Expression of the Forkhead box transcription factor Foxo3a in human periapical granulomas

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Abstract: It has been reported that Forkhead box transcription factor class O3a (Foxo3a) is expressed in rheumatoid arthritis, a chronic inflammatory condition accompanied by bone resorption, and plays a role in its pathology. However, it has remained unclear whether Foxo3a is involved in the pathogenesis of periapical granulomas. The present study was performed to compare the expression of Foxo3a in periapical granulomas and healthy gingival tissues. Samples were obtained surgically from patients, and subjected to hematoxylin-eosin staining for histopathologic diagnosis. Two-color immunofluorescence staining was also performed using antibodies against Foxo3a and markers for three types of inflammatory cells: neutrophils, T lymphocytes, and B lymphocytes. This revealed that Foxo3a was expressed in all three cell types in periapical granulomas but not in healthy gingival tissues. Foxo3a was expressed in 82.1%, 78.3%, and 77.5% of neutrophils, T lymphocytes, and B lymphocytes, respectively, and statistical analysis using the Kruskal-Wallis test followed by the Steel-Dwass test showed no significant difference of Foxo3a expression among the three cell types. Our results suggest that Foxo3a transcription factors may be involved in the pathogenesis of periapical granulomas.

Keywords: Foxo3a; periapical granuloma; neutrophil; B lymphocyte; T lymphocyte.

Introduction

Periapical lesions are caused by microorganisms and their products in the infected root canal, leading to clinical symptoms such as alveolar bone resorption (1-5). It has been reported that root apical lesions form as a result of a chronic inflammatory immune response in the periapical tissue induced by microbial antigens that cannot be eliminated (6). Root canal treatment is expected to assist healing of periapical lesions by removal of such microorganisms and infectious agents in the root canal (7,8).

Previously, we confirmed that the heparin-binding growth factor midkine, which plays an important role in chronic inflammation (9), is involved in immune responses in human periapical granulomas (10). However, the roles of inflammatory mediators in the pathogenesis of periapical periodontitis are still not fully understood.

Forkhead box transcription factor class O (Foxo) proteins are transcription factors that share a DNA-binding Fox domain and belong to a family comprising 18 Fox subgroups. Previous studies have reported that four Foxo proteins (Foxo1, Foxo3a, Foxo4, and Foxo6) are expressed in various tissues, and are associated with cell survival, cell proliferation, and DNA damage repair (11-17). In particular, Foxo3a acts as a tumor suppressor in leukemia and cancers of the liver, breast, and prostate.
Conversely, Foxo3a is overexpressed in the synovium, T lymphocytes, mononuclear cells, and polymorphonuclear cells in peripheral blood from patients with rheumatoid arthritis, a chronic inflammatory disease (23). In addition, Foxo3a has latent roles in the regulation of T cell proliferation and apoptosis, polymorphonuclear cell survival, and stress resistance (18,24). Furthermore, it has been reported that Foxo3a binds to the Fas ligand (FasL) promoter and suppresses cell apoptosis induced by FasL, which is involved in the onset and progression of rheumatoid arthritis (25-27). FasL is a member of the tumor necrosis factor (TNF) superfamily of cell death receptors, which can augment the inflammatory response through the expression of interleukin-1 beta (IL-1β) produced by apoptotic cells (28-30).

Thus, there is a possibility that Foxo3a expression is associated with chronic inflammation in periapical granulomas. In the present study, to determine whether Foxo3a is involved in the development of periapical granulomas, we examined the expression of Foxo3a proteins in inflammatory and healthy tissues using two-color immunofluorescence staining.

**Materials and Methods**

**Sample collection**
Periapical lesions were obtained from 30 patients (15 males and 15 females; age range 20-68 years) diagnosed as having refractory chronic periapical periodontitis based on the standard clinical criteria, during endodontic surgery at the Department of Endodontics, Nihon University Dental Hospital. Before sampling, informed consent was obtained from all patients, as stipulated by the Ethics Committee of Nihon University School of Dentistry (EP2014D6-1), based on the Declaration of Helsinki.

Five samples of healthy gingival tissue were also collected from patients who were referred to the Department of Oral Surgery for extraction of impacted third molars. These patients had no symptoms such as swelling or pain.

**Preparation of tissue sections**
Samples with an average diameter of 0.82 cm were collected surgically from the patients by apicoectomy, and immediately fixed in 10% buffered formalin for 24 h. The samples were then embedded in paraffin and cut into sections 5 μm thick.

The healthy gingival tissues obtained during extraction of impacted third molars were also subjected to the same preparation methods.

**Histological examination of the specimens**
Histological examination of paraffin sections was performed after hematoxylin-eosin staining. The sections were first deparaffinized using xylene and rehydrated using a graded ethanol series. The rehydrated sections were then stained with hematoxylin and eosin, and examined histologically using a light microscope (BH-2; Olympus, Tokyo, Japan).

**Two-color immunofluorescence staining**
To identify the Foxo3a-expressing types of inflammatory cells in periapical granulomas, we performed two-color immunofluorescence staining with a Foxo3a antibody and specific markers for three types of inflammatory cells: neutrophils, T lymphocytes, and B lymphocytes. The deparaffinized and rehydrated sections were boiled at 98°C for 5 min in citrate phosphate buffer (pH 6.0) for antigen retrieval. In brief, non-specific binding was blocked with normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 90 min. The primary antibodies used in the present study are shown in Table 1. Co-incubation of the anti-human Foxo3a monoclonal antibody with each one of the three cell markers separately was performed for 1 h. After co-incubation, further incubation was performed for 1 h with rhodamine isothiocyanate (RITC)-conjugated goat anti-mouse IgG antibodies (Thermo Fisher Scientific, Waltham, MA, USA) for Foxo3a expression and with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (Abcam, Cambridge, UK) for each of the cell markers. Nuclear counterstaining was performed using 4’,6-diamine-2-phenylindole (DAPI) (Roche Diagnostics, Mannheim, Germany). Joint expression of Foxo3a with each of the respective cell markers was analyzed quantitatively by two-color immunofluorescence using a fluorescence microscope (Eclipse E600, Nikon, Tokyo, Japan).

**Table 1 Primary antibodies used in the present study**

| Antibody          | Clone          | Dilution | Manufacturer                  |
|-------------------|----------------|----------|-------------------------------|
| Foxo3a            | Mouse monoclonal | 1/100    | Abcam, Cambridge, UK          |
| Neutrophil Elastase (NE) | Rabbit polyclonal | 1/200    | Abcam, Cambridge, UK          |
| CD3               | Rabbit monoclonal | 1/50     | Nichirei Bioscience Inc., Tokyo, Japan |
| CD79α             | Rabbit monoclonal | 1/50     | Nichirei Bioscience Inc., Tokyo, Japan |
gingival tissue were examined as described above.

**Statistical analysis**
Statistical analysis was performed using BellCurve for Excel version 2.12 (Social Survey Research Information Co., Ltd., Tokyo, Japan). In the examination of immunofluorescence, samples were scored by counting the number of Foxo3a-expressing neutrophils, T lymphocytes, and B lymphocytes in three different fields in each section by two specialists at a magnification of ×400. The percentage was calculated as the number of Foxo3a-expressing round cells divided by the total number of cells positive for each marker. Statistical analysis of the percentage values obtained from two-color immunofluorescence staining was performed using the Kruskal-Wallis test followed by the Steel-Dwass test.

**Results**

**Histological examination of the specimens**
Paraffin sections (n = 30) of periapical lesions stained by hematoxylin and eosin for histological examination revealed tissues with granulomas that comprised many inflammatory cells. A total of 26 periapical lesions had no epithelial cells, and these samples were diagnosed as periapical granulomas (Fig. 1A). The remaining 4 periapical lesions were diagnosed as radicular cysts due to the presence of epithelium and a cavity (Fig. 1B), and were excluded from the study. Healthy gingival tissues exhibited stratified squamous epithelium at the surface and a smaller number of inflammatory cells than was the case in the periapical lesions (Fig. 1C).

**Two-color immunofluorescence staining**
To characterize the types of Foxo3a-expressing round cells (neutrophils, T lymphocytes, or B lymphocytes), two-color immunofluorescence staining was performed using a Foxo3a antibody and three types of cell markers. Foxo3a-expressing neutrophils (Fig. 2), T lymphocytes (Fig. 3) and B lymphocytes (Fig. 4) were observed. However, not all inflammatory round cells expressed Foxo3a. Interestingly, Foxo3a was not expressed in healthy gingival tissues (data not shown). The percent-
ages of Foxo3a-expressing neutrophils, T lymphocytes, and B lymphocytes in periapical granulomas were 82.1%, 78.3%, and 77.5%, respectively (Fig. 5). However, statistical analysis using the Kruskal-Wallis test followed by the Steel-Dwass test revealed no significant differences in these percentages among the various inflammatory cell types.

**Discussion**

In order to clarify Foxo3a localization in periapical lesions, the expression of Foxo3a in three types of inflammatory round cells in periapical granulomas was examined by two-color immunofluorescence staining using a Foxo3a antibody and three types of cell markers. Foxo3a was found to be expressed in neutrophils, T lymphocytes, and B lymphocytes in periapical granulomas, and especially in their nuclei. On the other hand, Foxo3a was expressed weakly in the cytoplasm. The percentages of Foxo3a-expressing neutrophils, T lymphocytes, and B lymphocytes were 82.1%, 78.3%, and 77.5%, respectively. However, Foxo3a expression did not differ significantly among these three cell types. A previous study reported that Foxo3a was expressed in fibroblast-like synovial tissues in rheumatoid arthritis (30). In the present study, however, no expression of Foxo3a was evident in fibroblast-like tissues in periapical granulomas. This result appears to reflect the different origins of rheumatoid arthritis and periapical granulomas.

Akt (protein kinase B) contributes to the release of Foxo transcription factors from the DNA into the cytoplasm by phosphorylating the three serine/threonine residues (Thr32, Ser253, and Thr32) of Foxo3a, and 14-3-3 protein binds to this phosphorylated Foxo3a (19-21). This is believed to mask the nuclear localization signals of Foxo3a protein, retaining it in the cytoplasm by preventing its reentry into the nucleus. However, in quiescent cells without proliferation or survival signal stimulation, Akt is inactivated. This inactivation indicates that Foxo3a is retained in the nucleus, promoting the expression of the specific target gene FasL, which regulates metabolic state pathways and induces cell apoptosis (19-21). It has been reported that Foxo3a is phosphorylated by adenosine phosphate-activated protein kinase (AMPK), inhibiting NF-κB signal transduction by transfer from the nucleus into the cytoplasm, and suppressing the expression of inflammatory factors (22,26,27,31).

In conclusion, the present study has demonstrated Foxo3a-expressing inflammatory round cells (neutrophils, T lymphocytes, and B lymphocytes) in periapical granulomas. These results suggest that Foxo3a is involved in the pathogenesis of periapical granulomas. Further studies are needed to investigate the mechanism of inflammation inhibition and the signaling pathway involved in periapical granulomas expressing Foxo3a.
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Conflict of interest
The authors have no potential conflict of interest to declare with respect to the authorship and/or publication of this article.

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