Tumor necrosis factor-alpha and receptors for it in labial salivary glands in Sjögren's syndrome

H. Koski[1], A. Janin[2], M.G. Humphreys-Beher[3], T. Sorsa[4], M. Malmström[5], Y.T. Konttinen[6]

[1]*Institute of Biomedicine, Department of Anatomy, University of Helsinki, Finland;*
[2]*Service Central d'Anatomie Pathologique, Hopital Saint Louis, Paris, France;*
[3]*Department of Oral Pathology, College of Dentistry, University of Florida, Gainesville, FL, USA;*
[4]*Department of Periodontology, Institute of Dentistry, University of Helsinki;*
[5]*Department of Oral Medicine, Institute of Dentistry, University of Helsinki;*
[6]*Department of Oral Medicine, Surgical Hospital, Helsinki University Central Hospital, Finland.*

Abstract

Objective

Modulation of TNF- by neutralizing antibodies, soluble receptors and TNFR: Fc fusion proteins are being developed for the therapeutic modulation of immune inflammation. It is becoming increasingly important to understand the state and involvement of the TNF- /TNFR system in various rheumatic diseases.

Tumor necrosis factor-alpha (TNF-) affects its target cells through binding to two different receptors, TNFR-p55 and TNFR-p75. Mitogenic, cytostatic and cytotoxic effects of TNF- on various cells have been reported. In Sjögren's syndrome (SS) focal sialadenitis leads to salivary gland destruction and loss of function. Although TNF- is one possible mediator in these processes, nothing is known about the spatial distribution of TNF- in relation to its receptors/ target cells in salivary gland tissue.

Methods

Labial salivary glands (LSG) were obtained from 16 SS patients and 13 healthy controls and stained using the immunohistochemical peroxidase-anti-peroxidase (PAP) method for TNF- , TNFR-p55 and TNFR-p75.

Results

TNF- , TNFR-p55 and TNFR-p75 staining was absent, weak or relatively inextensive in controls compared to SS patients. Infiltrating mononuclear inflammatory cells in SS patients displayed moderate to strong TNF- and TNFR expression. In addition, resident vascular endothelial cells, ductal epithelial cells and fibroblasts co-expressed TNF- and TNFR. In contrast, acinar end piece cells did not express TNF- or TNFR-p75 although TNFR-p55 was expressed.

Conclusion

The interrelated localization of TNF receptors and their ligand TNF- in inflammatory and in endothelial cells suggests a proinflammatory role of TNF- in SS. The expression of TNF- and its receptors in fibroblasts and ductal cells may contribute to ductal hyperplasia and glandular fibrosis. However, in contrast to expectations, the cellular localization of the TNF- /TNRF system argues against its role in acinar cell atrophy.

Key words

Sjögren's syndrome, TNF- , receptor, apoptosis, atrophy, acinar cell.

Hannele Koski, MD; Anne Janin, MD; Michael G. Humphreys-Beher, PhD; Timo Sorsa, DDS; Maria Malmström, Professor; Yrjö T. Konttinen, MD, Professor.

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Please address correspondence and reprint requests to: Dr. Hannele Koski, Institute of Biomedicine, Department of Anatomy, PO Box 9 (Siltavuorenpenger 20 A), FIN-00014 University of Helsinki, Finland.
E-mail: hannele.koski@helsinki.fi

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Introduction

Immune abnormalities in Sjögren's syndrome (SS) include a reduced peripheral blood CD4+ cell count, hypergammaglobulinemia and specific autoantibodies against SS-A/Ro, SS-B/La and -fodrin. Mononuclear cell infiltrates/focal adenitis, regularly seen in the exocrine glands of the patients, seem to lead to disturbances of the acinar end piece cells and, later on, to irreversible destruction of the parenchymal components of the glands (1). Ultimately, salivary flow rates are affected. The initial event in SS pathogenesis is thought to be the activation of CD4+ cells by antigen presenting cells carrying a complex of the HLA II molecule and an unknown antigen on their surface (2). The CD4+ cells can be divided into two subtypes, T-helper 1 and 2 cells (T_h1 and T_h2) (3). CD4+ cell activation leads to the production and release of many cytokines, including TNF- , which is a product of a T_h1 subtype response (4).

The active form of TNF- is produced by proteolytic cleavage of a 17 kD fragment from the extracellular carboxyterminal domain of the 25 kD membrane-bound proform of the protein and subsequent formation of a homotrimer of the cleaved fragments (5). The biological (autocrine, paracrine, endocrine and juxtacrine) functions of TNF- are mediated through two different TNF receptor populations, TNFR-p55 and TNFR-p75 (CD120a and CD120b, respectively).

In addition to lymphocytes, TNF- is produced by macrophages, endothelial cells and by some epithelial cells, e.g. keratinocytes and salivary gland epithelial cells (6-8). TNF- plays an important role in host defense against infection and in inflammation. It increases the adhesive properties of endothelial cells for inflammatory cells (9) and is able to activate T- and B-lymphocytes and monocytes (10-12). It has well known cytotoxic/cytostatic properties not only for various malignant cells (4), but also for some salivary cell lines, e.g. for a human salivary epithelial cell line (5, 13). These effects have been shown to be dependent both on the TNF- concentration (5) and on the cooperative action of other cytokines (e.g. PDGF, EGF, IL-1, 2 and 6 and IFN-) (14, 15). In addition, cellular expression and properties of the two types of TNF receptors,TNFR-p55 and TNFR-p75, modulate TNF- actions (16).

An additive effect between the two TNF receptors has been proposed to be involved in the cytotoxity and adhesive properties of TNF- (9). TNFR-p55 mediates most of the cytotoxic effects, fibroblast proliferation and prostaglandin synthesis. The TNFR-p75 mediated effects are more restricted; it potentiates the cytotoxic effects of TNFR-p55 and stimulates T-cell proliferation synergistically with IL-1, IL-2, and IL-6 (16); it is more abundant in lymphoid cell aggregates than TNFR-p55 (15).

In SS the salivary gland epithelial cells produce over 40-fold more TNF- m-RNA than is produced by non-inflammatory control glands (6). Nothing is known, however, about TNF receptor expression or distribution in SS salivary glands. To better understand the local targets and potential effects of TNF- , we studied the presence, cellular expression and localization of TNF- and its two receptors in the labial salivary glands (LSGs) of SS patients compared to healthy controls.

Patients and methods

Patients and biopsies

Six to eight LSGs were obtained from a total of 16 patients with SS. The diagnosis was based on the European criteria for SS (17); in addition, the focus score index in all patient LSG biopsies was > 2 (criteria require a focus score ≥ 1) showing they all had sialadenitis; 12 of the patients had 1°SS, 4 had 2°SS with underlying systemic lupus erythematosus, and one had 2°SS with rheumatoid arthritis. Similarly, LSGs were obtained from 13 healthy volunteer controls with no symptoms or clinical signs of SS; all had a focus score index < 1 (i.e., they did not have focal sialadenitis). All LSG biopsies were fixed in 10% formalin, dehydrated in ethanol, cleared in xylene and embedded in paraffin. 4 m tissue sections were cut and mounted on APE (3-aminopropyltrietoxysilane; Sigma Chemical Co., St.

Louis, MO, USA) coated slides and stored at +4°C until staining.

Antisera and immunostaining

Tissue sections were deparaffinized in xylene, rehydrated through graded alcohol series and washed in 20 mM Tris-HCl, 150mM NaCl, pH7.5 (TBS). Immunoreactive epitopes hidden by aldehyde cross-links were disclosed by pretreatment in 0.4% pepsin in 1N HCl for 20 min at +37°C. The intrinsic peroxidase activity was abolished by pretreating the tissue sections in 0.1% H_2O_2 in methanol for 30 minutes. The tissue antigens were demonstrated with the unlabelled antibody enzyme method PAP (peroxidase-anti-peroxidase) (18). Briefly, tissue sections were treated sequentially with: 1) normal rabbit serum (dilution 1:5; Dakopatts a/s, Glostrup, Denmark), 2) primary polyclonal goat anti-human TNF- antibody, dilution 1:50 (R&D Systems, Abingdon, Oxford, UK) and monoclonal mouse anti-human TNF- (1:50; Dakopatts a/s); or monoclonal mouse anti-human TNFR-p55 (CD 120; 1: 100) and TNFR-p75 (CD 120b; 1:150) antibodies characterized for their specificity in detail elsewhere (19, 20); 3) rabbit anti-goat or rabbit anti-mouse IgG, heavy and light chain specific, respectively (1:50; Dakopatts a/s), and 4) with the appropriate horseradish peroxidase - anti-horseradish peroxidase complexes (1:100; Dakopatts a/s). For colour development, the sections were incubated for 3 min in 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2. Between each step the specimens were washed for 3 x 5 min in TBS. Finally, thesections, withouthematoxylin counterstain, were dehydrated in graded ethanol series, cleared in xylene and mounted in Diatex. The specificity of the reaction was tested by omitting the primary antibody, which was replaced with the corresponding normal serum or mouse IgG (Dakopatts a/s).

All samples were stained at the same time for each primary antibody and the conditions for each separate staining procedure were similar every time in order to get the best possible comparability. The reaction of polyclonal TNF- antibody was confirmed using monoclonal TNF- antibody. The results were analyzed by two members of the group in a blinded manner.

Table I. Expression of tumor necrosis factor-alpha (TNF-) and its receptors p75 and p55 in labial salivary glands (LSG) in primary (1°) and secondary (2°) Sjögren's syndrome (SS) and in healthy controls.

Samples		TNF-alpha	TNFR-p75	Samples		TNFR-p55
1°SS	1	bv dc ic f	bv dc ic	1°SS	9	ic f
	2	dc ic f	dc ic		10	ac dc f
	3	bv dc ic	bv dc ic		11	ic bv ac dc
	4	ic f	ic		12	bv ac dc f
	5	bv ic	bv ic	2°SS	13	ic bv ac
	6	dc ic f	dc ic		14	ic ac dc f
	7	bv dc ic	bv dc ic		15	bv ac dc f
	8	bv dc ic f	bv dc ic		16	bv ac dc
Contr.	1	dc	bv dc	Contr.	10	bv ac dc
	2	-	dc		11	bv ac dc
	3	dc	bv dc		12	ac dc
	4	-	dc		13	bv ac dc
	5	dc	Dc			
	6	-	-			
	7	Dc	bv dc			
	8	-	dc			
	9	-	-			

ac: acini; bv: blood vessel; f: fibroblasts; dc: ductus epithelial cells; ic: inflammatory cells; f: fibroblasts.

Results

General assessment

The PAP method gave a good signal-to-noise ratio for TNF- , TNFR-p55 and TNFR-p75 in LSGs in SS and in healthy controls. In all samples there was some intra-sample variation in the staining intensity, but by and large it was similar throughout the sample. Staining controls confirmed the specificity of all immunoreactions reported. 8 of the 16 SS samples and 9 of the 13 healthy specimens underwent a TNF- staining procedure; 6 of the 8 SS samples and all 9 of 13 healthy LSGs used in TNF- staining were also stained with TNFR-p75 antibody. All 8 of these patients had primary SS. The remaining 8 of the 16 SS samples and the remaining 4 of the 13 healthy LSGs were stained with TNFR-p55 antibody (Table I).

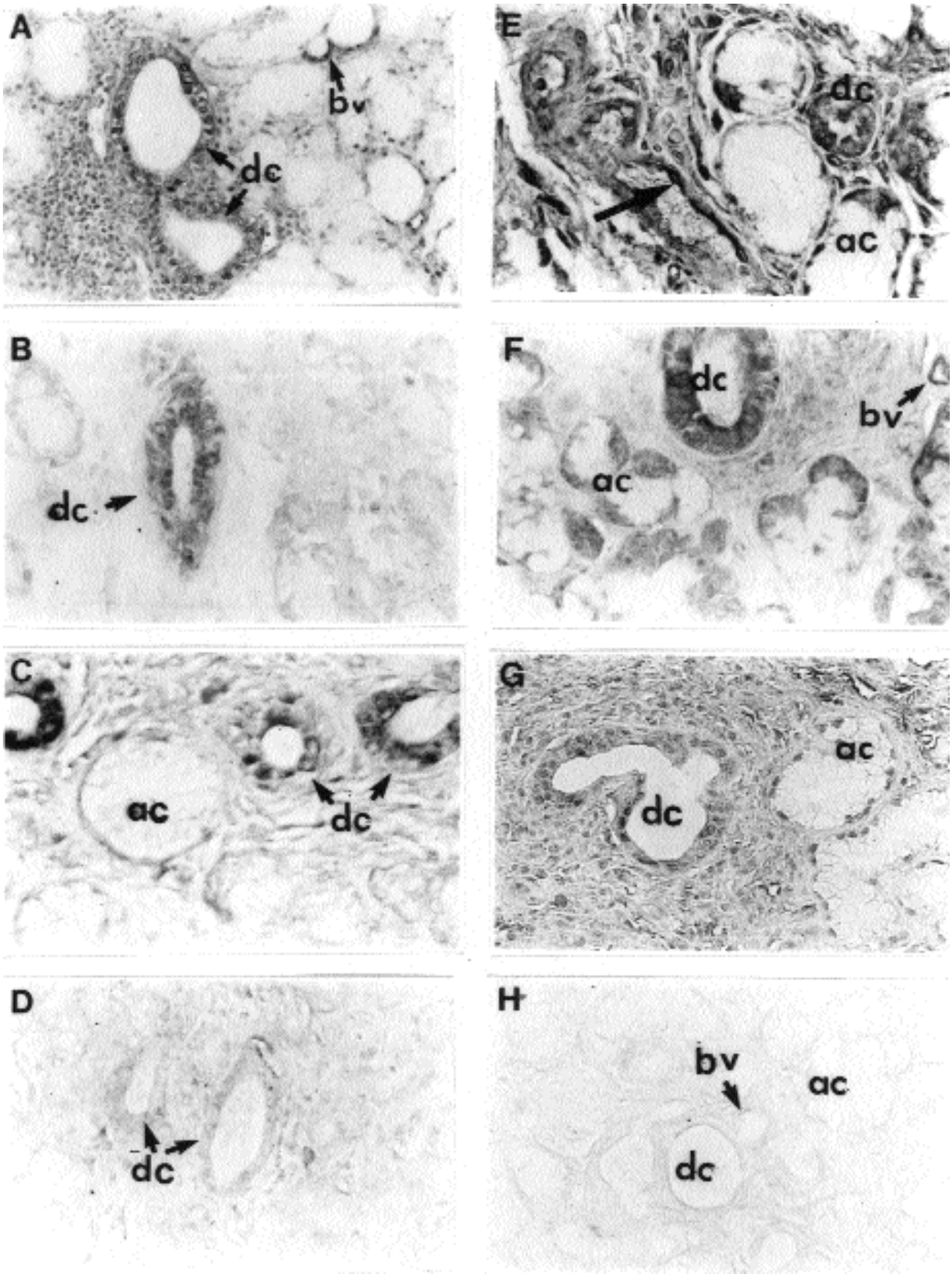
Tumor necrosis factor-alpha

Healthy control glands did not contain mononuclear cell infiltrates. Half of the healthy specimens did not show any immunoreaction for TNF- (although the positive sample controls were positive) but TNF- staining, when found, was moderate to strong as in all SS specimens. In SS samples infiltrating cell lymphocytes and macrophages in the mononuclear cell infiltrates also showed a strong immunoreaction (Figure 1, panel A, SS patient; Table I).

In addition, in some SS biopsy samples endothelial cells of the arterioles, capillaries and postcapillary venules stained strongly for TNF- (Fig. 1, panel A). Fibroblasts of fibrotic areas of the SS glands showed a strong immunoreaction for TNF- (TableI). Finally, in control samples staining of the salivary duct epithelial cells was more occasional than in SS samples; both displayed a relatively strong TNF- reaction (Fig. 1, panels B and C). Acinar cells did not display any immunoreaction for TNF- in either the control or SS samples.

Tumor necrosis factor receptor-p55

All samples contained TNFR-p55 posi-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tive cells. Inflammatory mononuclear cells in SS samples stained occasionally for TNFR-p55 (Table I). In contrast to the cytoplasmic TNF- staining, TNFR-p55 staining was most intense on the cell surfaces of inflammmatory mononuclear cells.

Some of the fibroblasts found in the inflamed and fibrotic areas were less intensively positive (Fig. 1, panel E). TNFR-p55 staining of vascular endothelial cells was strong in both SS and in control specimen. In both groups, ductal epithelial cells were strongly TNFR-p55 positive (Fig. 1, panels E and F; Table I). In acinar cells the TNFR-p55 immunoreaction was found to be restricted to the basal cytoplasm of the acinar cells, and sometimes several such cells formed half-moon like structures (Fig. 1, panels E and F).

Tumor necrosis factor receptor-p75

The immunoreaction for TNFR-p75 was strong in SS LSGs compared to the healthy controls and two of the healthy samples showed hardly any reaction for TNFR-p75 (Table I). All of the mononuclear cells in the infiltrates observed in SS glands stained positively with this receptor antibody. The reaction was as intense as that for TNF and TNFR-p55 (Fig. 1, panel G).

Blood vessel endothelium of both control and SS samples showed some positive reaction with more intense and widespread staining observed in SS than in control samples. Ductal cells showed positive reaction for TNFR-p75 with similar intensity as that for TNFR-p55 and TNF- . This reaction was strong in SS samples and less intense and more occasional in healthy specimens (Fig. 1, panel G).

In contrast, no immunoreaction for TNFR-p75 was detectable in any of the control or SS acinar cells.

Discussion

Sjögren's syndrome, clinically characterized by xerostomia and dry eyes, is an autoimmune disease where a complex cytokine network plays a significant role in the pathogenesis. The effects of TNF- on its target cells depend not only on its concentration (5), but also on receptor molecule expression and on the affinity of the receptors for TNF- (16). One way to modulate TNF- binding is via structurally related receptors in soluble form instead of the usual cell surface receptors. The soluble receptors compete for TNF- with the membrane bound receptors, thus inhibiting TNF activity. Interactions between many other cytokines further modulate TNF- mediated effects.

In addition to the local autocrine and paracrine effects of TNF- , juxtacrine stimulation of monocytes/macrophages is supposed to be possible and to contribute to TNF- mediated killing of tumor cells and virus-infected cells. IFN- has well known anti-viral effects and it is detected in normal salivary glands (21, 22). Salivary gland ductal epithelial cells may harbor some viruses in healthy individuals after the primary infection. Normally, however, viruses are kept latent and are not reactivated or shed except in some autoimmune diseases and in immunosuppressed patients (2). This is assumed to emphasize the T-cell mediated and/or IFN- dependent control of the infected cells (23, 24). Therefore, the basal production of TNF- and the receptors for it, now detected in normal salivary glands as well, may modulate normal cellular immune responses. TNF- may together with IFN- have useful functions in host defense in normal salivary glands.

In SS most of the local lymphocytes are T lymphocytes of the CD4+ subtype (2). The T_h1 subtype of CD4+ cells, rather than the T_h2 cells, are according to many studies in SS responsible for local lymphokine production (6, 25-27). Some of these cells are IL-2 receptor positive, proliferating T lymphoblasts producing IFN- and IL-2 (22, 28). TNF- has been shown to be produced by activated Th1 cells (4). TNF- has a significant ability to activate inflammatory cells. In combination with IFN- it increases HLA-II expression on antigen presenting cells and together with IL-2 it increases T-lymphocyte proliferation and activates T- and B-lymphocytes (5). TNF- production and expression have been shown to be increased in lymphoproliferative conditions and in lymphoid cell aggregates in rheumatoid arthritis (15, 29). Moreover, TNF receptor production and expression, mostly that of p75, is increased in activated lymphocytes (29). Interestingly, IFN- has in some studies been more consistently found in healthy controls than in SS patients (6, 25, 30). This suggests that IFN synthesis may be selectively downregulated in SS and perhaps shift toward that of TNF- . Expression of TNF- and of both p55 and p75 type receptors in mononuclear inflammatory cells, as shown in this study, most evidently indicates a proinflammatory role for TNF- in SS. The proliferation and activation of inflammatory cells might

Fig. 1. Immunohistochemical peroxidase-anti-peroxidase staining of tumor necrosis factor-alpha (TNF-) and its receptors p55 and 75 in labial salivary glands.

TNF- (panels A-D). A typical staining pattern for TNF- was as follows: In Sjögren's syndrome (SS) lymphocytes and macrophages in the periductal mononuclear cell infiltrates were TNF- positive (panel A). TNF- expression of the ductal epithelial cells was strong in SS samples (panels A and B), but usually more weak although still discernable in about half of the healthy control samples (panel C). In addition,TNF- was seen in the endothelial cells of local blood vessels (bv) in both SS samples (strong staining, panel A) and occasionally in healthy control samples (weak staining, not shown).

TNFR-p55 (**panel E**, a SS sample and **panel F**, a healthy specimen) was found in SS in the inflammatory mononuclear cells as surface staining and was in addition expressed in salivary duct (dc) epithelial cells, blood vessel (bv) endothelium and acinar cells (ac), often in half-moon like structures. TNFR-p55 expression was intensive both in SS and in healthy control samples.

TNFR-p75 (panel G) was found in inflammatory mononuclear cells and in ductal epithelial cells. TNFR-p75 (and its ligand TNF-) were never observed in the acinar cells.

Negative staining controls (**panel D** for TNF- and **panel H** for its receptors),in this instance after the omission of the primary antibody, which for control purposes was replaced with goat and mouse IgG, respectively, demonstrates the specificity of the staining. Original magnification x 400 in all panels; all samples without counterstaining.

thus partly occur *in situ* through TNF- actions.

TNF- increases the adhesive properties of vascular endothelial cells by inducing ICAM-1, VCAM-1 and E-selectin (9), which are also induced by IFN- (31). In our study the endothelial cells showed marked immunoreactivity for TNF- and for both of its receptors in SS samples, whereas in healthy specimens only TNFR-p55 immunoreaction was strong. IFN- is decreased in LSGs in SS compared to normal salivary glands (30), but TNF- is more abundant in the disease compared to controls (6). It is therefore possible that in normal salivary glands the regulation of adhesive properties of the endothelial cells occurs through IFN- actions, but is shifted toward TNF- actions in SS. These findings support the thought that migration of inflammatory cells from the circulation through endothelium to glandular tissue is enhanced by TNF- in SS.

One interesting point of view is that TNF- of endothelial cells is locally produced (9, 16). It converts the endothelium into a procoagulant surface (9), thus disturbing the normal function of endothelium. In the context of SS, an interesting question is whether TNF- , through its local effects on endothelial cells, is involved in the regulation of the transport of liquids to saliva and thus in decreased salivary flow, which is regularly seen in patients of SS.

TNF- takes part in release of collagenases from fibroblasts and endothelial cells (14, 26, 32). In this study, TNF- and its receptor p55 were frequently found in fibroblasts in SS samples. In this respect TNF- might, at least in part, explain the induction and increased expression of collagenase and other matrix metalloproteinases in SS (33).

It has also been suggested that TNF- has a role in parenchymal damage of salivary glands in SS (34). The cytotoxic properties of TNF- are highly dependent on TNF- concentrations and on environmental conditions such as the presence of other growth factors and the level of TNFR expression. TNF- increases the antiproliferative effects of IFN- on human salivary cell line (13) and on mesangial cells (35). In SS the salivary gland epithelial cells produce 40-fold more TNF- mRNA than epithelial cells from individuals with histologically normal salivary glands, as discussed above (6). All these findings bring up the thought that TNF- could have growth arrest/cytotoxic effects on acinar cells in SS. However, acinar cells did not contain TNF- or TNFR-75. Because high TNF- concentrations and possibly synergistic TNFR p55/p75 interactions are required for cytotoxic effects, the present findings do not support the hypothesis of TNF- induced cytotoxicity as a cause of acinar cell atrophy/loss in SS. It is also noteworthy that TNF- , which also has cytotoxic effects and is able to bind to both TNF receptors, has not been found in acinar cells either (11). However, it can not be excluded that the high production of TNF- , perhaps in concert with some other cytokines, in inflammatory areas can via TNFR-p55 induce acinar cell damage. Indeed, TNFR-p55 showed strong immunoreaction in acinar cells. Lack of neurogenic trophic stimuli, as a result of the loss of the anatomical peripheral nerve-acinar cell coupling, has been proposed as an alternative cause for acinar cell atrophy (36-38).

In previous studies (6, 34) the ductal epithelial cells have been shown to express TNF- with higher intensity than normal salivary gland ductal cells. Our study supports this finding and is the first that locates both TNF receptors in ductal epithelial cells, partly in the same cells that express TNF- itself. This finding suggests that both autocrine and paracrine growth factor-like mitogenic effects can be exerted by TNF- on ductal cells in SS. Again,this possible mitogenic effect probably requires interactions with other growth factors, as discussed in the context of mitogenic effects on fibroblasts in the previous chapter. Indeed, TNF- has been shown to be mitogenic for mesangial cells in the presence of PDGF (35) and it increases EGF-R expression on fibroblasts (14). Furthermore, transcription factor NF-kappa B is not inhibited by 1 kappa B alpha in the human salivary gland ductal cell line in the presence of TNF- (39). Normally, the growth arrest of salivary epithelial cells happens through IFN- actions, which are strengthened by TNF- present at the same time (13). In SS, though, the lack of IFN- discussed in the previous chapters and thus its inhibitory action on ductal epithelial cells might be involved in the marked ductal cell hyperplasia frequently seen in SS.

The localization and the increased expression of TNF- and receptors for it shed light on its role as a proinflammatory cytokine and as a growth factor in SS. Recently, promising results have been published on the use of soluble TNFR-p55 in NOD mice (40), which is widely used as a model for focal adenitis/Sjögren's syndrome.

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