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Diversity of collagen expression in the pleomorphic adenoma of the parotid gland

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Summary. The high diversity of collagen expression and its qualitative and quantitative aspects are demonstrated in pleomorphic adenoma using electron microscopy and specific histochemical methods. Great variability was observed in the amount, distribution and characteristics of the collagen found in the various types of tissue normally present in this tumour. Both deficient polymerization and hyperpolymerization of collagen and the occurence of desmoplasia were observed. Evidence is presented to suggest a role for localized collagenolysis in the invasiveness of this neoplasia.

Key words: Collagen – Extracellular matrix – Pleomorphic adenoma – Parotid gland – Histochemistry

Introduction

Pleomorphic adenoma, the most frequent tumour of the salivary glands, is characterized by great diversity of morphological expression (Foot and Frazell 1953; Welsh and Meyer 1968; Thackray and Lucas 1974). Thus, despite the fact that most authors consider it to be derived from epithelial tissue, more precisely from myoepithelial cells (Dardick et al. 1983; Dardick et al. 1983a; Erlandson et al. 1984; Lam 1985; Kahm et al. 1985) it may have regions with the characteristics of mixoid, cartilaginous, osteoid or bone tissue (Yates and Paget 1952) apart from the areas of epithelial cells. The typical growth patterns (tubular, trabecular and solid cell groups) have been described by Seifert et al. (1976).

The tumour has been the subject of many papers dealing with its histogenesis and intermediate filament expression (Nakazato et al. 1982; Caselitz et al. 1982; Krepler et al. 1982; Palmer et al. 1985; Caselitz et al. 1986). Despite the recent increased interest regarding the extracellular matrix and cell interactions in normal and neoplastic processes (Liotta et al. 1983; Iozzo 1983; Martinez-Hernandez and Amenta 1983; Jiwa and van der Hoof 1985) the fibrous and amorphous components of its matrix have so far received very little attention. Studies have reported that pleomorphic adenomas are rich in elastic tissue fibers, mainly in epithelial areas (Azzopardi and Zayid 1972; David and Buchner 1980; David and Buchner 1982). Biochemical and histochemical observations related to the presence of proteoglycan components have also been published (Azzopardi and Smith 1959; Quintarelli and Robinson 1967; Lovell et al. 1966; Takeuchi et al. 1975; Rovasio et al. 1980) showing the high content of chondroitin sulfate in the tumour.

Although collagen is the major protein of extracellular matrix and the most abundant protein in mammals, constituting about 30% of their total protein, there is, to our knowledge, no paper which deals specifically with its expression and distribution in the different types of tissue present in pleomorphic adenoma of the parotid.

The main purpose of this work is to study collagen expression in pleomorphic adenoma of the parotid gland. Knowledge of the nature of collagen expression may contribute to a better understanding of some aspects of the basical and clinical pathology of the tumour.

Material and methods

Surgical specimens from 24 patients provided by the Departments of Pathology of the A.C. Camargo Hospital (Antonio Prudente Foundation), of the University of São Paulo School

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of Medicine, and from the Heliopolis Hospital were fixed in 10% formalin and embedded in paraffin. Five μm sections were stained by the picrosirius-haematoxylin method. The method consists in staining the sections for 1 h in a 0.1% solution of Sirius Red (Sirius Red F_3B_{200}, Mobay Chemical Corporation, New Jersey, USA) in saturated picric acid followed by rapid washing in tap water and counterstaining with Harris' haematoxylin for 6 min, followed by dehydration and mounting in synthetic resin. The principle of this method is based on the reaction of Sirius Red, an elongated strongly acid dye, with collagen. This reaction stains collagen red and enhances the normal birefringence, since the dye molecules are attached to the collagen fibrils in such a way that their long axes are parallel. The mechanism of staining and the quantitation of the increased birefringence, as well as the conditions for optimal staining, have been previously published (Junqueira et al. 1979). The enhancement of birefringence observed when studying tissue sections by the Picrosirius-polarization method is specific for detection of oriented collagen molecules.

For comparative studies, sections were also stained with haematoxylin and eosin (HE). As a further control, tissue sections were digested with collagenase (Collagenase CLS IV, Worthington Biochemical Co., Freehold, New Jersey, USA) 0.5% dissolved in 0.05 M Tris buffer at pH 7.4 containing 1 mM $CaCl_2$. Incubation was performed for 48 h at 37° C. The collagenase solution was changed after 24 h. Under these conditions collagenase degrades collagen specifically. Control section were incubated under the same conditions without the enzyme.

Several small fragments of four cases, approximately 1 mm in size, were fixed in 2% glutaraldehyde in 0.15 M phosphate buffer, followed by post fixation in 1% osmium tetroxide dissolved in 0.9% sodium chloride. Block staining in 1% aqueous uranyl acetate was followed by embedding in a polyester resin (Polilyte, from Resana S.A., São Paulo, Brazil). Sections were cut in a LKB ultratome and were double-stained with uranyl acetate and lead citrate. Thin sections were studied and micrographed in a Philips EM 400 electron microscope.

Results

Collagen showed great variability in its distribution, molecular orientation and grade of polymerization in the various types of tissues present in this neoplasia and will therefore be considered separately in each tissue type. In epithelial regions collagen appeared as thin, irregularly distributed, or thick and well oriented fibers, forming bundles separating distinct cell nests or sheets, giving a trabecular aspect to these areas. Another common appearance was the presence of thick fiber bundles surrounding many of the tubular structures, solid cell groups and squamous metaplastic foci (Fig. 1). This finding was particularly prominent around some dilated ductal structures, and in some instances formed a compact mass of strongly birefringent oriented fibers with the typical aspect of collagen. When stained by HE, these compact masses displayed a hyalinized appearence which probably corresponds to the hyaline areas described by other authors (Foot and Frazell 1953; Thackray and Lucas 1974).

Electron microscopical studies revealed the presence of three main types of cells in epithelial regions. These cells were described previously by David and Buchner (1980) as myoepithelial-like cells with an electron dense, fibril rich cytoplasm; an epidermoid type cell which was characterized mainly by the presence of tonofilaments, and the duct-like cell, characterized mainly by a cubical form and ovoid centrally located nuclei. The collagen in these areas appeared as thick fibers composed of closely packed fibrils of variable thickness, or as thin and irregularly disposed fibrils (Fig. 2). Electron microscopical studies also revealed the presence of large and irregular forms of collagen hyperpolymerization (Fig. 3 see also Campbell et al. 1985).

The distribution of the collagenous fibers in myxoid tissue was extremely variable. In most cases the myxoid areas contained a mesh of very thin irregularly disposed collagen fibers, but in some instances these thin fibers were practically absent. The epithelial sheets limiting the myxoid areas or in the myxoid zones were usually surrounded by a band of variable width, formed by oriented collagen. When observed under electron microscopy the collagen fibrils were arranged to form thin fibers in the vicinity of the stellate cells which appeared in the myxoid tissue.

Two distinct types of cartilage with characteristics of hyaline and fibrous cartilage were observed. The fibrous cartilage matrix was formed by thick bundles of strongly birefringent and predominantly oriented collagen fibers (Fig. 4), while the hyaline cartilage matrix was characterized by loosely disposed, weakly birefringent collagen fibrils surrounding the chondrocytes (Fig. 5). When seen by the electron microscope the collagen of these areas showed the typical feature of apparently randomly disposed thin collagen fibrils (Fig. 6). In some cases small areas of hyaline cartilage appeared interspersed in a fibrous cartilage region.

Intermediate characteristics between the myxoid aspect and hyaline cartilage pattern were frequently observed. These areas show typical isolated chondrocytes surrounded by small portion of cartilaginous matrix enclosed in myxoid tissue. These features suggest that there are transitional areas between both tissues. There were also morphological findings suggesting the transition of epithelial to cartilaginous tissue. Dispersed areas of calcification were observed in the hyaline cartilage matrix. In three cases features of the so called asbestoid degeneration of the cartilage, composed of radially or interwooven mass of collagen fibers resembling asbestos (Line et al. 1988) were also observed.

Typical osteoid formation previously characterized (Junqueira et al. 1986) as a "three dimen-

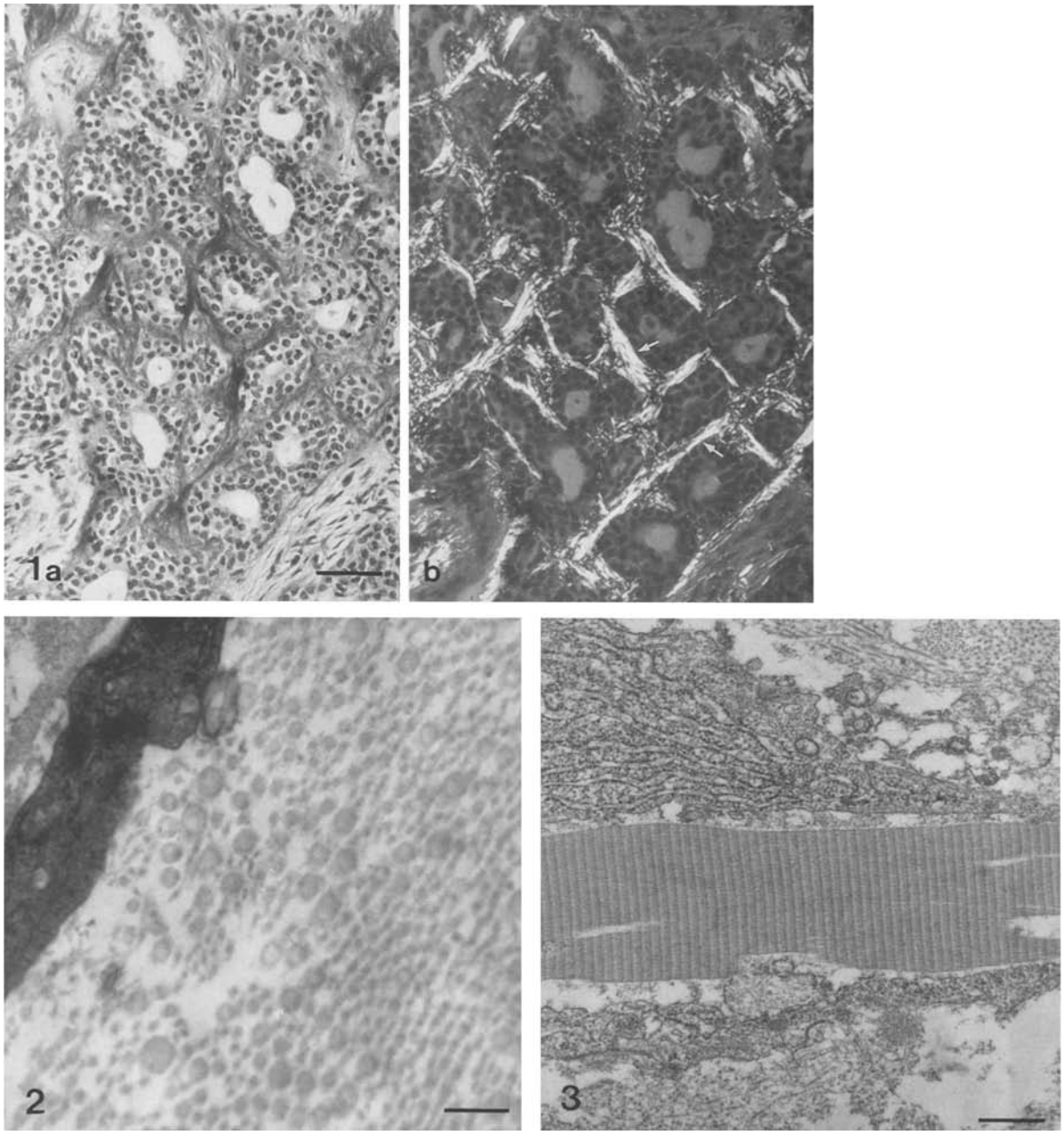


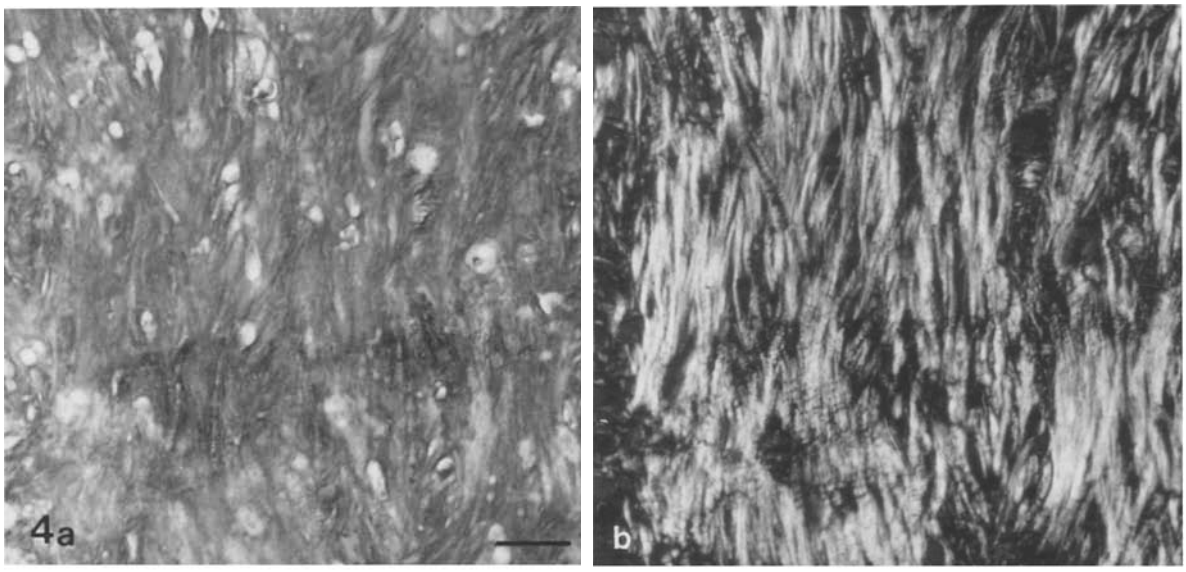
Fig. 1. **A** Picrosirius-haematoxylin. **B** Picrosirius-polarization. Observe the presence of strongly birefringent and oriented collagen fibers surrounding tubular structures and solid cell groups (*arrows*) ×280. bar 35 nm

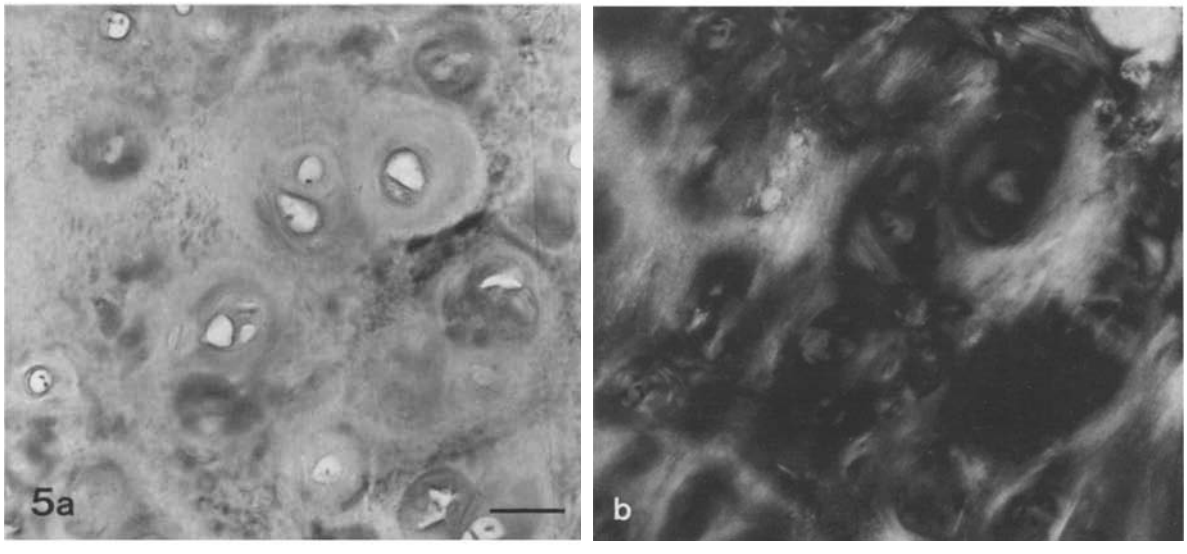
Fig. 2. Electron micrograph evidencing the variability in the collagen fibril diameter present in the compact masses of collagen. ×39850. bar 250 nm

Fig. 3. Electron micrography showing a giant collagen "fibril" presenting its characteristic cross banding. ×21450. bar 465 nm

tional network of randomly oriented, thin, short, weakly birefringent fibers" were identified between cellular epithelial regions of three cases.

The capsule of the tumour was usually formed by a continuous thick band of strongly birefringent oriented parallel collagen bundles surrounding the neoplasm. When observed by electron microscopy, the capsule was formed by closely packed and oriented collagen fibrils. However, in focal regions the collagen showed an eroded aspect with areas of thin weakly birefringent irregular disposed fibers contrasting with the thick portions of this same capsule (Fig. 7). These irregular eroded focal regions of the capsular collagen suggest areas of





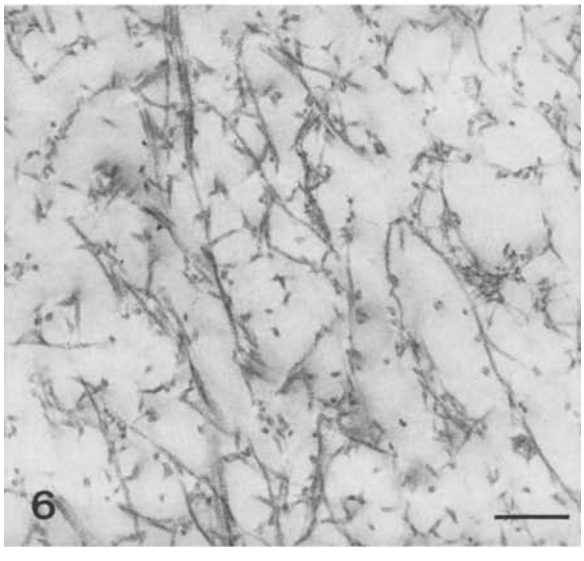


Fig. 4. A Picrosirius-haematoxylin. **B** picrosirius-polarization. Fibrous cartilage region showing the well oriented collagen bundles. × 770. bar 13 μm

Fig. 5. A Picrosirius-haematoxylin. **B** picrosirius-polarization. Hyaline cartilage region. Observe the weak birefringence of the cartilage matrix. × 740. bar 13 μm

Fig. 6. Electron micrography showing the irregular distribution of the collagen fibril in the hyaline cartilage. × 32750. bar 300 nm

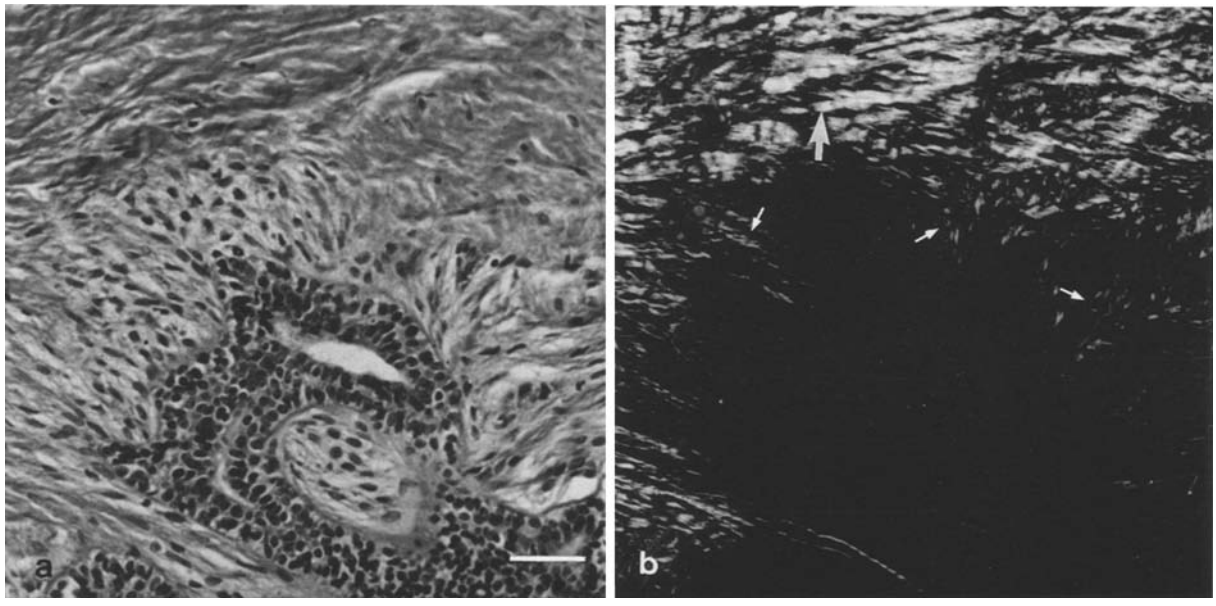


Fig. 7. **A** Picrosirius-haematoxylin. **B** picrosirius-polarization. Observe the eroded aspect of the collagen in the region near an epithelial bud of this tumour (*small arrows*), contrasting with the thick bundles of strongly birefringent and oriented collagen fibers of the tumour capsule (*large arrow*). × 385. bar 26 μm

Table 1. Incidence of cases with the corresponding collagen pattern

Tissue type	Number of cases/ incidence %	Collagen pattern
epithelial solid cell groups	14/50%	variating from small amounts of thin, irregularly distributed and weakly birefringent to large amounts of thick, well oriented and strongly birefringent fibers
epithelial tubular and trabecular	11/39%	moderate amounts of thick bundles of strongly birefringent fibers
myxoid	14/50%	small amounts of thin, irregularly disttributed and weakly birefringent fibers
hyaline cartilage	9/32%	moderate amounts of loosely disposed weakly birefringent fibrils
fibrous cartilage	6/21%	large amounts of thick bundless of strongly birefringent fibers
osteoid	3/11%	small amounts of randomly disposed short and weakly birefringent fibers

local degradation. This eroded aspect was also observed in other models in which collagen degradation has been described, such as in the uterine cervix during parturition (Junqueira et al. 1980). The importance and implications of this finding will be discussed later.

The identification of collagen as stained with Picrosirius was confirmed in all cases described above by the use of collagenase. This digested all red stained structures seen in the tumours.

The collagen pattern in each lesion and the incidence of cases are presented in Table 1.

Discussion

Our results emphasize the great variability of collagen expression in pleomorphic adenoma of the parotid gland. While in the hyaline cartilage collagen is expressed as thin and loose fibrils, those in the tumour capsule are thick and oriented, and in other areas bizarre forms of polymerization are formed. It is improbable, in our opinion, that such a wide variation of collagen expression occurs only on the basis of genetic changes, because it is known that the shape of collagen fibril is influenced by its interaction with other extracellular molecules (Nemeth Csoka and Kovacsay 1979). Our assumption finds support in the fact that pleomorphic adenomas can express all types of proteoglycan molecules and each type seems to be related to a particular tumour area (Lovell et al. 1966; Takeuchi et al. 1975).

In spite of its great variability in the tumour as a whole, the collagen presents defined characteristics in different areas of the tumour. This is particularly evident in the mesenchymal components where collagen expression is a differential characteristic between hyaline and fibrous cartilage.

Collagen production may contribute significantly to the tumour mass. It is noteworthy to mention that the cartillaginous areas are composed mainly by collagenous matrix and that large compact masses of collagen observed surrounding ductal structures were a common finding in our material.

It has been stated in previous reports that tumour cells may alter extracellular matrices through the direct synthesis or degradation of its components, or, indirectly, by promoting increased production of extracellular matrix by neighboring stromal cells, a process known as desmoplasia (Liotta et al. 1983).

Collagen desmoplasia has been referred in a variety of malignant (Barsky et al. 1982; Valensi 1979; MacCurley et al. 1986) and benign tumors (Brounstein and Shapiro 1977). Our results strongly suggest that collagen desmoplasia is frequently associated with epithelial areas in pleomorphic adenomas where it is particularly prominent around ectasic ducts. Apparently the ductal neoplastic cells are able to enhance normal stromal activity. The strongly hyalinized dense areas described by Foot and Frazell (1953) and Tackray and Lucas (1974) very probably correspond to the desmoplastic regions observed in our study.

Degradation of the collagenous capsule that encompasses the tumour may have important clinical implications. In fact, the high incidence recurrence after surgical removal when the tumour mass is dissected at the capsule-tumoral tissue inteface, has been ascribed in part to the presence of microscopical prolongations that cross and extend beyond the limiting capsule. The degradation of the tumours capsule may also be resposible for the typical multinodular pattern (Thackray and Lucas 1974), since after degrading and crossing the capsule the satellite nodule may give rise to a new focus. Though degradation of extracellular matrix components in malignant neoplasms and its implication in the processes of invasion and metastasis have been discussed (Liotta et al. 1983; Tryggvason et al. 1987), this is the first study where evidence of degradation of the capsular extracellular matrix components is correlated with local invasion in benign neoplasms.

Collagen molecules, being rich in basic aminoacids, react strongly with acidic dyes. Sirius Red, an elongated strongly acidic dye, reacts with collagen and promotes the enhancement of its normal birefringence due to the fact that many (about 120) dye molecules are attached to each collagen molecule in such a way that their long axes are parallel (Junqueira et al. 1979). The enhancement of birefringence promoted by the picrosirius-polarization method is specific for collagen. This method, specific for collagen detection in tissue sections, has proved to be more useful for studying collagen distribution in histological slides than the routinely used trichrome techniques. Additionally, the method has been shown to be useful for the study of collagen degradation and production (Junqueira et al. 1980; Junqueira et al. 1981) or detection of defective collagen organization in tissue sections (Junqueira et al. 1985; Line et al. 1988). Studies have shown the existence of a positive correlation between the intensity of birefringence and the localization of collagens type I, II and III (Junqueira et al. 1978; Montes et al. 1984). The method, however, does not permit any suggestions regarding this correlation in pathological material and immunohistochemistry has to be used to obtain reliable data.

The recently published paper of Caselitz et al. (1988) studying the expression of basement membrane associated substances (laminin, collagen type IV and fibronectin) in human salivary glands and salivary gland tumors, showed that the expression of these components in pleomorphic adenomas were variable and exhibited anomalous distribution and arrangement when comparing with normal salivary glands. These findings also stress the high diversity of expression of the extracellular matrix components in pleomorphic adenoma of the parotid gland.

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