in the germinal centers of palatine tonsils in young subject but not in the older subjects. Dimethylated histone H3 was not detected in either group of subjects. These modification patterns of histones were presumed to reflect the influences of inflammation and aging.

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