

Extracellular matrix molecules in chronic obstructive sialadenitis: an immunocytochemical and Western blot investigation

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Abstract: The exact pathomechanism of inflammation progress and fibrosis in chronic sialadenitis is unknown. Connective tissue growth factor (CTGF), matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) have been implicated in the pathogenesis of various fibrotic conditions. These factors are thought to be essential in the regulation of extracellular matrix turnover and the development of tissue fibrosis. In the present study, the expression of CTGF, MMP-2, -3, -9, -13 and TIMP-3 was examined in chronic obstructive sialadenitis. Tissue samples of 13 patients with chronic sialadenitis of the submandibular gland associated with sialolithiasis and 4 normal tissue samples of the submandibular gland were analyzed immunohistochemically and by Western blot analysis. An intense CTGF immunoreactivity was observed in the ductal system of inflamed salivary glands, whereas in normal glands no reactivity or a very low CTGF immunoreactivity was present. Immunohistochemical studies revealed a low to strong reactivity of MMP-2, -3, -9, -13, and TIMP-3 in the ductal system, in acinar cells and in lymphomonocytic infiltrates in normal and inflamed tissues. The expression of MMP-2, -3, -9, -13, and TIMP-3 was confirmed by Western blotting in all cases. Over-expression of CTGF in chronic obstructive sialadenitis suggests that this factor

may play a role in glandular fibrosis. However, the physiological role of MMP-2, -3, -9, -13, and TIMP-3 in normal glands, as well as their possible role in inflammation progress and fibrosis in chronic obstructive sialadenitis, remains to be elucidated. (*J. Oral Sci.* 46, 227-233, 2004)

Key words: connective tissue growth factor; matrix metalloproteinases; chronic obstructive sialadenitis; fibrosis.

Introduction

At about 35%, chronic obstructive sialadenitis represents the most frequently occurring type of chronic sialadenitis (1). Salivary gland cysts and lymph node diseases play an important pathogenetic role, especially mechanic obstruction by intraductal concretions and extraductal factors such as benign and malignant neoplasms of the salivary glands. The most common cause of an obstruction in the salivary gland system are sialoliths, which occur with a frequency of about 1.2% in the population (2).

Histologically, this disease is characterized by periductal lymphocytic infiltrates, acinar atrophy and increasing periductally limited fibrosis of the glandular parenchyma. Persistent obstruction leads to a diffuse lymphocytic infiltration and extended periductal and intralobular fibrosis with destruction of the lobular architecture and sclerosis of the whole gland in the late stages of the disease (3).

The inflammation progress and increasing fibrosis of the glandular parenchyma in chronic obstructive sialadenitis are likely the result of interactions of different cytokines

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of glandular cells and lymphomonocytic infiltrates which have not been studied in great detail. For a better understanding of the mechanism of glandular fibrosis in chronic obstructive sialadenitis, an exact knowledge of the cytokine profile involved in the inflammation process is essential.

In a previous study, we demonstrated an increased expression of transforming growth factor beta (TGF- β) in chronic obstructive sialadenitis, which suggests a possible role of this factor in the inflammation progress and fibrosis of this disease (4). Based on *in vitro* and *in vivo* studies, it has been proposed that connective tissue growth factor (CTGF) is a mediator of the fibrogenic effects of TGF- β (5,6). In the present study, the possible involvement of CTGF as a fibrogenic factor in chronic sialadenitis was examined.

The net accumulation of extracellular matrix in tissue depends on the balance between the synthesis and the degradation of matrix components by matrix metalloproteinases (MMPs) and their specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) (7). The aim of the present study was to investigate the expression pattern of MMP-2, -3, -9, -13, and TIMP-3 as potential modulators of tissue fibrosis in this disease.

Materials and Methods

Tissue specimens

Tissue specimens of the submandibular glands were taken from a total of 17 patients who had undergone extirpation of the gland at the Department of Otolaryngology, Head and Neck Surgery of Philipps University. In 13 cases (8 female, 5 male; mean age 51 years) the histopathological examination resulted in the diagnosis of chronic obstructive sialadenitis associated with sialolithiasis. The remaining 4 examined tissue samples represented unaltered tissue samples of the submandibular gland that were obtained during neck dissection as controls (4 male, mean age 60.8 years). A 1 cm³ part of the samples was fixed in 10% neutral formaldehyde solution and embedded in paraffin wax for histological staining. Approximately 70 mg was removed from the glands, frozen immediately in liquid nitrogen, and stored at -80°C until protein extraction.

Histology and immunohistochemistry

From the tissue samples, 4 μ m slices were cut, mounted on 3-aminopropyltriethoxysilane (APES)-coated slides and dried overnight at 37°C. The following day, the sections were dewaxed in xylene and rehydrated in a series of graded alcohol. The sections were stained with haematoxylin and eosin and with Giemsa. The intensity

and distribution of lymphocytic and monocytic infiltrates, the occurrence of germinal centres, and the degree of fibrosis were reviewed. The extent of these features was graded according to Seifert's staging system (Table 1).

The immunohistochemical investigations were performed using mouse monoclonal anti-CTGF (1:100, Acris, Bad Nauheim, Germany), anti-MMP-2 (1:100, R&D, Wiesbaden, Germany), -3 (1:50), -9 (1:100), and -13 (1:50), and TIMP-3 (1:200) antibodies (Chemicon, Hofheim, Germany). A streptavidin-biotin complex/horseradish peroxidase kit, with 3,3'-diaminobenzidine tetrahydrochloride, was used as a chromogenic substrate (Dako, Hamburg, Germany).

The protein detection required microwave treatment in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched by incubating the slides in 0.3% hydrogen peroxidase and methanol. Sections were counterstained with hemalaun. Negative controls in each staining series included sections in which the primary antibody was replaced by the buffer. Immunohistochemical staining was evaluated by comparing the staining intensity and the estimated proportion of the positively stained cells with the total number of glandular cells. The immunoreactivity of antigens was graded into four groups: no, low, moderate, and strong reactivity.

Table 1 Classification of chronic obstructive sialadenitis [3]

Stage 1	Slight focal and periductal lymphocyte infiltration, slight periductal fibrosis
Stage 2	Moderate diffuse and periductal lymphocyte infiltration, moderate periductal fibrosis and slight intralobular fibrosis, focal acinus atrophy
Stage 3	Accentuated diffuse lymphocyte infiltration, periductal and intralobular lymph follicle formation, accentuated periductal and intralobular fibrosis, accentuated acinus atrophy and duct epithelium metaplasia
Stage 4	Destruction of lobular architecture, marked parenchymal loss and sclerosis

Western blot analysis

Expression of MMP-2, -3, -9, -13, and TIMP-3 was evaluated by Western blot analysis. For completion of the immunohistochemistry, a Western blot analysis of CTGF was performed, but the mentioned anti-CTGF antibody was not suitable for frozen tissue. Thus, we were not able to perform Western blot analysis with this antibody.

The tissue was washed twice in cold PBS and resuspended in lysis buffer (1% NP-40, 66mM EDTA, 10mM Tris/HCl). Thirty-five μ g of each sample was run on a 15% SDS-PAGE gel, electrophoretically separated,

and then transferred to a nitrocellulose membrane. The membranes were blocked with 3% milk/PBS to prevent non-specific binding of the antibody and then incubated with MMP and TIMP-3 antibodies. These antibodies were detected by the enhanced chemiluminescence technique using the ECL kit (Amersham Biosciences, Freiburg, Germany). A mouse anti-actin monoclonal antibody (Chemicon, Hofheim, Germany) was used as a control.

Results

Histological studies showed that 4 morphologically unaltered tissue samples were obtained during neck dissection. A total of 13 submandibular glands were removed because of chronic sialadenitis, 3 of which were classified as stage 2, 5 as stage 3, and 5 as stage 4.

All analysed specimens of the inflamed salivary glands revealed a strong positive cytoplasmatic reaction with the CTGF antibody, the positive cells being localized in the ductal system (Fig. 1a, b). Immunohistochemically, there was no difference in the various divisions of the ductal system. In normal glands, no or a very low CTGF immunoreactivity was present in only a few ducts.

In specimens of the normal submandibular gland, a positive cytoplasmatic reactivity with the MMP-2, -3, -9, -13, and TIMP-3 antibodies was observed. The positive cells were localized in the various divisions of the ductal system and in acinar cells. The immunoreactivity of

antigens was weak or moderate. In chronic sialadenitis, MMP-2, -3, -9, -13, and TIMP-3 immunostaining was expressed by all of the stromal cells, and the staining was weakly to strongly positive in the ductal system and acinar cells in all specimens (Fig. 2a, b). All tissue factors investigated were expressed in glandular cells in close proximity to areas of fibrosis. Their expression was also evident in cells at a distance from the scar tissue and independent of the presence of lymphomonocytic infiltrates. The immunohistochemical analysis of all studied cytokines revealed no correlation between the staining intensity of the cytokines and stages of chronic sialadenitis.

In the Western blots, immunoreactive bands specific for MMP-2, -3, -9, -13, and TIMP-3 were observed in normal and also in inflamed samples, confirming protein expression in all tissues (Fig. 3). The molecular weights of the various MMP's and TIMP-3 corresponded to their latent forms.

Discussion

The exact aetiopathology and mechanism of atrophy of the glandular cells and lymphocytic infiltration associated with an increase in the extracellular matrix in chronic obstructive sialadenitis are unknown. The histomorphological changes of the glandular parenchyma and the progredient character of the inflammatory progression in this disease cannot be explained merely by mechanic secretory congestions and overpressure in the salivary

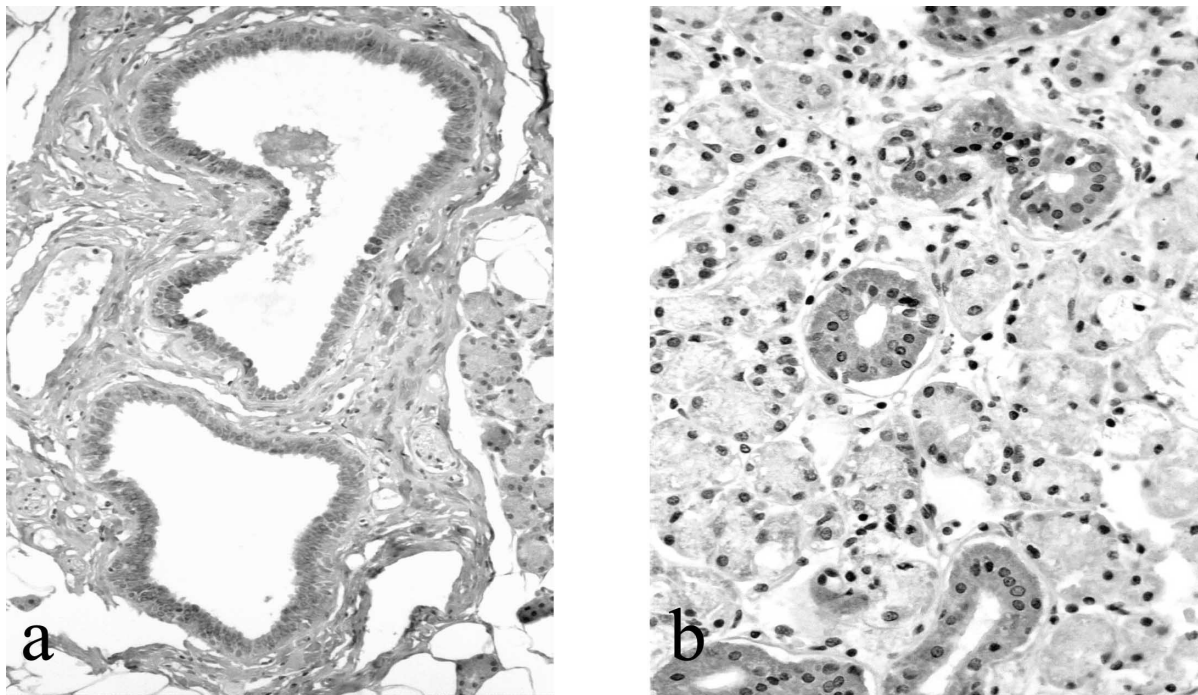


Fig. 1 Ductal system of submandibular gland tissue in chronic obstructive sialadenitis showing CTGF-positive epithelial cells. a) stage $\times 3$, 100, b) stage $\times 2$, 200.

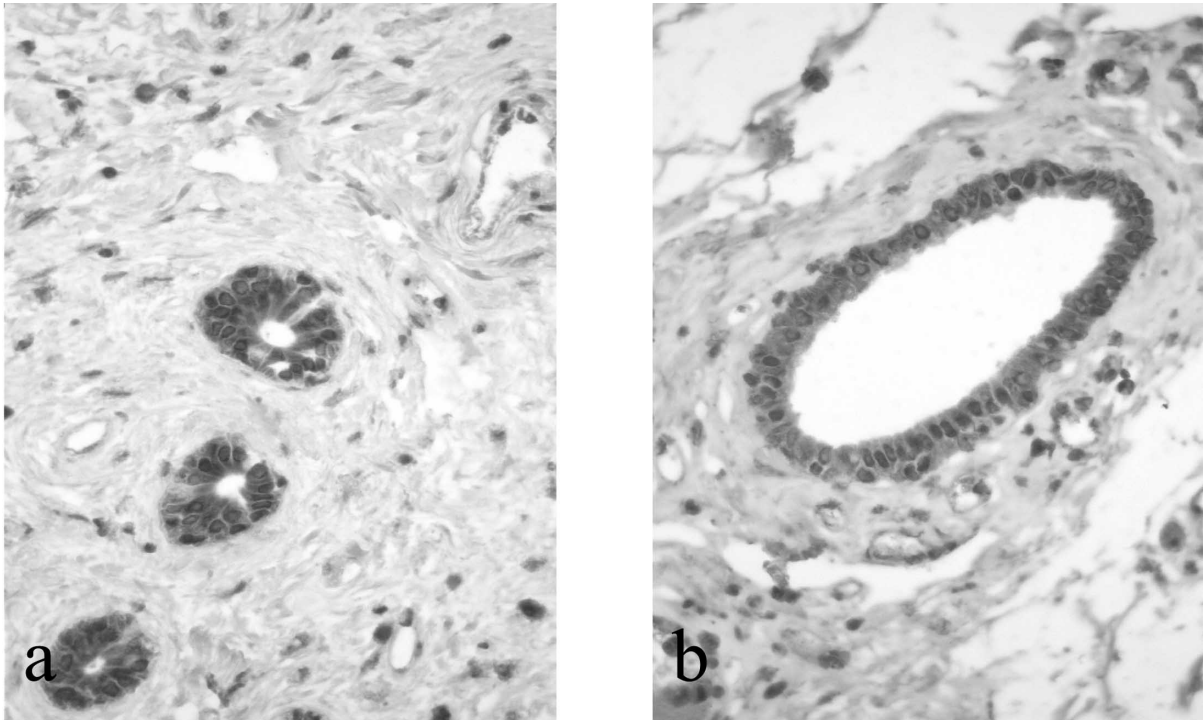


Fig. 2 Ductal system of submandibular gland tissue in chronic obstructive sialadenitis. a) TIMP-3 positive cells in stage $\times 3$, 200 , b) MMP-9 positive cells in stage $\times 3$, 200.

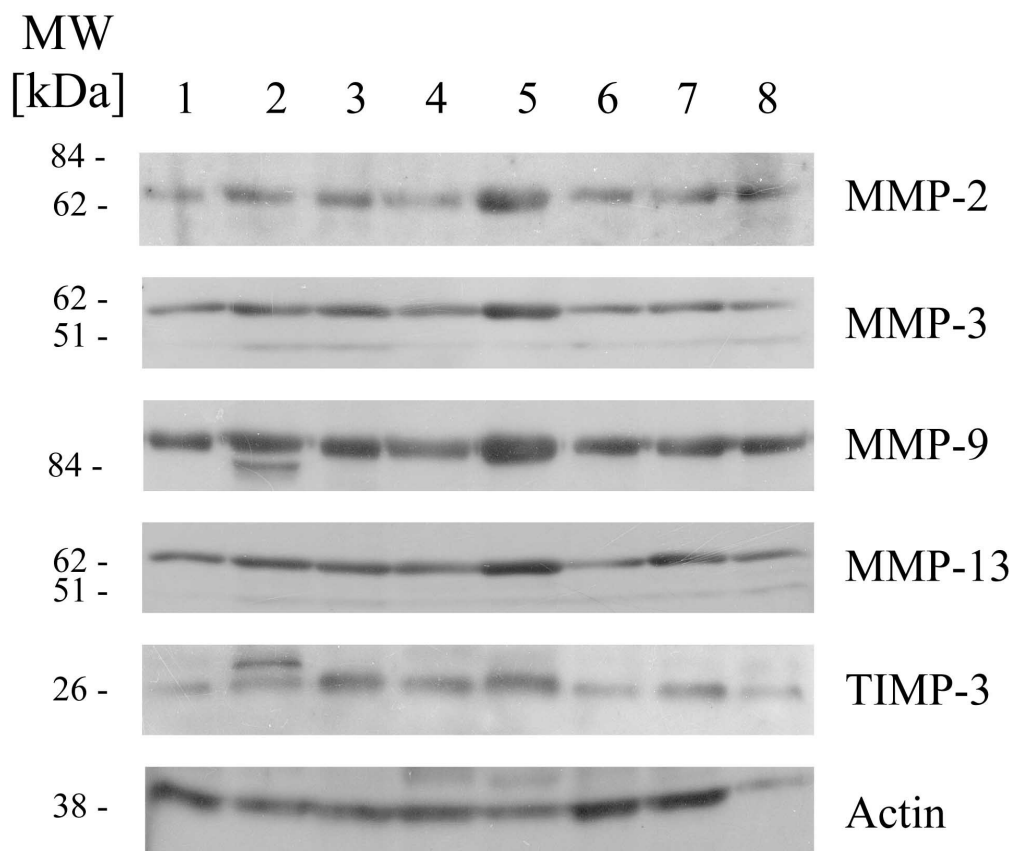


Fig. 3 Western blot analysis of MMP-2, -3, -9, -13 and TIMP-3 in 4 normal samples of the submandibular gland (lanes 1 - 4) and in 4 samples of chronic obstructive sialadenitis of the submandibular gland (lanes 5 - 8). The additional bands in case 2 are presumed to be the result of protein degradation in this sample and are not consistently specific.

gland ductal system. Animal studies show an extended acinar atrophy with progressive secretory dysfunction of the glandular parenchyma and alterations of the ductal epithelium after ligation of the main duct (8,9). Experimental ligation of the ducts of the salivary glands revealed that after complete obstruction, the expected increasing lymphocytic infiltrates were not seen within the parenchyma (10).

The recent histomorphologic studies of chronic obstructive sialadenitis associated with sialolithiasis revealed a periductal origin of the inflammatory reaction and a close relation between the lymphomonocytic infiltrates and the ductal cells. This supports the central role of the ductal cells in the inflammatory process. The phenotype of the periductal inflammatory infiltrations showed a clear dominance of the CD4 positive cells. Activated cytotoxic cells were detected in different stages of the disease in association with ductal destruction. The immunologic profile of the inflammatory infiltrations revealed that intraepithelial, inflammatory factors are likely a cause for the inflammatory reaction. In chronic obstructive sialadenitis, a progression of the inflammation obviously occurs because of an infection-induced immune response (11).

Fibrogenesis develops as a result of an imbalance between extracellular matrix synthesis and matrix degradation. There are a number of cytokines secreted by tissue cells that stimulate fibroblast proliferation and regulate the synthesis of extracellular matrix components. Among these cytokines, TGF- β plays a major role in regulating extracellular matrix synthesis. TGF- β stimulates the synthesis of extracellular matrix proteins, inhibits matrix degradation by both an increase of the activity of matrix protease inhibitors and a decrease of matrix proteases, and stimulates the synthesis of receptors for extracellular matrix proteins (12,13).

Glandular fibrosis in chronic obstructive sialadenitis is likely the result of the interaction of many inflammatory mediators between ductal cells and lymphomonocytic infiltrates. To increase our understanding of the pathogenesis of glandular fibrosis, we previously examined the expression of TGF- β in specimens of chronic obstructive sialadenitis associated with sialolithiasis. We demonstrated an increased level of TGF- β 1 mRNA and strong TGF- β immunoreactivity of the ductal system in cases with progressive fibrosis (4).

CTGF has become known for its involvement in a variety of fibrotic processes (14-16). *In vitro*, CTGF stimulates extracellular matrix production by fibrotic

competent cells (17). CTGF is a cysteine-rich peptide originally identified as a growth factor that is secreted by vascular endothelial cells in culture (18). However, the physiological function of CTGF has not been elucidated. Previous studies showed that CTGF may be one of the downstream effectors of the fibrogenic effects of TGF- β . *In vitro* and *in vivo* studies revealed that TGF- β -induced collagen synthesis is blocked with anti-CTGF antibodies or antisense to CTGF oligonucleotides in fibroblasts (5,6). Furthermore, there is a TGF- β responsive element in the CTGF promoter gene sequence (19). A direct relationship between CTGF and fibrotic changes in liver cirrhosis and chronic pancreatitis was confirmed (20,21). In the present study, we found a similar expression pattern for CTGF in comparison to TGF- β expression in tissue samples of submandibular glands with chronic obstructive sialadenitis. An important role of CTGF can be expected in the fibrogenesis of the chronic obstructive sialadenitis of the submandibular gland. Further studies with anti-CTGF antibody suitable for Western blotting are necessary for completion of the immunohistochemical analysis.

The key enzymes involved in extracellular matrix turnover are MMPs (7). Currently, more than 25 different members of these proteolytic enzymes are known. MMPs are a group of structurally related zinc- and calcium-dependent enzymes that degrade extracellular matrix macromolecules, such as collagens, gelatins, fibronectin, tenascin and laminin (22,23). They participate in the destruction of the extracellular matrix and the development of fibrosis associated with inflammatory processes as well as tumor invasion and metastasis (24-26). Recent findings suggest that MMP functions are not limited to the digestion of matrix components, but also are involved in the production and organization of matrix molecules (27). The activity of MMPs are regulated at the levels of transcription, proenzyme activation, or inhibition of activated enzymes by TIMPs (28). There is also evidence that the expression of these enzymes is influenced by TGF- β (29).

In the present study, it was demonstrated that tissue samples of the normal submandibular gland have the capacity to synthesize MMP-2, -3, -9, -13 and TIMP-3. These factors were also expressed in chronic obstructive sialadenitis and their reactivity did not differ from normal glands. The precise role of the expression of these proteins, as well as other MMPs and TIMPs in salivary glands and chronic sialadenitis, will have to be elucidated in further studies.

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