

Stephan Ihrler · Sabine Blasenbren-Vogt ·
Andrea Sendelhofert · Matthias Rössle ·
John D. Harrison · Udo Löhrs

Regeneration in chronic sialadenitis: an analysis of proliferation and apoptosis based on double immunohistochemical labelling

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Abstract The understanding of regeneration in salivary glands as a finely tuned balance of cellular proliferation, differentiation and apoptosis has been limited by the difficulty of identifying proliferating cells. This has been overcome in the present investigation by double immunohistochemical labelling for the proliferation-associated antigen Ki67 and for different cell-type-specific antigens applied to 8 specimens of normal parotids and 16 specimens of chronic parotid sialadenitis with particular reference to acini and intercalated ducts. In comparison with low baseline rates of proliferation in normal parotids, proliferative indices were significantly increased in chronic sialadenitis in mature acinar cells, intercalated ductal cells and myoepithelial cells without evidence of proliferation by an additional population of cells. In accordance with findings in glands of experimental animals, the present data do not support the previously postulated concept of regeneration of acini and intercalated ducts by a hypothetical population of uncommitted ductal stem cells. The demonstration of a profound capacity for intrinsic glandular regeneration from differentiated cells represents a biological basis for the good results obtained from conservative therapy of chronic sialadenitis and offers hope for novel therapies designed to reconstitute impaired salivary flow.

Keywords Salivary glands · Regeneration · Proliferation · Apoptosis · Glandular atrophy

Introduction

Chronic sialadenitis is a common cause of removal of salivary glands, although, recently, interest in conservative procedures has increased due to their excellent results and the morbidity associated with surgery, especially parotidectomy [1, 20]. Preliminary animal studies with gene transfer or transplantation of stem cells are being undertaken with the aim of improving salivary flow rates in severely atrophic glands [3]. However, the understanding of the glandular regeneration that is important for the development of these therapies is limited.

Regeneration of the salivary glands has, for a long time, been predominantly attributed to a putative population of uncommitted stem cells believed to be situated in the ducts [2]. However, investigations on experimental animals have failed to support this concept, and a growing body of evidence indicates the importance of a proliferative capacity of differentiated cells in diverse segments of the salivary duct [7, 8, 10, 29]. The major obstacle for an analysis of the proliferative capacity has been the difficulty of accurately identifying types of proliferating cells in the complex cellular composition of the salivary glands, which is aggravated in sialadenitis by an additional intimate admixture of intensely proliferating inflammatory and stromal cells (Fig. 3a).

The implementation of double immunohistochemical labelling for the proliferation-associated antigen Ki67 and for different cell-type-specific antigens has greatly improved the accurate localisation and quantification of proliferating cells. This technique recently enabled us to demonstrate a quantitatively diverse, but generally low, baseline proliferation in all five types of parenchymal cells of the normal human salivary gland [18]. These findings indicate that acinar cells, intercalated ductal cells and myoepithelial cells regenerate independently, without cellular transition and without the participation of an additional population of cells, while the renewal of luminal columnar cells in striated and excretory ducts is maintained by proliferation and differentiation of basal cells. In conditions of chronic damage, these basal cells

S. Ihrler (✉) · S. Blasenbren-Vogt · A. Sendelhofert · M. Rössle ·
U. Löhrs
Institute of Pathology,
Ludwig-Maximilians-University,
Thalkirchnerstraße 36, 80337 Munich, Germany
e-mail: Stephan.Ihrler@patho.med.uni-muenchen.de
Tel.: +49-89-51604011
Fax: +49-89-51604043

J. D. Harrison
Department of Oral Pathology,
Guy's, King's and St. Thomas' Dental Institute,
London, England

manifest an additional capacity for a pluridirectional morphogenetic differentiation into several types of reactive ductal metaplasias [18, 24]. However, despite this prominent role of basal cells in the striated and excretory ducts, there is no evidence for an active participation of basal cells in the cellular regeneration of the acini and intercalated ducts.

Investigations on the effects of chronic noxious stimuli on the acini and intercalated ducts have shown a limited reaction pattern that is largely confined to atrophy of the acini [11, 12, 13, 15, 21, 23, 25, 26, 27, 28, 29]. The role of cellular proliferation and apoptosis in parenchymal atrophy in chronic sialadenitis is not understood, and it is not clear to what extent cellular regeneration can compensate for the loss of cells in the atrophic glands. The aim of the present investigation is, therefore, to examine proliferation and apoptosis in chronic parotid sialadenitis to provide a biological basis for modern therapies and is focused on the alterations in the acini and intercalated ducts to complement the previous investigation, in which the striated and excretory ducts were examined [18].

Materials and methods

Materials

Surgical specimens ($n=24$) from human parotids were retrieved from the archives of the Institute of Pathology, Ludwig-Maximilians-University, Munich. They had been fixed in buffered formaldehyde (4%; pH 7), embedded in paraffin wax and cut into 4- μ m serial sections. Normal parenchyma lacking inflammation was obtained from 8 of the specimens that had been resected for small Warthin tumours or pleomorphic adenomas (male 5; female 3; ages 36–51 years). Of the specimens, 16 had been diagnosed as chronic sialadenitis with or without sialolithiasis (male 9; female 7; ages 28–58 years) and did not include cases of benign lymphoepithelial lesion.

Methods

The degree of inflammation and parenchymal atrophy in these cases ranged from minor to severe and often demonstrated a variable focal distribution. The 16 cases of chronic sialadenitis were divided into a subgroup of 10 cases with minor acinar atrophy, which varied from mild to moderate and was often focal, and a subgroup of 6 cases with predominantly severe acinar atrophy. An accurate enumeration of cellular proliferation and apoptosis could not be accomplished in the latter subgroup because so few acinar cells were preserved. Previous data on the cellular proliferation of the normal parotids [18] served as a basis for the present investigation.

Quantification of proliferation

The protocols of double immunohistochemical labelling for Ki67–CK7 (labelling intercalated ductal cells) and Ki67–CK18 (labelling acinar cells) are as follows. Deparaffinised slides were subjected to microwave pretreatment (750 Watt) with target retrieval solution (TRS 6; pH 6; 30 min). Endogenous peroxidase was blocked with peroxidase solution (7.5%). An avidin-biotin peroxidase complex method was applied (ABC-kit; Vector). The slides were incubated with normal horse serum for 20 min. After avidin-biotin blocking, the slides were incubated (room temperature; 60 min) for either monoclonal anti-CK7 (1:500 dilution) or anti-CK18 (1:450 dilution). Biotinylated universal antibody was applied as secondary antibody followed by avidin-biotin complex (Elite; 30 min each). Diaminobenzidine+ (Dako; 3 min) was used as chromogen. For staining with Ki67, the alkaline phosphatase–antialkaline phosphatase (APAAP)-method was used (APAAP-ChemMate; Dako). The monoclonal anti-Ki67 (1:50 dilution) was applied at room temperature for 60 min. Visualisation of the bound anti-Ki67 was obtained with a secondary antibody of mouse immunoglobulin followed by APAAP-immunocomplex (30 min each). Fast red (Sigma) was used as the chromogen (10 min) and Vector haematoxylin (Gill's formula) as the counterstain. For double staining with Ki67 and α -actin (labelling myoepithelial cells), the same procedure was used as outlined above with the following exceptions: a labelled streptavidin biotin kit was applied (LSAB-Kit, Dako) and anti- α -actin was used in a dilution of 1:400. Table 1 summarises the protocols.

The labelling-index was determined by calculating the percentage of Ki67-positive nuclei within a total of 400 acinar cells, intercalated ductal cells and myoepithelial cells, each identified according to cytological criteria and the immunohistochemical staining pattern (Fig. 4) [5, 16, 17, 18, 22]. The mean percentage of cellular proliferation and the standard error of the mean were calculated. For comparison of normal glands with inflamed glands, statistical analysis of the primary data of the three different types of cells was undertaken, using the χ^2 test.

Quantification of apoptosis

Caspase-mediated cleavage of cytoplasmic intermediate filaments is an early event in apoptosis of epithelial cells. The immunohistochemical detection of a specific caspase-cleavage site in CK18 filaments as a neo-epitope (M30) can be applied to formalin-fixed material for the detection of apoptosis in certain types of epithelial cells [9, 19]. Although M30 does not detect apoptosis in myoepithelial cells, due to the absence of CK18 filaments, it is associated with a better morphological result than when other markers of apoptosis are used, and the type of parenchymal cell that is labelled by M30 can be identified morphologically without confusion with the numerous inflammatory and stromal cells in sialadenitis, in which apoptosis is also not detected due to the lack of CK18 filaments. Cytological criteria for apoptosis (especially nuclear condensation and fragmentation) were used as a control for positive M30 labelling. Double labelling for M30 and cell-type specific markers analogous to Ki67 could not be established.

Due to the relatively small number of apoptoses demonstrated by M30, they were counted in 20 microscopical fields (0.25 mm²

Table 1 Monoclonal mouse antibodies (mAb) used in this study were applied to the antigens listed below. CK cytokeratin, MW microwave, TRS 6 target retrieval solution pH 6, ABC-E avidin-biotin complex method Elite, APAAP alkaline phosphatase anti-alkaline phosphatase method, LSAB labelled streptavidin biotin method

Antigen	mAb clone	Source	Pretreatment	Dilution of mAb	Method
CK18 ¹	Ks18.04	Progen	MW 30 min TRS 6	1:450	ABC-E ²
CK7 ¹	Ks7.18	Progen	MW 30 min TRS 6	1:500	ABC-E ²
α -Actin ¹	asm-1	Cymbus Biotec	MW 30 min TRS 6	1:400	LSAB ²
Ki67 ¹	MIB-1	Dako	MW 30 min TRS 6	1:50	APAAP ³
M30 CytoDeath	M30	Roche Diagn	MW 15 min Citrat	1:60	APAAP ³

¹ As part of double immunohistochemical labelling (see Methods)

² With biotin blocking system

³ Dako ChemMate

with a 10× eyepiece and 20× objective) separately for acinar cells and intercalated ductal cells. The mean number of cellular apoptoses and the standard error of the mean were calculated.

Results

The striated and excretory ducts contain columnar cells and were easily distinguished from the intercalated ducts, which are smaller and contain cuboidal or flattened lining cells, in normal and moderately atrophic glands. The columnar structure was often maintained in very atrophic glands, but sometimes all the intralobular ducts were very atrophic and lacked columnar cells, although there was a difference in size that enabled the smaller ducts to be interpreted as intercalated ducts.

Double immunohistochemical labelling accurately demonstrated proliferation in the different types of cells of the salivary parenchyma, even with a background of intensely proliferating inflammatory and stromal cells in sialadenitis. The proliferation indices in chronic sialadenitis were increased about twofold in acinar cells, fourfold in intercalated ductal cells and more than tenfold in myoepithelial cells (Fig. 2a, b, c, Fig. 3b, c; Table 2). In the statistical analysis, the proliferation indices were significantly higher in the sialadenitis subgroups, with minor atrophy and severe atrophy compared with normal glands for intercalated ductal cells (both $P < 0.001$) and myoepithelial cells ($P < 0.02$ and $P < 0.01$, respectively). The proliferation indices for intercalated ductal cells and myoepithelial cells were slightly higher in the sialadenitis subgroup with severe atrophy than in the subgroup with minor atrophy, although this was not statistically significant. In contrast to an absence of apoptoses in normal parotids (Fig. 1d), small numbers of apoptoses were seen in acinar and intercalated ductal cells in chronic sialadenitis (Fig. 2d, Fig. 3d; Table 3).

Table 2 Proliferation indices in normal parotids and in glands with chronic sialadenitis (percentage of Ki67-positive cells and standard error of the mean)

	Normal glands (n=8)	Chronic sialadenitis	
		With minor atrophy (n=10)	With severe atrophy (n=6)
Acinar cells	2.0% (± 0.9) ²	3.6% (± 1.2)	- ¹
Intercalated ductal cells	0.8% (± 0.3) ²	3.4% (± 0.4)	4.1% (± 0.8)
Myoepithelial cells	0.2% (± 0.1) ²	2.3% (± 0.3)	3.5% (± 1.2)

¹ Too few acinar cells are preserved in severe atrophy for an accurate calculation

² Data from Ihrler et al. [18]

Table 3 Apoptotic indices in normal parotids and in glands with chronic sialadenitis [mean of apoptotic cells in 20 high power fields (HPF) and standard error of the mean]; apoptoses are not identifiable in myoepithelial cells in the M30 technique owing to the lack of intermediate filaments of low molecular weight (CK 18)

	Normal glands (n=8)	Chronic sialadenitis	
		With minor atrophy (n=10)	With severe atrophy (n=6)
Acinar cells	0.0/20 HPF	2.0/20HPF (+/-0.3)	- ¹
Intercalated ductal cells	0.0/20 HPF	1.5/20HPF (+/-0.2)	0.8/20HPF (+/-0.1)

¹ Too few acinar cells are preserved in severe atrophy for an accurate calculation

Discussion

A precise identification and quantification of proliferating cells facilitated by double immunohistochemical labelling has recently enabled us to establish a low, yet quantitatively diverse, proliferative capacity in the five types of differentiated parenchymal cells of the human salivary gland that maintain the gland in a steady state [18]. The significant increase of proliferation demonstrated in the present investigation in mature acinar cells, intercalated ductal cells and myoepithelial cells in chronic sialadenitis is consistent with several experimental animal investigations, which indicate a proliferative capacity in differentiated glandular cells [7, 8, 10, 11, 29]. Previous investigations on proliferation in salivary glands have mainly been performed on the animal model of experimental ductal obstruction, followed by removal of obstruction [7, 8, 10, 25, 27, 29] and are of value in helping our understanding of chronic sialadenitis in which ductal obstruction, which is often intermittent, is one of the noxious stimuli [14].

Burgess et al. [7, 8] used a similar immunohistochemical technique to that of the present investigation in rodent glands and found that an increased proliferation predominantly of myoepithelial cells during ductal ligation was followed by an increased proliferation predominantly of acinar cells after removal of the ligation. Thus, the increased proliferation of both of these types of cells in sialadenitis found in the present investigation possibly represents a reaction to intermittent obstruction, in which there is increased proliferation of myoepithelial cells during obstruction in an attempt to overcome the obstruction followed by proliferation of the acini during relief from the obstruction, when the environment is more conducive to secretory activity.

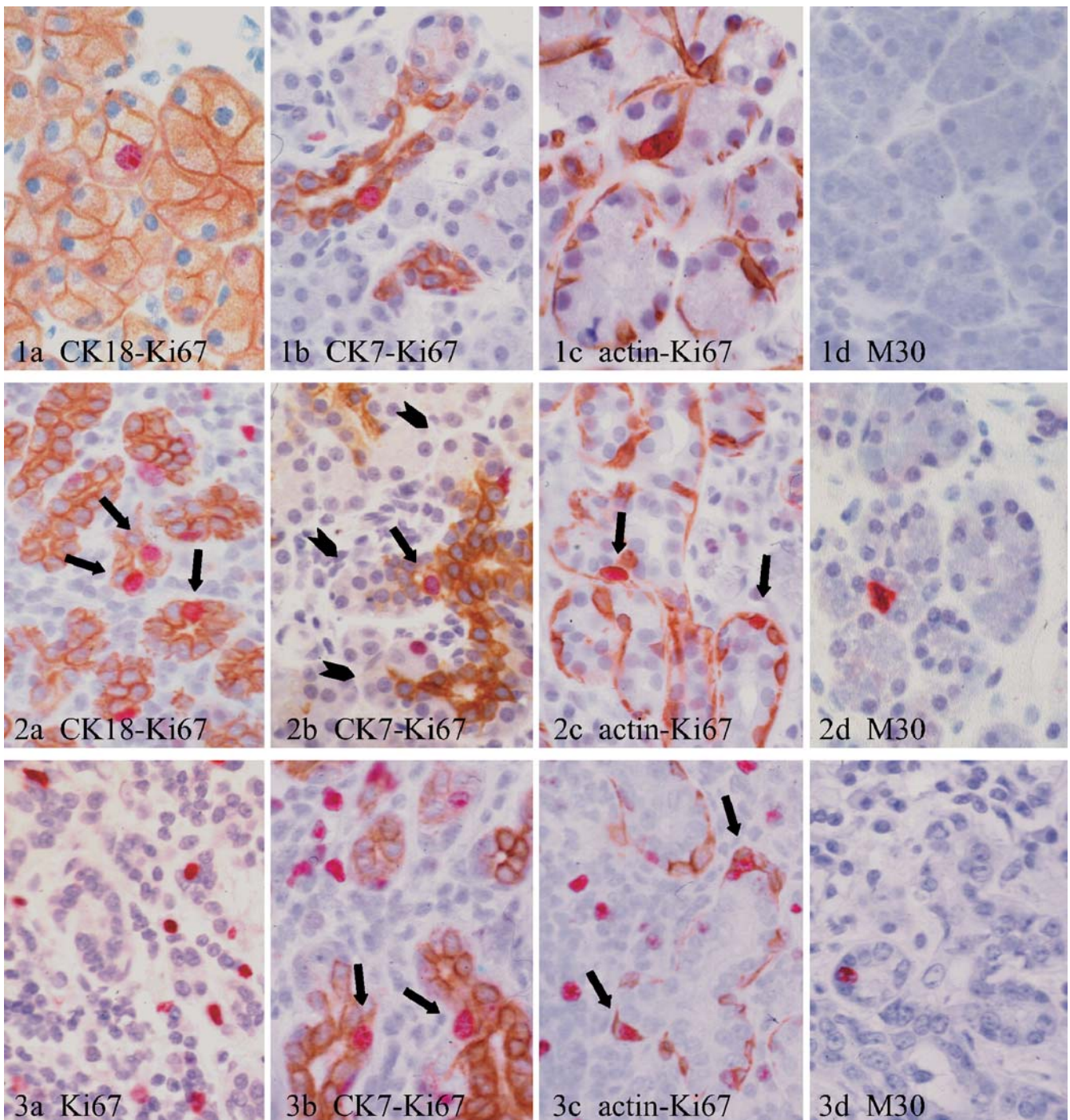


Fig. 1 Cellular proliferation and apoptosis in acini and intercalated ducts illustrated in representative cases of normal parotid. Original magnification: $\times 160$. Double immunohistochemical labelling applied to normal parotids demonstrates proliferation in a CK18-positive acinar cell (**a**), a CK7-positive intercalated ductal cell (**b**) and an α -actin-positive myoepithelial cell (**c**). Apoptoses are absent (**d**; M30)

Fig. 2 Cellular proliferation and apoptosis in acini and intercalated ducts illustrated in representative cases of chronic sialadenitis with minor atrophy. Original magnification: $\times 160$. In chronic sialadenitis with minor atrophy, cellular proliferation is markedly increased. Applying double labelling, increased proliferation can be attributed to CK18-positive acinar cells (**a**; *arrows*), CK7-positive intercalated ductal cells (**b**; *arrow*; remnants of CK7-negative acinar cells are

indicated by *arrowheads*) and myoepithelial cells (**c**; *arrows*). One apoptosis is seen and is attributable to an acinar cell (**d**; M30)

Fig. 3 Cellular proliferation and apoptosis in acini and intercalated ducts illustrated in representative cases of chronic sialadenitis with severe atrophy. Original magnification: $\times 160$. In chronic sialadenitis with severe atrophy, **a** illustrates that an accurate demonstration of proliferation is difficult when staining for Ki67 alone, owing to the complex cellular composition. Double labelling demonstrates the increased proliferation in intercalated ductal cells (**b**; *arrows*) and myoepithelial cells (**c**; *arrows*), while recognisable acinar cells are absent. The apoptosis demonstrated by M30 (**d**) is attributable to an intercalated ductal cell

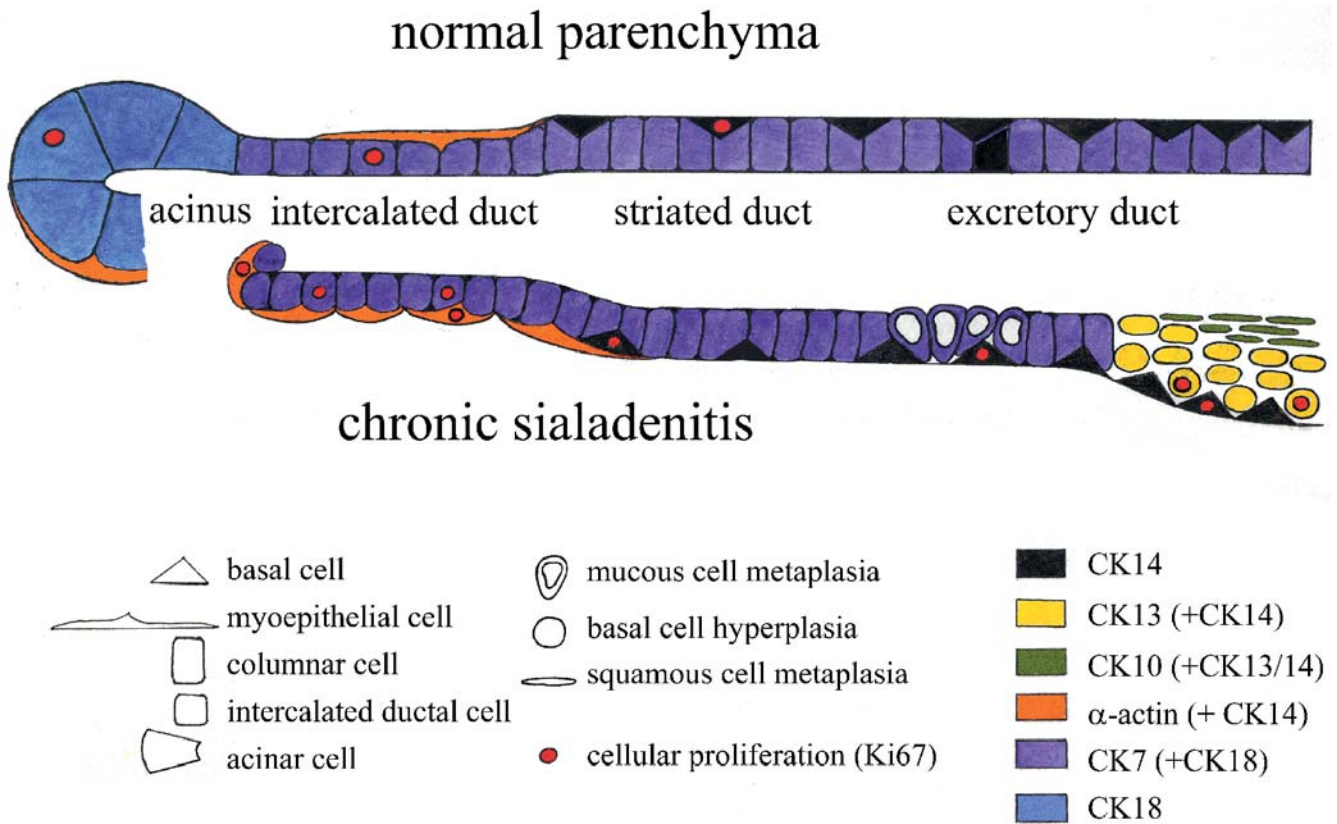


Fig. 4 Schematic illustration of the cellular alterations and proliferation in chronic sialadenitis compared with the normal salivary gland, focusing in the present investigation on acini and intercalated ducts

In addition to increased proliferation, the present investigation demonstrates an increased rate of apoptosis (Fig. 1d, Fig. 2d, Fig. 3d) in acinar and intercalated ductal cells in chronic sialadenitis compared with normal glands, in which apoptosis was not demonstrated. This does not mean that there is no apoptosis of these cells in normal glands, for there is a rapid phagocytic removal of apoptotic cells, which restricts the immunohistochemical demonstration of apoptosis to a much shorter time [19] than the demonstration of cellular proliferation by an antibody to Ki67, which appears to last for about one to several days [6]. Therefore, in contrast to Ki67-based demonstration of proliferation, which includes events that occur during one or more days before removal of the glands, only a relatively small fraction of the apoptoses that occur during this time will be demonstrable. Nevertheless, the presence of M30 labelling indicates that there is an increase in apoptosis in sialadenitis. However, the immunohistological detection of the dynamic processes of cellular proliferation and apoptosis that last for very different lengths of time does not allow a direct quantitative comparison between the proliferation and apoptotic indices. Possibly, the cell loss in chronic sialadenitis is partly compensated by increased cellular proliferation of differentiated cells, which is compatible with partial glandular recovery after removal of experimental ductal ligation [25]. The rates of apoptosis in chronic sialadenitis

are much lower than the high rates reported by Walker et al. [29] and Scott et al. [25] in ductal ligation, and this is possibly due to adaptation to a chronic damaging process and to an obstruction that is intermittent and partial in sialadenitis.

Despite the capacity for intrinsic cellular proliferation, chronic sialadenitis eventually leads to progressive glandular atrophy, characterised by a loss of acinar cells with preservation of the intercalated ducts and myoepithelial cells (Fig. 4) [11, 12, 13, 14, 15, 21, 23, 25, 26, 28]. A gradual transformation of acinar cells during severe glandular damage into a less differentiated type of cell, similar to the intercalated ductal cells, has long been postulated. Convincing support for this kind of dedifferentiation of acinar cells is derived from ultrastructural studies [12, 13, 15, 21, 23, 27, 28, 29] and from the gradual gain of CK7 expression, which is seen in atrophic salivary parenchyma of man [18]. This gradual dedifferentiation, together with the increased apoptotic loss of acinar cells, is likely to contribute to the predominant atrophy of acini, which occurs in spite of increased acinar proliferation [25, 29].

The synthesis of data from animal models [7, 8, 10, 25, 29], our previous investigation on reactive metaplasia in human glands [18] and the present investigation of sialadenitis supports the following principles for glandular regeneration: (1) The physiological regeneration of the

acini and intercalated ducts is based on a low baseline proliferation of mature acinar cells, intercalated ductal cells and myoepithelial cells with a capacity for greatly increased proliferation in the presence of noxious stimuli. (2) The basal cells harbour an important role in the central duct segment as reserve cells for the physiological regeneration of luminal columnar cells and for a pluridirectional differentiation into several types of reactive epithelial metaplasia. This function of basal cells, however, obviously is restricted to the striated and excretory ducts. (3) There is a lack of support for the previously prevailing postulate of a predominant role for putative uncommitted stem cells in parenchymal regeneration.

Although the basic principles of cellular proliferation are likely to be universal [4, 6, 18], the complex mechanisms of physiological cellular regeneration, reactive metaplasias and, presumably, also pluriform tumorigenesis of salivary glands are likely to be a consequence of their unique and complex cellular composition. With regard to clinical implications, the low baseline and profound inducible capacity for intrinsic glandular regeneration represents a biological basis for the good functional results from conservative therapies of chronic sialadenitis [1, 3, 20]. This offers hope for novel therapeutic strategies designed to restore impaired salivary function.

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