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ORIGINAL ARTICLE

Immunohistochemical study of fibroblasts and mast cells in chronic submandibular sialadenitis

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AIM: To further our understanding of the processes involved in fibrosis that occurs in chronic submandibular sialadenitis by investigating the distribution of myofibroblasts, CD34-positive fibroblasts and tryptase-containing mast cells.

MATERIALS AND METHODS: Thirty specimens of chronic submandibular sialadenitis with varying degrees of fibrosis and five normal submandibular glands were examined immunohistochemically for the presence of CD34, α-smooth-muscle-actin, desmin and tryptase.

RESULTS: Myofibroblasts were not demonstrated by the techniques for α-smooth-muscle-actin or desmin. CD34-positive fibroblasts were found around normal and moderately atrophic acini, but were not found around extremely atrophic acini and duct-like structures or in periductal and interlobular fibrous tissue. Tryptase-containing mast cells were found around vessels in normal submandibular glands. They were found in increased numbers in chronic submandibular sialadenitis, particularly in glands with widespread fibrosis, in which they were found in the fibrous tissue, and in which the increase was statistically significant.

CONCLUSIONS: The results of this investigation suggest that tryptase-containing mast cells are likely to be involved in the fibrosis of chronic submandibular sialadenitis, but myofibroblasts and CD34-positive fibroblasts are not.

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Keywords: chronic sialadenitis; myofibroblasts; CD34-positive fibrocytes; mast cells; fibrosis; submandibular gland; salivary glands

Introduction

A radical change in our understanding of the natural history of chronic submandibular sialadenitis and sialolithiasis is the outcome of extensive clinical and experimental research that took place in the final quarter of the last century and which has been recently reviewed (Harrison, 2006). Essentially, secretory inactivity in a normal gland leads to an accumulation of sialomicroliths that lead to foci of obstructive atrophy that are havens for microbes that have ascended Wharton's duct and cause chronic sialadenitis, which may subsequently lead to dialolithiasis.

A major clinicopathological investigation of chronic submandibular sialadenitis (Harrison *et al*, 1997) revealed that there was increasing fibrosis with increasing duration of symptoms and that fibrosis was related to inflammation, atrophy and sialolithiasis. However, there was no indication of the processes involved in fibrosis, and these have been seldom investigated and are still poorly understood (Teymoortash *et al*, 2003, 2004). The present investigation is an attempt to remedy this situation by examining the distribution of three types of cells that are involved in glandular fibrosis, namely myofibroblasts, CD34-positive fibroblasts and tryptase-containing mast cells (Yamazaki and Eyden, 1996; Braganza, 1998; Sekine *et al*, 1999; Tsuneyama *et al*, 2000) in normal submandibular glands and in chronic submandibular sialadenitis.

Materials and methods

Thirty cases of chronic submandibular sialadenitis were selected from archival material that had been used in a previous clinicopathological investigation (Harrison *et al*, 1997) to give the entire range of fibrosis and to give associated sialolithiasis in 16 of the cases. The patients' symptoms consisted of pain, swelling and discharge, and there had been no evidence of Sjogren's syndrome. Histological features and the aetiology of our selected cases are presented in Table 1. Five normal submandibular glands were selected from the post-mortem material from the University of Thessaloniki used in previous investigations (Epivatianos and Harrison, 1989; Harrison *et al*, 1997). The investigations were approved by the local research ethics committees.

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Table 1 Histological features of 30 cases of chronic submandibular sialadenitis

Degrees of fibrosis (Fib.) and associated total inflammatory infiltrate (Inf.)	*Number of cases without sialolithiasis*	*Number of cases with sialolithiasis*
Fib. 0, Inf. 1	1	2
Fib. 1, Inf. 1	7	6
Fib. 2, Inf. 2	2	3
Fib.2, Inf. 3	2	2
Fib. 3, Inf. 3	2	3
Total number of cases	14	16

Serial sections were cut at a thickness of 4 μm from paraffin-embedded tissues and were stained with haematoxylin and eosin and for immunohistochemistry. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 10 min at room temperature. Sections were pretreated for antigen retrieval (Table 2). Sections that were intended for the detection of desmin, CD34 and tryptase were incubated with normal goat serum and those intended for the detection of α-smooth-muscle-actin were incubated with normal swine serum at a dilution of 1:20 for 20 min at room temperature. Sections were incubated with monoclonal primary antibodies (Table 2). Sections incubated with normal mouse serum were used as negative controls. The streptavidin-biotin-complex method was performed for the detection of all antigens using the autostainer Ventana (Ventana Med Systems Inc., Tuscon, AZ, USA) and the reaction was developed using diaminobenzidine. Haematoxylin was used as counterstain.

Alpha-smooth-muscle-actin and desmin were used in conjunction with morphology to identify myofibroblasts, which are usually spindle shaped and sometimes stellate. CD34 was used in conjunction with morphology to identify CD34-positive fibroblasts, which are usually spindle shaped. Tryptase was used to identify tryptase-positive mast cells.

Fibrosis was graded as: 0, small amount of fibrous tissue; 1, increase of fibrous tissue in parts only; 2, widespread moderate increase of fibrous tissue; and 3, widespread great increase of fibrous tissue. The total inflammatory infiltrate was graded as: 0, scattered inflammatory cells interstitially as seen in normal glands and no focal collections; 1, scattered inflammatory cells interstitially and occasional focal collections as in normal glands; 2, widespread moderate increase of inflammatory cells; and 3, widespread great increase of inflammatory cells (Harrison *et al*, 1997).Cases with grades of fibrosis of 0 and 1 were grouped together as cases with no or localized fibrosis and cases with grades of fibrosis of 2 and 3 were grouped together as cases with widespread fibrosis.

The number of mast cells in relation to the degree of fibrosis was measured as the arithmetic mean of the number of mast cells per high-power field using a $\times$40 microscope-objective in 20 randomly selected fields for every case. The measurements were analysed using the one-way ANOVA test and statistical significance was determined at $P < 0.001$. Sections were examined by two of the authors (A.E. and T.Z.) independently of each other. Sections were re-examined when there were differences in the measurements and discussion was occasionally necessary to establish uniformity.

Results

Expression of α-smooth-muscle-actin was seen in myoepithelial cells and muscular blood vessels and staining for desmin was seen in a leiomyoma used as a control but neither stain was seen in myofibroblasts in normal glands or in chronic sialadenitis.

CD34-positive fibroblasts were found in small numbers as spindle-shaped cells around acini in normal glands and around acini that were similar to normal or moderately atrophic in chronic sialadenitis (Figures 1 and 2), but they were not found around extremely atrophic acini and duct-like structures in cases with widespread fibrosis (Figure 3). They were not found in fibrous tissue around striated and collecting ducts or in interlobular septa in normal glands or in chronic sialadenitis. The endothelial cells of blood vessels were CD34 positive.

Tryptase-containing mast cells were found interstitially and were sparsely distributed in normal glands (Figure 4) whereas were seen in increased numbers in chronic sialadenitis with no or localized fibrosis (Figure 5) and in greatest numbers in chronic sialadenitis with widespread fibrosis (Figure 6). The mean and standard deviation of tryptase-containing mast cells per high-power field was 0.49 $\pm$ 0.383 for the five

Table 2 Clone, source and pretreatments for antigen retrieval, dilutions and incubation time of primary antibodies

Antibody	*Source*	*Clone*	*Pretreatment*	*Dilution*	*Incubation (min)*
CD34	Novocastra, Lab. Ltd, Newcastle upon Tyne, UK	Qbend/10	Microwave; citrate buffer 0.01 M; pH 7.2; 95°C; 15 min	1:50	30
Alpha-smooth-muscle-actin	Dakocytomation, Glostrup, Denmark	1A4	No treatment	1:100	30
Desmin	Biocare, Carmino Diablo, CA, USA	D33	Microwave; citrate buffer 0.01 M; pH 7.2; 95°C; 15 min	1:100	30
Tryptase	Novocastra	AA1	Protease I (Sigma, St Louis, MO, USA) 0.05% in 0.1 M phosphate buffer; pH 7.8; 37°C; 8 min	1:100	30

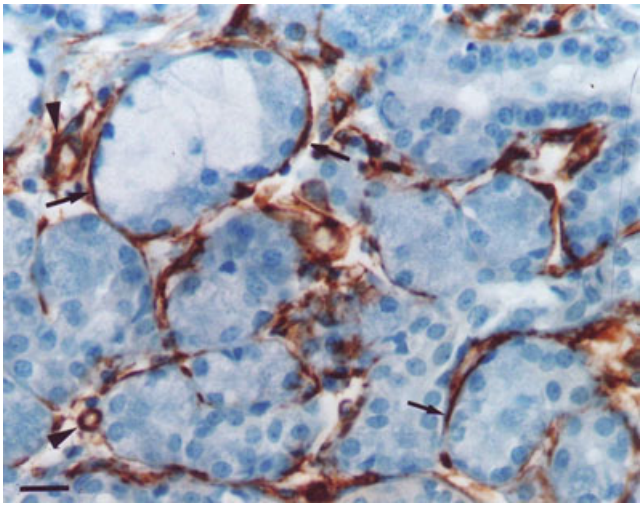

Figure 1 Normal submandibular gland. CD34-positive fibroblasts are seen around acini (arrows). Small blood vessels are also stained (arrowheads). Streptavidin-biotin-complex method. Bar = 20 μm

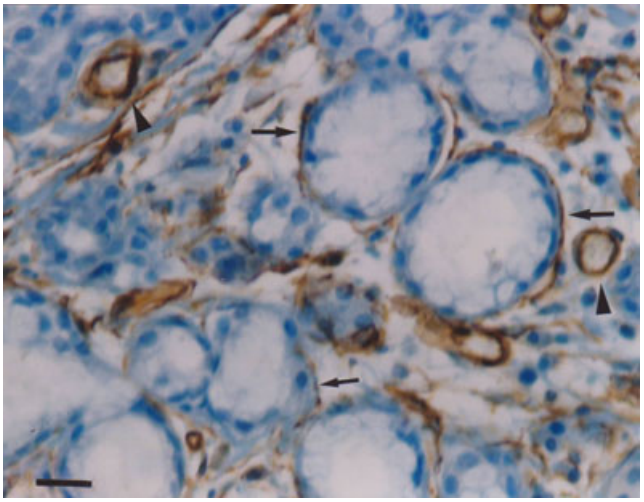

Figure 2 Chronic submandibular sialadenitis with localized or no fibrosis (grade 1). CD34-positive fibroblasts are seen around moderately atrophic acini (arrows). Small blood vessels are also stained (arrowheads). Streptavidin-biotin-complex method. Bar = 20 μm

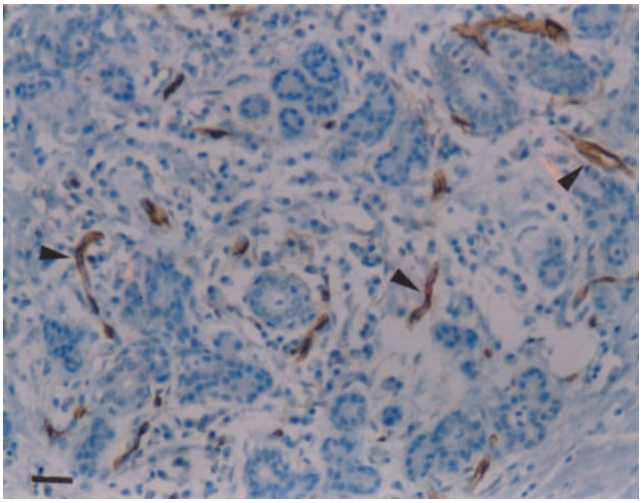

Figure 3 Chronic submandibular sialadenitis with widespread fibrosis (grade 3). Small blood vessels are stained for CD34 (arrowheads) but there are no CD34-positive fibroblasts associated with extremely atrophic acini and duct-like structures. Streptavidin-biotin-complex method. Bar = 30 μm

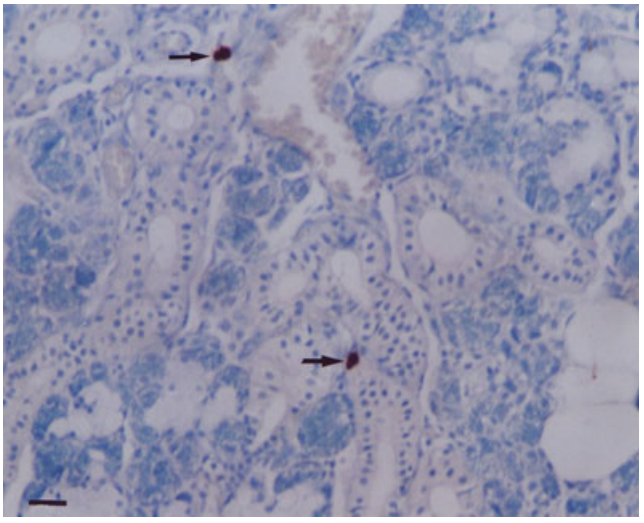

Figure 4 Normal submandibular gland with very few tryptase-containing mast cells (arrows) seen in the field. Bar = 50 μm

normal glands and the results for chronic sialadenitis are presented in Table 3. Data of statistical results are presented in Table 4. The differences between the numbers of tryptase-containing mast cells in normal glands and in sialadenitis and between the numbers in sialadenitis with localized fibrosis and in sialadenitis with widespread fibrosis were statistically significant at $P < 0.001$. There were no differences between the numbers in sialadenitis with sialolithiasis and in sialadenitis without sialolithiasis.

Discussion

The absence of myofibroblasts from normal submandibular glands is similar to the situation in the mammary gland (Barth *et al*, 2002a). However, they are seen in the normal pancreas, where they are scantily distributed, and are increased in number in chronic pancreatitis (Barth *et al*, 2002b; Fukumura *et al*, 2006), where they are associated with fibrosis (Apte *et al*, 1998; Haber *et al*, 1999). The difference between the pancreas and the submandibular gland is possibly related to differences in the distribution of transforming growth factor-β, which plays an important role in the recruitment of cells and the transformation of them into myofibroblasts (Schurch *et al*, 1998). Transforming growth factor-β in the pancreas is present in stellate cells that are transformed into myofibroblasts (Shek *et al*, 2002), whereas in chronic submandibular sialadenitis, it is distributed in the ductal cells and duct-like structures and apparently not in cells in the stroma

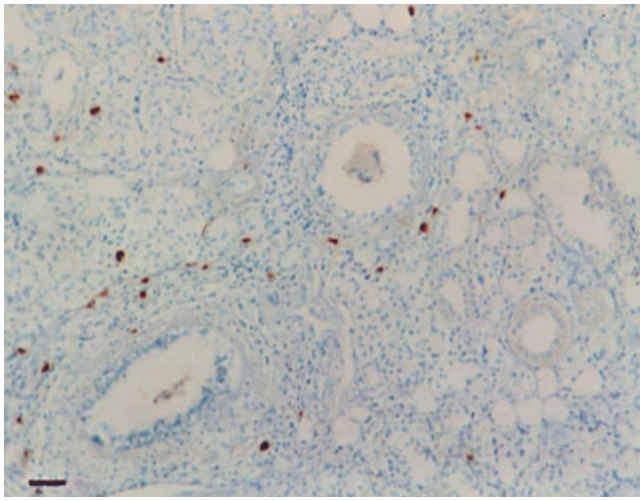

Figure 5 Chronic submandibular sialadenitis with localized or no fibrosis (grade 1). A moderate number of tryptase-containing mast cells are scattered interstitially. Streptavidin-biotin-complex method. Bar = 50 μm

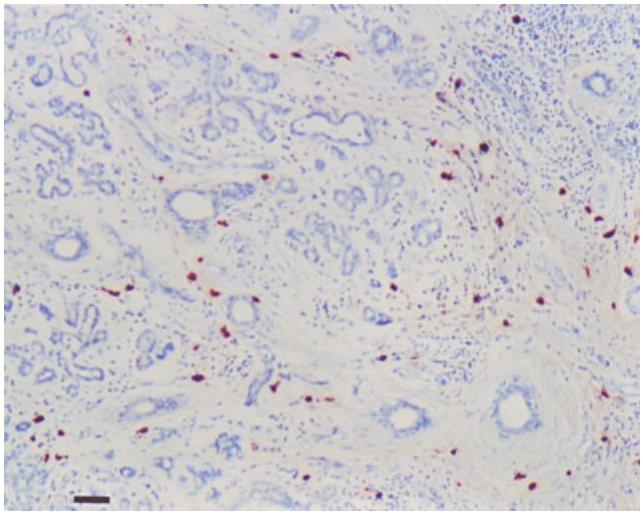

Figure 6 Chronic submandibular sialadenitis with widespread fibrosis (grade 3). Many tryptase-containing mast cells are seen interstitially. Streptavidin-biotin-complex method. Bar = 50 μm

(Teymoortash *et al*, 2003). The presence or absence of myofibroblasts possibly reflects different functional needs. Thus, there are myoepithelial cells in the submandibular and mammary glands but not in the pancreas (Garrett *et al*, 1970), and possibly myoepithelial cells give the parenchyma sufficient muscular support without the need for the additional support of myofibroblasts.

The absence of myofibroblasts from chronic sialadenitis contrasts with the presence of these cells in the rare fibrosis of the submandibular gland that has been reported in association with idiopathic retroperitoneal fibrosis (Sekine *et al*, 1999) and serves to differentiate between the two diseases and to emphasize that the changes in the submandibular gland in idiopathic retroperitoneal fibrosis are of a completely different aetiology from, and nature to, those of the usual, nonspecific type of chronic submandibular sialadenitis, which appears to be secondary to ascendant infection by commensal microbes (Harrison *et al*, 1997; Harrison, 2006).

The distribution of CD34-positive fibroblasts found in the normal submandibular gland is similar to that described by Yamazaki and Eyden (1996). The absence of these cells from around extremely atrophic acini in glands with widespread fibrosis indicates a lack of involvement in fibrosis. The periacinar CD34-positive fibroblasts are apparently replaced by other mesenchymal cells or lose CD34 expression. Yamazaki and Eyden (1996) suggested that CD34-positive fibroblasts form part of an immunosurveillance mechanism in the submandibular gland between the outside world and the vessels. Possibly this mechanism is no longer needed once fibrosis in chronic sialadenitis is widespread.

The finding of significant increases in the number of tryptase-containing mast cells in chronic submandibular sialadenitis and with increasing fibrosis is in agreement with the findings of Tsuneyama *et al* (2000) in a case of bilateral chronic submandibular sialadenitis and primary sclerosing cholangitis, which is possibly an example of the rare, immunologically mediated, IgG4-related form of chronic sialadenitis (Kitagawa *et al*, 2005). Mast cells play an important part in the development of chronic pancreatitis and the attendant fibrosis (Braganza, 1998) and appear to do so also in chronic submandibular sialadenitis. Mast cell tryptase can contribute to connective tissue breakdown by activating pro-collagenase directly (Gruber *et al*, 1988) and by setting in motion a cascade of matrix metalloproteinases by activating prostromelysin-1 to stromelysin (Gruber *et al*, 1989). Breakdown of connective tissue matrix induced by mast cell tryptase may facilitate the infiltration of tissues by leucocytes during the development of inflammation (Walsh, 2003) Thus, the tryptase-containing mast cells are likely involved in primary inflammatory changes in chronic submandibular sialadenitis. It seems that primary inflammatory changes are possibly important in the aetiology of chronic submandibular sialadenitis, as it has been reported that the principal factor in the progress of the disease appears to be

Table 3 Degree of fibrosis and number of tryptase-containing mast cells (mean ± s.d.) in chronic sialadenitis

Degree of fibrosis	*Mast cells in cases without sialolithiasis*	*Mast cells in cases with sialolithiasis*	*Mast cells in all cases*
No or localized fibrosis	3.73 ± 1.60 (8)	2.99 ± 1.13 (8)	3.36 ± 0.40 (16)
Widespread fibrosis	8.63 ± 1.25 (6)	9.58 ± 1.46 (8)	9.17 ± 0.55 (14)
All degrees of fibrosis	5.83 ± 2.86 (14)	6.28 ± 3.46 (16)	6.07 ± 3.24 (30)

Numbers in parentheses indicate number of cases.

Table 4 Significance of differences of the number of tryptase-containing mast cells between the degrees of fibrosis and normal glands

Comparison between normal glands and the degrees of fibrosis and between the degrees of fibrosis		*Mean difference*	*Significance (*) of difference*
Normal glands	No or localized fibrosis not associated with liths	−3.241	0.014*
	Widespread fibrosis not associated with liths	−8.135	0.000*
	No or localized fibrosis associated with liths	−2.497	0.007*
	Widespread fibrosis associated with liths	−0.9.091	0.000*
	All degrees of fibrosis not associated with liths	−5.183	0.000*
	All degrees of fibrosis associated with liths	−5.614	0.000*
	All cases with no or localized fibrosis	−2.897	0.000*
	All cases with widespread fibrosis	−0.8689	0.000*
	All cases with all degrees of fibrosis	−5.454	0.000*
No or localized fibrosis not associated with liths	No or localized fibrosis associated with liths	0.743	0.987
	Widespread fibrosis not associated with liths	−4.893	0.001*
No or localized fibrosis associated with liths	Widespread fibrosis associated with liths	−6593	0.000*
Widespread fibrosis not associated with liths	Widespread fibrosis associated with liths	0.956	0.946
All degrees of fibrosis not associated with liths	All degrees of fibrosis associated with liths	0.813	0.995

*Values are statistically significant.

inflammation, and sialolithiasis is secondary to sialadenitis (Harrison *et al*, 1997; Harrison, 2006).

In conclusion, the results of this investigation indicate that tryptase-containing mast cells are involved in the fibrosis of chronic submandibular sialadenitis, but that myofibroblasts and CD34-positive fibroblasts are not.

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