

# Expression of endothelin-1 in macrophages and mast cells in hyperplastic human tonsils

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**Abstract** In situ hybridization and immunohistochemical examinations of surgically resected specimens of human hyperplastic tonsils showed that macrophages in the germinal center and mast cells in the parafollicular and interfollicular areas expressed the transcript and protein of endothelin (ET)-1, but not ET-2 and ET-3. The macrophages appeared to be activated, since they possessed significant amounts of inducible nitric oxide synthase. None of these expressions was observed in normal tonsil. Our results suggest that the over-production of ET-1 by macrophages and mast cells may be involved in the pathogenesis of hyperplastic tonsils.

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**Key words:** Endothelin-1; Macrophage; Mast cell; Human tonsil; In situ hybridization

## 1. Introduction

The tonsils are the first line of defense against invasion of viruses, microbes, and irritants into the airways. Recurrent tonsillitis and reactive tonsil hypertrophy are sometimes a serious clinical problem even in healthy subjects, and tonsillectomy is inevitable for patients with serious symptoms, such as recurrent infections, sleep disturbance, and failure to gain weight. The pathological microflora is the primary cause of recurrent tonsillitis; however, the mechanism by which tonsils progressively become hyperplastic is unknown. Inflammatory and immune responses of tonsils are regulated by complex cytokine and chemokine networks, and the Th2 profile of cytokines was reported to be predominant in recurrent tonsillitis [1].

In this study, we focused on the expression of endothelin (ET)-1 in hyperplastic tonsils. ET-1 exerts a variety of biological actions, including vasoconstriction [2], neurotransmission [3], and cell proliferation [4,5], some of which are supposed to contribute to the development and progression of inflammatory processes. In fact, synthesis and release of ET-1 were reported to be up-regulated during the initial phase of airway inflammation, leading to proinflammatory effects in bronchial asthma [6]. ET-1 may also participate in the hyper-responsiveness of tonsils; however, ET-1 expression in normal and hyperplastic tonsils has never been studied in detail.

In normal human tonsil, the ET-1 transcript was scarcely detected by in situ hybridization and immunohistochemical techniques. However, surgically resected specimens of hyperplastic tonsils contained high levels of the transcript and pro-

tein in macrophages of germinal centers and in mast cells of parafollicular and interfollicular areas, suggesting a pathogenic role of ET-1 in hyperplastic tonsils.

## 2. Materials and methods

### 2.1. Tissues preparation

The present study was approved by the Ethics Committee, University of Tokushima. Four male and two female patients with hyperplastic tonsils (age 25–46) understood the purpose and protocol of this study and gave written consent to offer tonsil tissues after surgical resection. For in situ hybridization, these surgically resected tonsils were immediately fixed with ice-cold Bouin solution without acetic acid for 12 h. After fixation, these tissues were embedded in paraffin. Tissue slices of normal human tonsil, embedded in paraffin, were purchased from DAKO (Glostrup, Denmark).

### 2.2. In situ hybridization

Template DNA for cRNA synthesis was prepared by the polymerase chain reaction from the human ET-1 cDNA with the following primers: sense, T7 RNA promoter site (5'-GGATCCTAATACGACTCACTATAGGGCCGCGCTGGCCTGCAGA-3'); anti-sense, SP6 RNA promoter site (5'-GATACGATTTAGGTGACATATAGTGAAAGAAGAGACCAAG-3'). The reaction was performed in 35 cycles (5 min at 95°C followed by 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C) in a DNA thermal cycler [7]. Digoxigenin (DIG)-labeled antisense or sense cRNA was generated by in vitro transcription system from the above template DNA. Both probes were synthesized by T7 or SP6 RNA polymerase using DIG-UTP, according to the manufacturer's recommended protocol (Roche, Switzerland). The cRNA probes were hydrolyzed in 0.1 M sodium carbonate (pH 10.2) at 60°C for the appropriate time to give 150 bp. Hybridization and color development were carried out by the previously described method [8] with minor modifications.

RNase-pretreated sections were hybridized with the antisense cRNA probe to detect non-specific bindings, and the DIG-labeled sense cRNA probe was used to determine the specific hybridization reaction.

### 2.3. Immunohistochemistry

For immunohistochemistry, serial sections (6 µm thick) were dewaxed in xylene and dehydrated in a graded series of ethanol. Endogenous peroxidase activity was inhibited by incubation with 0.6% hydrogen peroxide in methanol. The slides were incubated overnight at 4°C with an antibody against anti-human iNOS (1:1000) (Wako Chemical Co., Tokyo, Japan), human ET-1, ET-3 (3 µg/ml) (IBL, Fujioka, Japan), human big ET-2 (1:500) (Peptide Institute, Osaka, Japan), human nuclear factor-κB components, p50 and p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the samples were incubated for 1 h at room temperature with a diluted second antibody (biotinylated anti-rabbit IgG) and then treated with Extra avidin-peroxidase (Sigma, St. Louis, MO, USA). Immunoreactive materials to these antibodies were visualized with Extra avidin-biotin-peroxidase staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Dojin, Kumamoto, Japan) and 0.01% H<sub>2</sub>O<sub>2</sub> as substrates. The other slides were incubated overnight at 4°C with macrophage antibody (HAM56; Enzo Diagnostics, San Francisco, CA, USA). They were treated for 1 h at room temperature with a diluted second antibody (alkaline phosphatase-conjugated anti-mouse IgG+M+A) (Zymed Lab. Inc., San Francisco, CA, USA), followed by treatment with alkaline phosphatase substrate (fast blue RR and naphthol AS-

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MX phosphate). Tonsil sections were also stained with 0.1% (w/v) alcian blue in 0.7 M HCl for 15 min at room temperature and mounted in AQUATEX (Merck, Darmstadt, Germany).

### 3. Results

As shown in Fig. 1A, germinal centers of hypertrophic tonsils contained abundant ET-1 mRNA signals which were not detected by the sense RNA (Fig. 1B). These strong signals were not observed in germinal centers of normal tonsils (Fig. 1E). Most cells in the centers of hyperplastic tonsils expressed ET-1 mRNA to varied extents, and cells expressing extremely high levels of the ET-1 transcript (indicated by arrows, Fig. 1C) were sporadically observed. Based on their distribution, size, and shape, these cells were likely to be macrophages. This was confirmed by immunohistochemical analyses of the serial sections with an antibody against macrophages (HAM56). The distribution of HAM56-positive macrophages (Fig. 1D) roughly coincided with that of the large macrophage-like cells having strong ET-1 mRNA signals (Fig. 1C).

The expression of ET-1 was also confirmed by immunohistochemistry. Cells positive for immunoreactivities to the ET-1 antibody were observed in the centers of hyperplastic tonsils (Fig. 2A), but not in normal tonsils (Fig. 2B). These cells (shown by arrows in Fig. 2A) were confirmed to be macrophages by immunohistochemical examination of a serial section with the anti-HAM56 antibody (data not shown). We also examined whether the other ET isoforms were expressed in hyperplastic tonsils. Significant amounts of immunoreactive materials to big ET-2 (Fig. 2C) or ET-3 antibody (Fig. 2D) were not detected.

The macrophages expressing ET-1 were characterized to be activated cells, since they contained immunoreactive materials to anti-iNOS antibody (Fig. 3A). In normal tonsil, the number of HAM56-positive macrophages, distributed in germinal centers (Fig. 1F), was about 30% less than in hyperplastic tonsil (Fig. 1D), and iNOS-expressing cells were not observed in normal tonsil (Fig. 3B).

Although the ET-1 mRNA-expressing cells were distributed mainly in the germinal centers of hypertrophic tonsils, small cells expressing the transcript were scatteredly observed in the

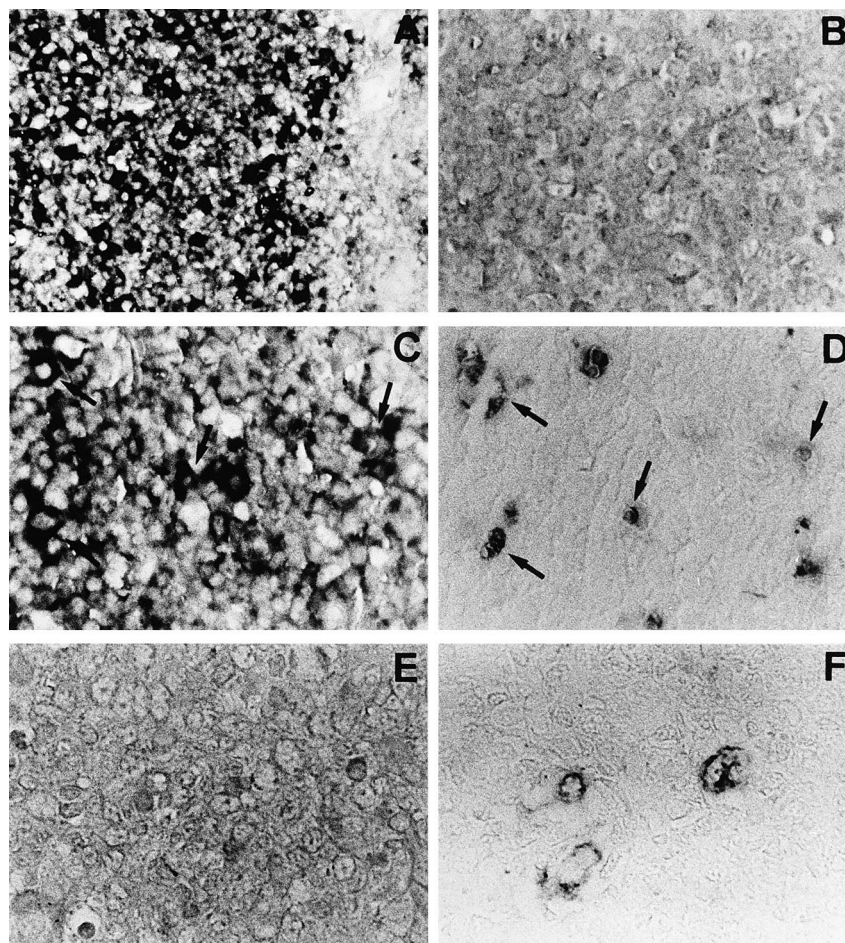


Fig. 1. ET-1 expression in germinal centers of hyperplastic tonsils. A: Germinal centers and interfollicular areas of hyperplastic tonsil were stained with the antisense cRNA probe of ET-1 mRNA. Abundant signals are located in the germinal centers. Magnification  $\times 200$ . B: Germinal centers of hyperplastic tonsil were stained with the sense probe of ET-1 mRNA. Magnification  $\times 400$ . C: Germinal centers of hyperplastic tonsil were stained with the antisense cRNA probe of ET-1 mRNA. Cells expressing strongly were sporadically observed in the germinal center (arrows). Magnification  $\times 400$ . D: Immunohistochemical detection of HAM56-positive macrophages in the germinal centers of hyperplastic tonsil (arrows). Fig. 1C,D and Fig. 3A were serial sections, and arrows indicate identical cells in the sections. Magnification  $\times 400$ . E: Germinal centers of normal tonsil were stained with the antisense cRNA probe of ET-1 mRNA. Magnification  $\times 400$ . F: Immunohistochemical detection of HAM56-positive macrophages in the germinal centers of normal tonsil. Magnification  $\times 400$ .

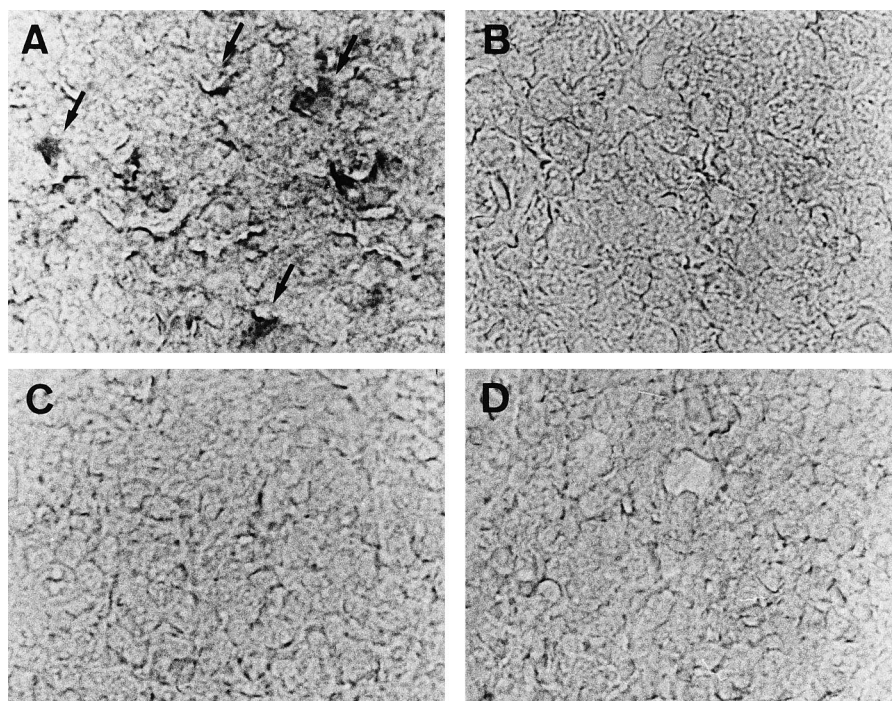


Fig. 2. Expression of ET isoforms in hyperplastic tonsils. Hypertrophic (A,C,D) or normal tonsils (B) were subjected to immunohistochemical analysis with the antibody against human ET-1 (A,B), big ET-2 (C), or human ET-3 (D), as described in Section 2. ET-1-positive cells are shown by arrows. Magnification  $\times 340$ .

interfollicular spaces of hypertrophic tonsils (Fig. 4A), but not in the normal tonsils (data not shown). These spindle-shaped cells were identified as mast cells positive for alcian blue staining (Fig. 4B), and ET-1 immunoreactivities were also observed in these cells (Fig. 4C).

#### 4. Discussion

ET-1 is a member of the ET family of structurally related 21 amino acid peptide hormones [2,9]. ET-1 exerts numerous biological actions and is widely distributed in different human tissues, including the brain [10], lung [11], kidney [11], and placenta [12].

In normal tonsils, resident macrophages in the germinal centers did not possess significant amounts of iNOS protein, and they expressed only low levels of the ET-1 mRNA. In addition, the macrophages did not possess detectable amounts

of ET-1 protein. In hyperplastic tonsils, the number of macrophages infiltrated into the germinal centers increased, and they appeared to be activated, since these cells contained higher levels of iNOS protein. Immunohistochemical study with antibodies against nuclear factor- $\kappa$ B components (p50 and p65) showed that these factors translated into nuclei of macrophages to varied extents in hyperplastic tonsils, while nuclear translocation was not observed in normal tonsil (data not shown). Thus, hyperplastic tonsil contained activated macrophages that appeared to produce abundant ET-1 as well as NO. Human lung macrophages, activated by lipopolysaccharide or stimulated by phorbol diesters, were also shown to markedly enhance synthesis and secretion of ET-1 [13], suggesting an important role for ET-1 in the microenvironment of tissue macrophages.

Activated tissue macrophages dramatically enhance or newly induce secretions of biologically active products, includ-

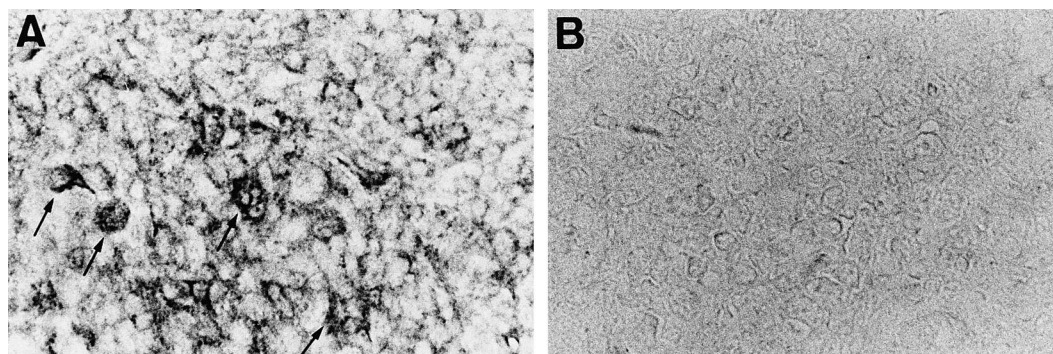


Fig. 3. Expression of iNOS in hyperplastic tonsils. Comparable sections of the germinal centers of human hyperplastic tonsil (A) and normal tonsil (B) were stained with anti-iNOS. The iNOS-positive cells can be detected in the germinal centers of hyperplastic tonsil (arrows). Magnification  $\times 340$ .

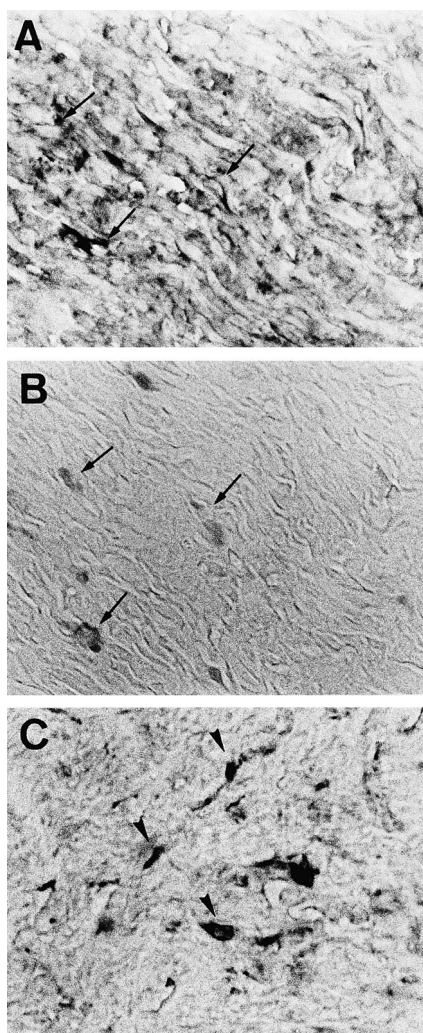


Fig. 4. Expression of ET-1 in mast cells. Sequential sections were stained with the antisense cRNA probe of ET-1 mRNA (A) and alcian blue (B). Mast cells can be detected in the interfollicular areas (arrows indicate identical mast cells). ET-1 immunoreactivity was also observed in mast cells (C) (shown by arrowheads). Magnification  $\times 340$ .

ing reactive oxygen metabolites, cytokines and other factors, all of which are important mediators for inflammatory and immune responses. Enhanced secretion of superoxide anion and NO produces more reactive metabolites, such as hydroxyl radical and peroxynitrate [14,15]. These metabolites are well known to cause cell and tissue damage that may result in increased hyperresponsiveness of tonsil. Compared to these oxygen intermediates and other cytokines, the exact meaning of the over-production of ET-1 in the pathogenesis of hyperplastic tonsils is unknown. ET-1 exerts its biological action through binding to distinct G protein-coupled receptors, known as ETA and ETB receptor subtypes [16]. Identification of specific target cells for ET-1 and demonstration of ET-1-dependent events are required to reveal a pathogenic role of ET-1 in hyperplastic tonsils.

We also show that connective tissue mast cells in hyperplastic tonsils expressed ET-1 mRNA. Fureder et al. [17] reported that the distribution of human tonsil mast cells was localized in parafollicular and interfollicular areas, where they exhibit functional and phenotypic properties that are similar to lung or uterus mast cells, but not to skin mast cells. Considering the potent activities of mast cells to produce cytokines or chemical mediators, distinct factors derived from the mast cells are likely to be involved in the pathogenesis of hyperplastic tonsil, and ET-1 might be one of the possible mediators.

Our histological approach may provide only limited information. However, to the best of our knowledge, the results of this study provide the first evidence that activated macrophages in the germinal centers, as well as mast cells in the parafollicular and interfollicular areas, contained high levels of ET-1 mRNA and its protein, suggesting an important role of ET-1 in the development and progression of hypertrophic tonsil. Our results also imply the possibility that administration of specific ET-1 receptor antagonists or iNOS inhibitors may be a novel approach for treatment of intractable hyperplastic tonsil.

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