Immunohistological analysis of tumour growth factor β1 expression in normal and inflamed salivary glands

Y Kizu, H Sakurai, S Katagiri, N Shinozaki, M Ono, K Tsubota, I Saito

Department of Oral Medicine, Tokyo Dental College, Chiba, Japan
Y Kizu
S Katagiri

Department of Ophthalmology
N Shinozaki
M Ono
K Tsubota

Division of Immunological Diseases, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan
H Sakurai
I Saito

Correspondence to: Dr Ichiro Saito, Department of Pathology, Tojushima University School of Dentistry, 3-18-15 Karamoto, Tokushima 770, Japan.

Abstract

Aim—To determine whether transforming growth factor-β1 (TGF-β1) has a pathogenic role in disease of the salivary glands.

Methods—An indirect immunohistochemical technique was used to analyse TGF-β1 expression in six specimens of normal salivary gland and 23 surgical specimens.

Results—TGF-β1 was strongly expressed in the ductal epithelial cells of normal salivary gland tissues (six of six cases) and in inflammatory conditions (eight of 11 cases). In contrast, TGF-β1 was not detectable in ductal epithelial cells expressing HLA-DR around infiltrating CD4+CD45RO+ activated T cells, in the salivary gland tissue of patients with Sjögren’s syndrome.

Conclusion—Because TGF-β1 has an essential role in the mucosal immunity of salivary glands, abnormal expression of this cytokine must be regarded as a candidate in the pathogenesis of Sjögren’s syndrome.

Keywords: TGF-β1, salivary gland, Sjögren’s syndrome.

Transforming growth factor-β (TGF-β) has several important biological effects, including cell growth and differentiation, embryonic development, extracellular matrix formation, bone remodelling, and wound healing. TGF-β is also important in haemopoiesis, inflammation, and immune regulation. Although TGF-β can function as a proinflammatory cytokine and is occasionally immune potentiating, considerable evidence suggests that it is a natural immunosuppressor.1 3 TGF-β was initially recognised by its ability, together with transforming growth factor-α (TGF-α), to induce the reversible transformation of rat fibroblasts in culture.2 4 There are three distinct molecular forms of TGF-β, designated TGF-β1, TGF-β2, and TGF-β3.5 The biologically active mature 25 kilodalton TGF-β polypeptide consists of two identical disulphide linked monomers.5 In their mature sequences human TGF-β2 and TGF-β3 genes are about 80% homologous with TGF-β1; TGF-β3 is 72% homologous with TGF-β2.6 TGF-β1 seems to have an important role in regulating mucosal immunity. This involves induction of precursor IgA secreting cells in mucosal sites, such as lacrimal and salivary glands, and the terminal differentiation of B cells for IgA which is important in mounting a defence against infection.7 Relatively little, however, is known about the presence of TGF-β1 in normal and inflamed salivary glands.

Sjögren’s syndrome is a chronic autoimmune disease characterised by dryness of the mouth and eyes. Impaired salivary and lacrimal gland function is caused by acinar or ductal epithelial cell destruction, or both, accompanied by lymphocytic infiltration that is thought to be immune mediated.7 8 Activated CD4+ T cells are the major subsets infiltrating the exocrine glands in Sjögren’s syndrome, and B cells subsequently appear in the lesion.9 10 Sjögren’s syndrome can also manifest as a generalised systemic lymphoproliferative disorder, with a tendency to progress to malignant lymphoma. The aetiology of Sjögren’s syndrome is obscure, with evidence implicating both genetic and environmental factors.11 12 Recently, it has been reported that mice bearing the TGF-β1 null mutation (-/-) develop lymphoid infiltrates in the heart, lungs, salivary glands, and other organs which are similar to those seen in the pseudolymphoma of Sjögren’s syndrome.11 We therefore studied the pattern of expression of TGF-β1 as part of mucosal immunity in normal and inflamed salivary glands, and compared this with TGF-β1 immunolocalisation in salivary gland biopsy specimens obtained from patients with Sjögren’s syndrome.

Figure 1 HLA-DR was faintly expressed in cytoplasm of ductal epithelial cells, a few lymphocytes, and postcapillary venules (original magnification ×250).
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Methods
All patients were seen at the Ichikawa General Hospital of Tokyo Dental College or Tokyo Medical and Dental University Hospital. Normal healthy subjects with no sicca symptoms underwent minor salivary gland biopsies as part of the diagnostic workup for other oral problems (n = 3). Histologically normal salivary gland tissues were also obtained at the time of necropsy (n = 3). Salivary gland tissues from sialoadenitis (n = 3) and ranula (n = 4) were also obtained at the time of surgery. Twelve patients with Sjögren's syndrome underwent minor salivary gland biopsies for the differential diagnosis of Sjögren's syndrome.17 Informed consent was obtained from all patients. All of the patients were women with a mean age of 45 years (range 21 to 65 years).

The biopsy specimens revealed a focus score of more than 2, but did not show evidence of lymphoma in routine histological and immunohistological analysis. Each of these patients showed keratoconjunctivitis sicca with decreased tear production: less than 5 mm on a Schirmer test with anaesthesia, and less than 10 mm on another Schirmer test with nasal stimulation.16 The cornea and conjunctival epithelia were positive for Rose Bengal and fluorescein staining. Decreased salivary secretion was confirmed by the gum test producing less than 5 ml. These patients had not received glucocorticoids or immunosuppressive agents for at least six months before biopsy. All patients exhibited raised serum titres of rheumatoid factor (>1:160) by latex agglutination and antinuclear antibodies, including anti-SS-A or anti-SS-B antibodies.

The unfixed tissue blocks were placed in OCT compound (Miles, Naperville, Illinois, USA), snap frozen in liquid nitrogen, and stored at −80°C. Cryostat sections (4 μm) from each block were transferred on to glass slides for immunohistochemical analysis. Sections were stained with haematoxylin and eosin using the standard method. Acetone fixed (−20°C acetone, 10 minutes) sections placed on gelatin coated slides were incubated with murine monoclonal antibodies directed against CD3, CD4, CD8, CD14, CD20, CD21, CD45RA, CD45RO, HLA-DR (Coulter Immunology, Hialeah, Florida, USA), cytokeratin (Becton-Dickinson, USA) or factor VIII (Cedarlane, USA). Antibodies of the same isotype with irrelevant antibody activity were used as negative controls. After rinsing, the sections were reacted with biotinylated goat anti-mouse (IgG + IgM) antibody (Tago, USA), followed by peroxidase conjugated avidin-biotin complex (Vector Laboratories, Burlingame, California, USA) and then the substrate, 3,3′-diaminobenzidine. When rabbit antibody to TGF-β1 C-terminal peptide was used, the sections were reacted with biotinylated goat anti-rabbit (IgG, H + L) antibody (Vector Laboratories), followed by avidin-biotin complex.

Rabbit antibodies against synthetic TGF-β1 C-terminal peptide were purchased from Santa Cruz Biotecnology (USA). This C-terminal peptide corresponds to residues 328–353 of the complete TGF-β1 molecule. This antibody detects intracellular TGF-β1.17 To define the specificity of the antiserum against TGF-β1, we screened a database of primary structure proteins to search for homology with the TGF-β1 C-terminal 25 polypeptide. As expected, there was no clinically relevant homology with known sequences. TGF-β1 peptide was obtained from Genzyme Diagnostics (USA).

Results
Only a few T and B cells were observed in normal salivary glands, and these were mostly located near acini and ducts and seldom within epithelial cells. TGF-β1 was detectable in these lymphocytes. A few intercalated ducts and

Figure 2 Numerous anti-TGF-β1+ epithelial cells (acini and ductal structures) were present in normal salivary gland tissue (original magnification ×120). The small vessels, stromal cells, and lymphoid cells were negative for TGF-β1.

Figure 3 HLA-DR expression on small numbers of epithelial cells and lymphoid cells was slightly increased in inflamed salivary glands, compared with normal salivary glands (original magnification ×250).
acini expressed HLA-DR (fig 1). TGF-β1 was expressed by most ductal and a few acinar epithelial cells in all the normal glands examined (fig 2). TGF-β1 positive cells seemed to be epithelial cells, based on their reactivity in serial sections of tissue stained with anticytokeratin antibody (epithelial cell marker), and their lack of reactivity with anti-factor VIII antibody (endothelial cell marker).

HLA-DR expression on small numbers of epithelial cells was slightly increased in inflamed salivary glands, compared with normal salivary glands (fig 3). Only a few clusters of CD4+CD45RO+ activated T cells showed a significant spatial relation to HLA-DR positive epithelial cells (fig 4). TGF-β1 was found in almost all intercalated ductal cells, but was rarely seen in acini (fig 5). Signs of decreased TGF-β1 expression were observed only in HLA-DR positive epithelial cells adjacent to the occasional activated T cell infiltrates. There was no difference in the pattern of TGF-β1 expression between normal and inflamed salivary glands.

Most infiltrative T cells seen on biopsy specimens from patients with Sjögren’s syndrome were reactive with anti-CD4 antibody. Most of these T cell aggregates were localised near acini and ducts, although some were also scattered in the perivascular space. CD45RO expression was extensive in most of the CD4+ T cell rich area. Most lymphocytes were these CD4+/CD45RO+ T cells (50% to 70%); fewer than 50% of lymphocytes were reactive with CD20 and CD21 antibodies. Numerous HLA-DR positive epithelial cells (acini or ductal structures, or both), vessels, stromal cells with dendritic morphology, and lymphoid cells were present (fig 6). Faint staining with anti-TGF-β1 antibody was detected in a few Sjögren’s syndrome salivary glands (two of 12 cases) (fig 7). In particular, decreased TGF-β1 expression was noted in epithelial cells located near HLA-DR positive epithelial cells around activated T cell rich areas. TGF-β1 expression in salivary gland tissue specimens from two cases of Sjögren’s syndrome was virtually negligible. TGF-β1 staining was abolished by pre-incubation of the antibody with the TGF-β1 peptide, and no cells stained for an isotype matched rabbit IgG.

**Discussion**

TGF-β1 inhibited the activation of T cells and cytokine production. Many recent studies, including a previous report of ours, have implicated cytokines, such as interferon-γ (IFN-γ) and tumour necrosis factor (TNF), and cell adhesion molecules, in perpetuating the inflammation of Sjögren’s syndrome. Furthermore, our finding that salivary glands from patients with Sjögren’s syndrome show extensive lymphocyte infiltration, predominantly of activated T cells, agrees with previous reports. On the other hand, mice bearing the TGF-β1 null mutation (−/−) also expressed increased IFN-γ and TNF. Taken together with our present data, we suggest that down-regulation of TGF-β1 may lead to dysfunction of the immune and inflammatory responses of Sjögren’s syndrome salivary glands. Autoimmunity may involve IFN-γ induced expression of mixed histocompatibility class II molecules in a variety of cells. TGF-β1 downregulates IFN-γ induced class II antigen expression on both lymphoid and non-lymphoid cell types, and is a potent inhibitor of IFN-γ production by peripheral blood mononuclear cells. Consequently, decreased TGF-β1 expression could also lead to the presentation of self antigens by inappropriate cells, thereby eliciting an autoimmune response.

Normal salivary glands from healthy subjects and those affected by inflammatory conditions, such as sialolithiasis, ranula, and acute supplicative sialoadenitis, reacted strongly with anti-TGF-β1 antibody. These results suggest that under these inflammatory conditions, TGF-β1 expression might be decreased.
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Figure 6 Numerous HLA-DR positive epithelial cells, vessels, stromal cells with dendritic morphology, and lymphoid cells were present in Sjögren’s syndrome salivary gland (original magnification ×120).

Figure 7 Faint staining with anti-TGF-β1 antibody was detected in cytoplasm of ductal epithelial cells of Sjögren’s syndrome salivary gland tissue (original magnification ×250). Small vessels, stromal cells, and lymphoid cells were also negative for TGF-β1.

conditions the functional characteristics of normal epithelial cells are retained in salivary glands.

TGF-β1 inhibits cell binding of lymphocytes to high endothelial venules in the mucosal immune system.28–30 A TGF-β1 irregularity could, therefore, result in aberrant lymphoctic infiltration of salivary glands. Local administration of TGF-β1 or introduction of the TGF-β1 gene, which enhances mucosal immunity, may therefore soon become a new mode of treatment for Sjögren’s syndrome.

Several properties that may be relevant to the initiation and perpetuation of the pathogenesis of Sjögren’s syndrome are now known to be present in Sjögren’s syndrome salivary glands, but not in other salivary gland condi-

tions, or in normal salivary glands. These include intense lymphocytic infiltration, destruction of epithelial cells, abnormally high concentrations of IL-1, IFN-γ, and TNF, de novo expression of HLA-DR on cell surfaces, and decreased TGF-β1 expression. These observations are consistent with several scenarios in which TGF-β1 has an important role in the initiation or continuation, or both, of an immune system attack on salivary and lacrimal glands.

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