

## Transforming growth factor $\beta$ 2 in labial salivary glands in Sjögren's syndrome

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### Abstract

**Objective**—To compare the distribution and the amount of transforming growth factor  $\beta$  (TGF $\beta$ ) in labial salivary glands (LSG) in patients with Sjögren's syndrome (SS) and healthy controls.

**Methods**—LSG from SS patients (n = 10) and healthy controls (n = 6) were labelled with peroxidase-antiperoxidase staining for TGF $\beta$ 2, which was quantitated in image analysis using Video Interactive Display System software.

**Results**—In all LSGs in SS and healthy controls, TGF $\beta$ 2 was found in endothelial cells of the capillaries and in the capsular and stromal fibroblasts. In LSGs in SS, TGF $\beta$ 2 was also found in some lymphocytes in the inflammatory cell foci and in fibroblasts in fibrotic areas. The TGF $\beta$ 2 staining index ( $\mu\text{m}^2/\text{mm}^2$  tissue) was greater in SS than in control LSGs (3670 (SEM 430) v 2061 (176);  $p < 0.01$ ), with no difference between the primary and secondary forms of SS ( $p > 0.05$ ).

**Conclusion**—The localisation and the level of expression of TGF $\beta$ 2 indicate its involvement in local tissue fibrosis, and may reflect attempts at immunosuppression.

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Lymphocyte infiltrates occur in labial salivary glands (LSG) in Sjögren's syndrome (SS), and in many patients local fibroblasts show signs of activation in the form of collagen synthesis leading to fibrosis.<sup>1</sup> Most of the lymphocytes in the infiltrates are activated CD4 T lymphocytes. Activation occurs when a peptide fragment of as yet unknown antigen binds to a major histocompatibility complex (MHC) class II molecule and is presented to the T lymphocyte.<sup>2</sup>

Transforming growth factor  $\beta$ s (TGF $\beta$ ) are multifunctional growth factors secreted mostly as an inactive form that is activated by cleavage of the active 25 kDa homodimeric TGF $\beta$  from the latent complex.<sup>3</sup> It is produced by epithelial and endothelial cells of many tissues, and by activated macrophages and lymphocytes;<sup>3-5</sup> in addition, some mesenchymal cells such as activated fibroblasts are reported to produce and secrete it.<sup>6</sup> All TGF $\beta$ s (1, 2, and 3) found in human tissues share most of their biological activities, although in some cases one form is reported to be more potent than the other, and sometimes only one form seems to take part in certain activities.

TGF $\beta$  is chemotactic towards fibroblasts and inflammatory cells, but has immuno-

suppressive properties in addition: it inhibits proliferation and certain differentiative functions of T and B lymphocytes. Both TGF $\beta$ 1 and TGF $\beta$ 2 exhibit activities in vitro that are consistent with an immunosuppressive action in vivo.<sup>3</sup> TGF $\beta$  inhibits the production of proteolytic enzymes in autoimmune diseases,<sup>7</sup> but increases the production of tissue inhibitors of matrix metalloproteinases (TIMPs)<sup>8</sup> and plasminogen activator inhibitor-1 (PAI-1).<sup>9</sup> Although TGF $\beta$  is a potent inhibitor of cell proliferation and differentiation of lymphocytes, it is mitogenic for endothelial and connective tissue cells, inducing platelet derived growth factor (PDGF) expression at low concentrations. It also increases the expression of many extracellular matrix (ECM) components, which leads to accumulation of matrix;<sup>3</sup> accumulation of ECM can also occur through chemotaxis of fibroblasts, endothelial cells and epithelial cells induced by TGF $\beta$ . Because of these properties, TGF $\beta$  is important during development and remodelling of various tissues,<sup>10</sup> fibrosis, and wound healing.<sup>3</sup> Both TGF $\beta$ 1 and TGF $\beta$ 2 have been reported to have a role in most of these conditions.<sup>11, 12</sup> The purpose of this work was to study the presence, localisation and level of expression of TGF $\beta$ 2 in LSGs in patients with SS, compared with healthy controls.

### Patients and methods

#### PATIENTS AND BIOPSIES

LSGs were obtained from 10 patients with SS; five had primary SS (four women, 40-74 years old, mean 58 years; one man, 62 years old) and five had secondary SS—three with an underlying systemic lupus erythematosus (SLE) (all women: 26, 28, and 37 years old) and two with rheumatoid arthritis (RA) (women: 47 and 48 years old). Six LSGs from healthy control subjects were studied as controls (two women, 43 and 62 years old; four men, 17-44 years old, mean 31 years). The biopsy specimens were fixed in 10% formalin, dehydrated in alcohol, cleared in xylene and embedded in paraffin. Tissue sections 4  $\mu\text{m}$  thick were cut, mounted on slides coated with APE (3-aminopropyltriethoxysilane; Sigma Chemical Co, St Louis, CA, USA), and stored at 4°C until required for staining.

#### ANTISERA AND IMMUNOSTAINING

Tissue sections were cleared of paraffin in xylene, rehydrated through graded alcohol series and washed in 150 mmol/l sodium

chloride in 20 mmol/l Tris-hydrochloric acid buffer, pH 7.5 (TBS). Immunoreactive epitopes hidden by aldehyde crosslinks were disclosed with pretreatment in 0.4% pepsin in 1 mol/l hydrochloric acid for 20 minutes at 37°C.<sup>13</sup> The intrinsic peroxidase activity was abolished by pretreating tissue sections in 0.1% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in methanol for 30 minutes. The tissue antigens were demonstrated with the unlabelled antibody enzyme method (horseradish peroxidase-anti-horseradish peroxidase (PAP)).<sup>14</sup> Briefly, the tissue sections were treated sequentially with: normal swine serum (dilution 1:5; Dakopatts a/s, Glostrup, Denmark); rabbit antihuman TGF $\beta 2$  IgG (dilution 1:120; British Bio-Technology, Oxon, UK; no cross reactivity with TGF $\beta 1$ , acidic or basic fibroblast growth factors (aFGF, bFGF), PDGF, or epidermal growth factor (EGF) as detected by Western blot or direct enzyme linked immunosorbent assay (quality control data from the supplier)); swine antirabbit IgG, heavy and light chain specific (1:50; Dakopatts a/s); and rabbit PAP complexes (1:100; Dakopatts a/s). The sections were finally incubated for three minutes in 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co) and H<sub>2</sub>O<sub>2</sub> (DAB 50 mg/100 ml TBS and 0.003% H<sub>2</sub>O<sub>2</sub>). Between each step, specimens were washed three times for five minutes in TBS. Finally, the sections, with or without haematoxylin counterstain, were dehydrated in a graded alcohol series, cleared in xylene and mounted in Diatex. All sections used for quantitation were stained at the same time. The specificity of the reaction was tested by the omission of primary antibody, which was replaced by normal swine or rabbit serum (Dakopatts a/s).

#### ASSESSMENT AND QUANTIFICATION OF IMMUNOHISTOCHEMICAL STAINING

The total area of positive reaction in PAP staining was measured for each section using Video Interactive Display System software (VIDAS) linked to a low light charge-screen coupled closed circuit television camera (Panasonic WV-CD 130L, Osaka, Japan) mounted on an Olympus BH-2 light microscope linked to a VIDAS image analyser (Kontron, Munich, Germany).<sup>15</sup> The total area of positive reaction was calculated in three representative samples in SS and in control groups to obtain an estimate for population

mean. Because of very intense staining, the capsule of each sample was excluded.

It was calculated that the mean of five high power fields was not significantly ( $p > 0.05$ ) different from the population mean, using the formula:

$$t = \frac{\bar{x} - \mu}{SEM}$$

where  $t = t$  statistics;  $\bar{x}$  = sample mean;  $\mu$  = population mean; SEM = standard error of mean. Five to 10 fields per section were analysed at  $\times 250$  magnification, starting from a randomly selected field. Results are expressed as a staining index (total area of staining in  $\mu\text{m}^2/\text{mm}^2$  of tissue).

#### STATISTICS

Statistical software of BMDP-PC 7.01 was used to calculate the mean (SEM) to describe the dispersion of the data. The significance of differences between means was analysed by Mann-Whitney  $U$  test.

#### Results

##### IMMUNOHISTOPATHOLOGY

The PAP method gave a good signal to noise ratio of TGF $\beta 2$  in both healthy human LSGs and in the biopsy specimens from SS patients.

The capsular lining cells were always quite intensively positive (fig 1A) and the capsules of the affected LSGs appeared to be thicker than those of the healthy LGSs (data not shown). In all specimens the endothelial cells of capillaries contained TGF $\beta 2$  (fig 2). In the connective tissue, TGF $\beta 2$  was observed mainly in the fibroblasts (fig 1C).

In all patients, staining was weak in some areas and more intense in others, with no correlation of this staining pattern to the amount of mononuclear cell foci, fibrosis, or variation between individual patients. Most positive cells were mononuclear cells (mostly macrophages), lymphocytes in the lymphocyte foci (fig 1D), or fibroblast like cells in fibrotic areas (fig 1C). No difference in this pattern was observed between the primary and secondary types of the disease.

Staining controls confirmed the specificity of all staining results (fig 1B).

##### IMAGE ANALYSIS

There was a significant difference between the level of TGF $\beta 2$  expression in samples from patients with SS compared with healthy controls (expressed as staining index— $\mu\text{m}^2$  positive staining/ $\text{mm}^2$  tissue: 3670 (430) *v* 2061 (176);  $p < 0.01$ ) (table), but the primary and secondary forms of SS did not differ as to TGF $\beta 2$  expression ( $p = 0.97$ ).

#### Discussion

Normal function and integrity of the salivary glands depend on the balance between

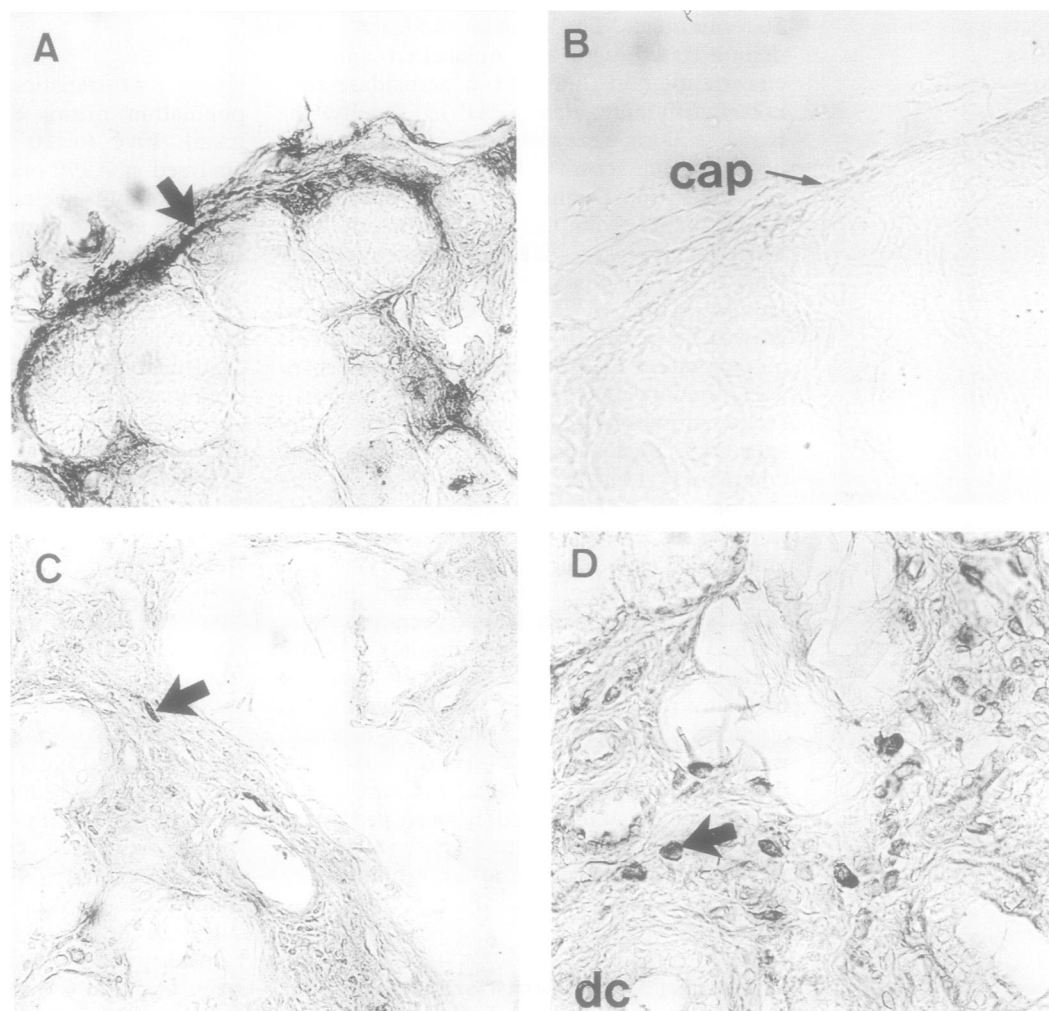
Staining index for transforming growth factor $\beta 2$  in labial salivary glands in primary and secondary Sjögren's syndrome (SS) compared with healthy controls

	Staining index ( $\mu\text{m}^2/\text{mm}^2$ tissue)		
	Primary SS	Secondary SS	Controls
	3555	4470	1737
	5648	3045	2512
	2646	2811	1439
	3437	4405	2228
	3109	3472	1948
	—	—	2506
Mean (SEM)	3679.0 (516.9)	3640.6 (342.3)†	2061.7 (176.4)**

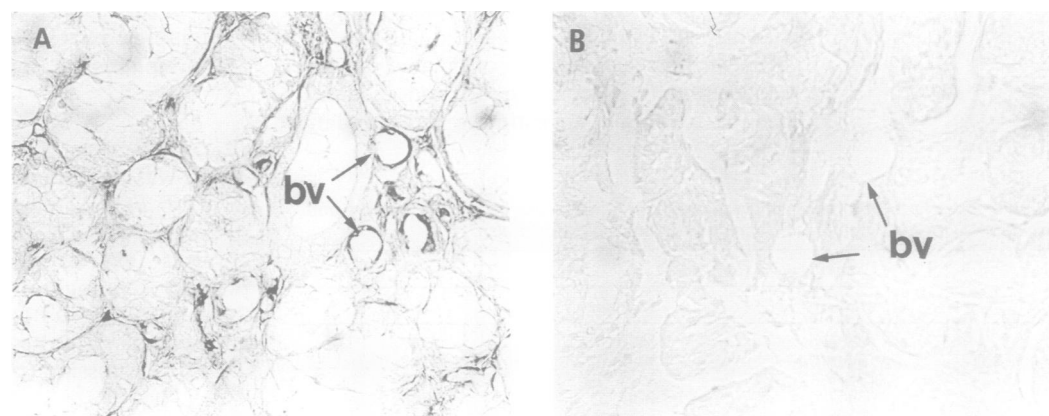
\*\* $p < 0.01$  compared with SS groups; †not statistically different from primary SS group (Mann-Whitney).

hormones, neurotransmitters, adhesion molecules, and cytokines/growth factors. TGF $\beta$ s have been reported to take part in the development, integrity, and remodelling of nearly all normal tissues, including salivary

glands of mouse embryos and rats.<sup>10</sup> The present study has demonstrated the presence and localisation of TGF $\beta$ 2 in the endothelial cells of capillaries and in the capsular and stromal fibroblasts in healthy human



**Figure 1** Immunohistochemical peroxidase-antiperoxidase staining of TGF $\beta$ 2 in labial salivary glands. **A:** Fibroblasts in the salivary gland capsule (arrow) of a patient with Sjögren's syndrome (SS) show a typical intense staining. **B:** Negative staining control of a sample from a healthy control shows no positive reaction in the capsule (cap). **C:** Fibroblasts (arrow) in the fibrous area from a patient with SS display relatively intense TGF $\beta$ 2 staining. **D:** A focus of mononuclear inflammatory cells, mainly lymphocytes and macrophages, in a patient with SS (dc = ductus). Original magnification  $\times 400$ ; no counterstaining.



**Figure 2** Endothelial cells in both capillaries and postcapillary venules in LSGs from patients with Sjögren's syndrome and from healthy controls contained TGF $\beta$ . **A:** Staining of a healthy control gland. **B:** Negative staining control, showing no positive reaction in the endothelium of the vessels (arrows). bv = blood vessels. Peroxidase-antiperoxidase staining; original magnification  $\times 400$ ; no counterstaining.

LSG, which would be compatible with the role of TGF $\beta$ s in maintaining the structure of the gland and its extracellular matrix.

In SS, the homeostasis is disturbed by local sialadenitis, which leads to production of proteolytic enzymes and tissue destruction, with the end result of fibrosis in some cases. Increased expression of TGF $\beta$  could, however, diminish local production of proteinases and their activators (the plasmin system<sup>9</sup>) and increase TIMPs.<sup>8</sup> In addition, TGF $\beta$ s might oppose local tissue destructive events by their ability to induce local accumulation of mesenchymal cells, by chemotactic and mitogenic properties, and by stimulating local cells to production of various connective tissue components.<sup>6</sup> Unfortunately, TGF $\beta$ s fail to restore the original structure of the gland. Accumulation of matrix and the continuous production of matrix components gradually lead to fibrosis of the affected glands, and to replacement of the secretory elements by scar tissue, with deleterious effects on glandular function. The amount of TGF $\beta$  has been reported to be increased in fibrotic processes; in the present study this was the case also in SS.

The mononuclear cell foci are a consequence of local sialadenitis in SS. Lymphocytes and macrophages have been reported to synthesise and secrete both TGF $\beta 1$  and TGF $\beta 2$ . TGF $\beta$  is a potent chemo-attractant and activator of monocytes, neutrophils and T lymphocytes. Although it seems to increase the amount of inflammatory cells, it has immunosuppressive properties in addition. It inhibits proliferation and some differentiative functions of T and B lymphocytes by as yet unknown mechanisms. When monocytes are transformed into macrophages, TGF $\beta$  decreases their hydrogen peroxidase production, thus inhibiting their ability to kill via a respiratory burst.<sup>4 5</sup>

The data revealed no significant difference in expression of TGF $\beta 2$  between primary and secondary SS, suggesting that the TGF $\beta$  mediated compensatory mechanisms, activated by local disease processes in these conditions, are the same and do not represent disease specific features. However, the difference between SS and the healthy controls

was significant, so it seems likely that TGF $\beta 2$  has a role as an immunoregulatory and a profibrotic factor in SS.

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