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Distribution of cytoskeletal and contractile proteins in normal and tumour bearing salivary and lacrimal glands

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Summary. We have evaluated by means of immunocytochemistry the distribution of various cytoskeletal and contractile proteins (cytokeratins, vimentin, desmin and α-smooth muscle actin) in 23 salivary or lacrimal gland primary tumours (15 pleomorphic adenomas and 8 carcinomas in pleomorphic adenoma), one third of which contained areas of normal gland. Normal epithelial luminal cells were stained by cytokeratin antibodies with a general specificity, while myoepithelial cells were selectively stained by a monoclonal antibody (SK2-27) reacting in immunoblots with cytokeratin polypeptides 14, 16 and 17, according to the classification of Moll et al. (1982) and by an antibody directed against α-smooth muscle actin (Skalli et al. 1986). In pleomorphic adenomas, both epithelial and myoepithelial cells displayed typical topographic distributions; moreover, myoepithelial cells showed two distinct cytoskeletal phenotypes. These findings could account in part for the heterogeneity of aspects observed in this tumour. In carcinomas, malignant cells were always positive to cytokeratin antibodies with general specificity and myoepithelial cells were absent as judged by anticytokeratin SK2-27 and anti-α-smooth muscle actin immunostainings. However, interestingly, there was in all cases a strong positivity for α-smooth muscle actin in stromal cells, similarly to what has previously been described for mammary carcinoma (Skalli et al. 1986). Our findings may be useful for the interpretation of the histogenesis of salivary and lacrimal tumour and stromal cells.

Key words: Pleomorphic adenoma – Actin isoforms – Stromal reaction – Myoepithelial cells

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Introduction

Pleomorphic adenoma is the most common benign tumour of salivary and lacrimal glands. Although it is a well defined entity on histological grounds, its histogenesis is still open to debate (Kahn et al. 1985; Brocheriou et al. 1986). In some cases, evidence of malignancy, such as cellular anaplasia and invasive growth, is present. In these cases, which are defined as carcinoma in pleomorphic adenoma, the diagnosis and the differential diagnosis may be difficult (Takata et al. 1987).

In this study, a series of pleomorphic adenomas and carcinomas in pleomorphic adenomas has been examined by immunohistochemical methods with a panel of monoclonal and polyclonal antibodies directed against various cytoskeletal and contractile proteins. Our purpose was to compare the immunohistochemical pattern in the tumours with that found in normal glandular tissue in order to contribute to the comprehension of histogenetic and diagnostic problems.

Materials and methods

We collected 23 non consecutive cases of neoplasms of salivary and lacrimal glands. The tumour tissue was obtained during surgery, fixed in ethanol 85% and paraffin embedded. 4 μm sections were cut and stained with haematoxylin and eosin. Histological typing was pleomorphic adenoma in 15 cases and carcinoma in pleomorphic adenoma in 8 cases. In the latter cases remnants of pleomorphic adenoma were always present. In 8 cases, samples of normal glandular tissue were present.

We used the following antibodies (their specificity will be discussed in Results, with the exception of those already published): SK56-23 (Sclavo R.C., Siena, Italy), a broad spectrum monoclonal antibody to cytokeratins; SK2-27 (Sclavo R.C.), a monoclonal anticytokeratin antibody specific for a family of small and acidic polypeptides (no. 14, 16 and 17 according to Moll's et al. [1982] catalog; SK60-61 (Sclavo R.C.), a monoclonal anticytokeratin specific for polypeptides of simple epithe-

lia (no. 8 and 18 of Moll's et al. [1982] catalog); monoclonal antivimentin (Sclavo R.C.); polyclonal antidesmin (Sclavo R.C.). In addition, we employed: keratin (Ortho D.S., Raritan, NJ, USA), a polyclonal broad spectrum anticytokeratin antibody which stains epithelial tissues and epithelial tumours; PKK1 (Labsystems, Helsinki, Finland), a monoclonal antibody which reacts with low molecular weight cytokeratins (52.5, 45 and 40 KD) present in simple epithelia; S-100 (Dakopatts, Copenhagen, Denmark), a polyclonal antiserum to S-100 protein, which stains a wide variety of tissues and cells, including myoepithelial cells in salivary glands, anti-α sm-1 (Skalli et al. 1986) which stains specifically the α-smooth muscle isoform of actin.

The specificity of the antibodies was determined by immunoblotting of 8% sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) of cytoskeletal preparation of human foot sole epidermis and mouse liver and of non-equilibrium pH gradient gel electrophoresis (NEPHGE/SDS-PAGE) (O'Farrell et al. 1977) of cytoskeletal preparations of HeLa cells. The proteins were transferred to nitrocellulose paper as described by Towbin et al. (1979), incubated with the first antibody at the appropriate dilution in Tris-buffered saline (TBS) containing 3% BSA and 0.1% Triton-X-100, for 2 h at room temperature. After three rinsings in TBS, a second incubation was done with rabbit anti-mouse Ig labelled with horseradish peroxidase (Cappel Laboratories, West Chester, Pa, USA) diluted 1:1000 in TBS containing 3% BSA and 0.1% Triton-X-100 for 1 h at room temperature. Peroxidase activity was revealed with 10 mM Tris HCl, pH 7.4, containing 0.025% dianisidine and 0.0075% H_2O_2.

Immunohistochemistry was performed on serial sections using the avidin-biotin system (ABC, Vectastain Kit, Vector Laboratories, Burlingame, Ca, USA), according to Hsu et al. (1981). The substrate for development of the peroxidase activity was 3-amino-9-ethyl carbazole (Graham 1965). Sections were weakly counterstained with Mayer's haematoxylin. Negative controls were obtained substituting primary antibodies with non immune serum. In all malignant cases and in some benign cases (no. 8, 12, 16), double immunostaining was performed for SK56-23 and anti-αsm-1, and for SK56-23 and vimentin, respectively (see below), using the biotin-avidin peroxidase technique (Butterworth et al. 1985). In these cases the first substrate was 4-chloro-1 naphtol and the second was 3-amino-9-ethyl carbazole.

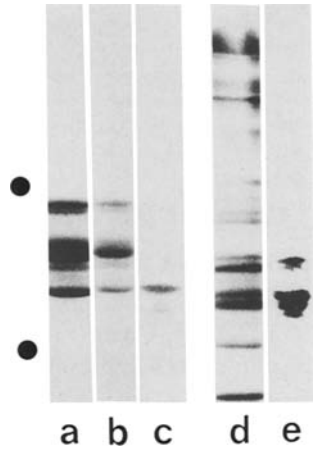

Fig. 1a–d. Immunoblotting with SK56-23 and SK2-27 of different cytoskeletal preparations. **a** Amido-black stained 8% SDS-PAGE of a cytoskeletal preparation of human foot sole epidermis. **b–c** Nitrocellulose sheet corresponding to SDS-PAGE after incubation with SK56-23 (**b**) and SK2-27 (**c**); note that the four bands separated by SDS-PAGE react with SK56-23 whereas only the lower band reacts with SK2-27. **d** Amido-black stained 8% SDS-PAGE of a cytoskeletal preparation of mouse hepatocytes. **e** Nitrocellulose sheet corresponding to SDS-PAGE after incubation with SK60-61 shows a positive reaction with cytokeratins "A" and "D" (52 and 49 kD). The dots indicate the molecular weight markers (68 and 42 kD)

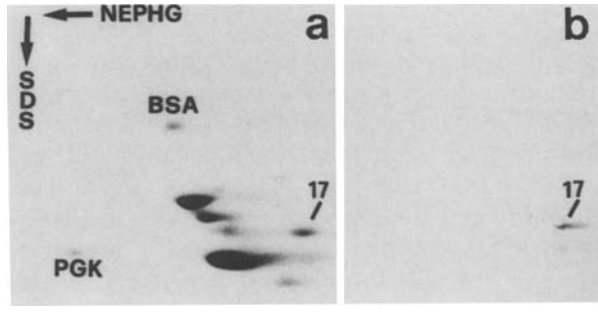

Fig. 2a–b. Immunoblotting with SK2-27 of cytoskeletal preparation of HeLa cells. **a** Coomassie blue stained NEPHGE/SDS-PAGE of a cytoskeletal preparation of HeLa cells using bovine serum albumine (BSA) and 3-phosphoglycerokinase (PGK) as markers. **b** Nitrocellulose sheet corresponding to NEPHGE/SDS-PAGE after incubation with SK2-27 shows a positive reaction only with cytokeratin Nr 17

Results

The specificity of our antibodies was tested by means of immunoblotting (Figs. 1 and 2) and immunofluorescence (Table 1). Immunoblotting with SK56-23 antibody of a cytoskeletal preparation of human foot sole epidermis showed a reaction at the level of all cytokeratin bands, whereas immunoblots with SK2-27 showed a single band (Fig. 1); this positive band is composed of two different polypeptides (48 and 50 kD, Nr 14 and 16 of Moll's et al. [1982] catalogue) as revealed by immunoblotting of 4-16% SDS-PAGE (data not shown). Moreover, immunoblotting of HeLa cells cytoskeletal proteins separated by NEPHGE/SDS-PAGE showed that SK2-27 reacts with another polypeptide (47 kD, acidic isoelectric point) identified as cytokeratin Nr 17 (Fig. 2). The specificity of SK60-61 was assayed on a cytoskeletal extract of mouse liver and was shown to be restricted to the two polypeptides named "A" and "D" (Franke et al. 1981) and corresponding to human cytokeratins Nr 8 and 18 (Moll et al. 1982) (Fig. 1).

Antibody specificity was further tested by means of immunofluorescence on different normal human tissues and the results are shown in Table 1. As expected from immunoblotting results, anti-

Table 1. Immunohistochemical staining of several human tissue by anticytokeratin monoclonal antibodies

Tissue and/or cells	SK56-23	SK60-61	SK2-27
Epidermis			
basal layer	+	−	+
suprabasal layers	+	−	−
Hair follicles	+	−	+
Eccrine sweat glands	+	−	+
Apocrine sweat glands	+	−	−
Esophageal epithelium			
basal layer	+	±	+
suprabasal layer	+	±	±
Salivary glands			
acinar cells	+	±	±
ductal luminal cells	+	+	±
myoepithelial cells	+	−	+
Gastric glands	+	+	±
Large bowel glands	+	+	−
Hepatocytes	+	+	±
Urothelium			
basal cells	+	−	+
umbrella cells	+	+	−
Vaginal epithelium			
basal layer	+	−	+
suprabasal layer	+	−	−
Exocervical epithelium			
basal layer	+	−	+
suprabasal layer	+	−	−
Endocervical glands	+	+	−
Endometrium	+	+	−

Table 2. Clinical features and pathological diagnosis of tumours

Case No.	Sex	Age	Site	Histological diagnosis
1	M	50	Parotid	Carcinoma on pleomorphic adenoma
2	F	70	Parotid	Carcinoma on pleomorphic adenoma
3	M	52	Parotid	Carcinoma on pleomorphic adenoma
4	M	73	Parotid	Carcinoma on pleomorphic adenoma
5	M	71	Minor salivary	Carcinoma on pleomorphic adenoma
6	M	70	Parotid	Carcinoma on pleomorphic adenoma
7	F	51	Parotid	Carcinoma on pleomorphic adenoma
8	M	30	Parotid	Pleomorphic adenoma
9	F	21	Minor salivary	Pleomorphic adenoma
10	M	45	Parotid	Pleomorphic adenoma
11	M	59	Parotid	Pleomorphic adenoma
12	F	85	Minor salivary	Pleomorphic adenoma
13	M	63	Parotid	Pleomorphic adenoma
14	M	42	Parotid	Pleomorphic adenoma
15	F	50	Lacrimal	Pleomorphic adenoma
16	F	30	Minor salivary	Pleomorphic adenoma
17	M	47	Lacrimal	Pleomorphic adenoma
18	F	37	Minor salivary	Pleomorphic adenoma
19	M	38	Minor salivary	Pleomorphic adenoma
20	M	41	Minor salivary	Pleomorphic adenoma
21	M	51	Parotid	Pleomorphic adenoma
22	F	47	Lacrimal	Pleomorphic adenoma
23	F	43	Lacrimal	Carcinoma on pleomorphic adenoma

body SK56-23 stained all the epithelial tissues examined. SK2-27 stained the basal layers of stratified epithelia intensely and stained the myoepithelial cells of salivary and eccrine sweat glands. It stained the suprabasal layer esophageal epithelium, acinar and ductal cells of salivary glands, gastric glands and hepatocytes weakly. It did not stain suprabasal layers of epidermis, vaginal epithelium, apocrine sweat glands, large bowel glands, umbrella cells, endocervical glands and endometrium. Antibody SK60-61 stained mainly epithelial cells of gastric and large bowel glands, hepatocytes and umbrella cells of the urothelium. It also stained the cells of the endocervical glands and endometrial epithelial cells.

Sex, age of patients, and location of the tumours examined are illustrated in Table 2. Microscopical examination of pleomorphic adenomas revealed a great variability in structure from tumour to tumour and in different areas of the same tumour. Most of the cases were cellular and myxoid in similar proportions, while 2 cases (no. 11 and 17) were prevalently myxoid. Trabecular and solid areas were always well represented. A variable number of duct-like structures was present in different cases. Squamous metaplasia was present in 2 cases (no. 4 and 6). In 1 case (no. 4) we have found "hyaline cells" similar to those described by Lomax-Smith and Azzopardi (1978).

The 8 cases of carcinoma were characterized by the coexistence of atypical cell nests, mostly arranged in irregular tubules, duct-like structures or solid sheets, and benign myxomatous or chondroid areas.

In normal glandular tissue, acinar cells and ductal structures generally showed a relatively intense stain with monoclonal large spectrum and polyclonal anticytokeratin antibodies (Geiger et al. 1987). Few cells were stained by restricted specificity cytokeratin antibodies (PKK1, SK2-27 and SK60-61). In particular, ductal luminal cells showed the most intense reaction with SK60-61 (Fig. 3a) and PKK1 antibodies, while the cells of the outer layer of excretory, salivary and intercalated ducts exhibited a strongly positive staining using SK2-27 (Fig. 3b), with increasing intensity from large to small ducts. Many basket cells surrounding the acini were also positive for SK2-27 (Fig. 3c). Moreover, some of the outer ductal cells

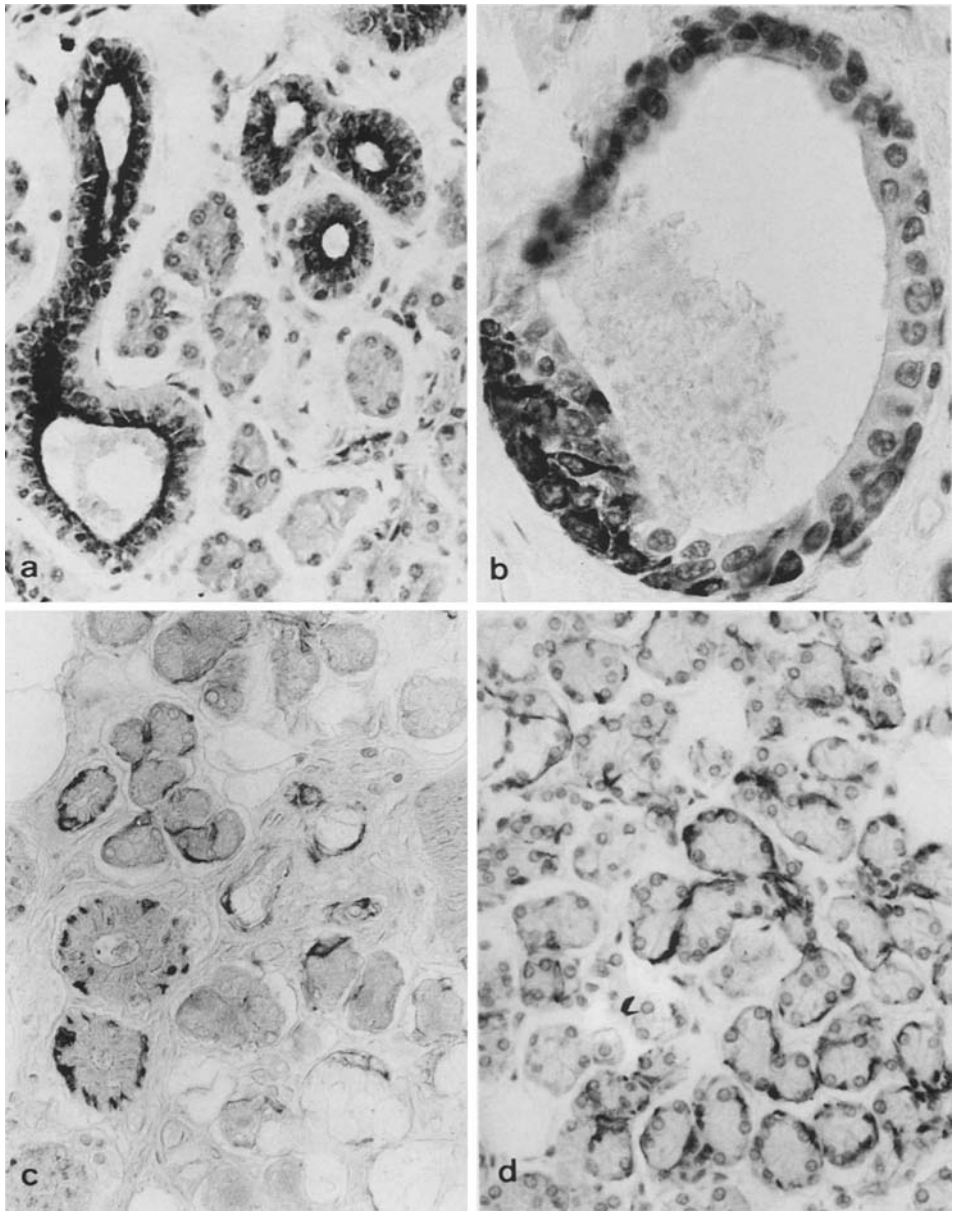

Fig. 3a–d. Immunoperoxidase staining of normal human salivary gland with SK60-61 (**a**), SK2-27 (**b–c**), anti-αsm-1 (**d**). SK60-61 stains the ductal luminal cells (**a**), whereas SK2-27 stains the cells of the outer layers of excretory, salivary and intercalated ducts (**b**) and the myoepithelial "basket" cells of the acini (**c**). These latter cells are also stained with anti-αsm-1 (**d**). (**a, c, d**: ×240; **b**: ×600)

were weakly stained by S-100 and vimentin but totally negative with SK60-61 and PKK1. A characteristic distribution was observed after staining with anti-αsm-1 which strongly reacted with basket cells around the acini of normal glandular tissue (Fig. 3d). The staining was particularly intense around serous acini. A continuous ring of positive cells was also present in small (intercalated) ducts with decreasing positivity in large ducts. No positive reaction was ever found for desmin with the exception of small vessels which were also always positive for anti-αsm-1.

In pleomorphic adenomas, large spectrum monoclonal and polyclonal anticytokeratins reacted diffusely with neoplastic cells. With the restricted specificity antibodies, pleomorphic adenomas showed variable staining from case to case and in different areas of the same case. In particular, positive stain for SK2-27 predominated in solid and trabecular areas (Fig. 4a); here, we found few

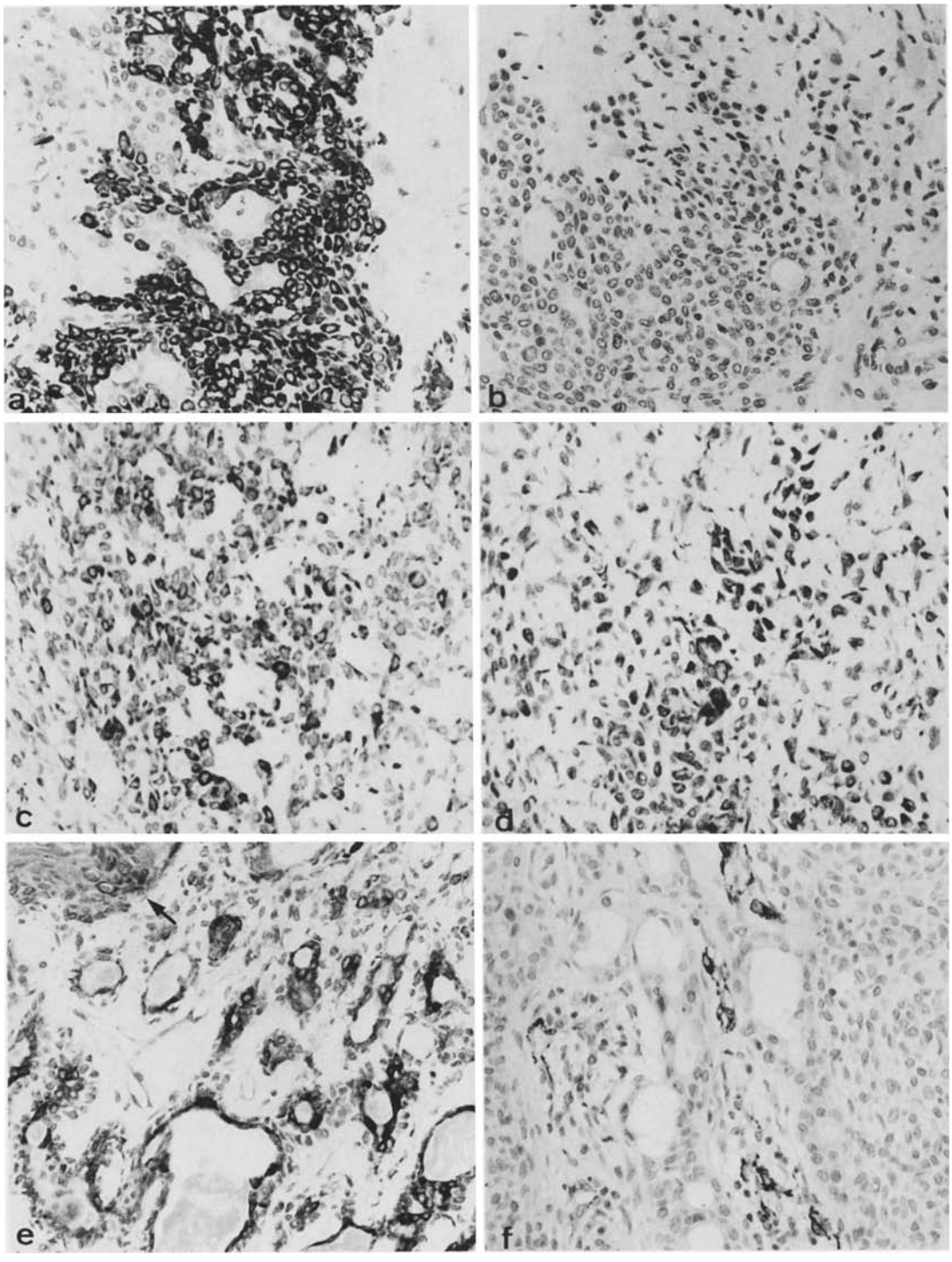

Fig. 4a–e. Immunoperoxidase staining of pleomorphic adenoma with SK2-27 (**a**), SK60-61 (**b, e**), antivimentin (**c**), S-100 protein (**d**) and anti-α sm-1 (**f**). In solid and trabecular areas most of the cells are positive for SK2-27 (**a**) but negative for SK60-61 (**b**); vimentin (**c**) and S-100 protein (**d**) are present in cells within solid and trabecular areas. Cells lining ductular and pseudoductular areas show strong reactivity with SK60-61 which also stains, although with lower intensity, several cells of an area of squamous metaplasia (*arrow*) (**e**). Anti-αsm-1 stains few cells isolated or in clusters (**f**) in relation to mixed areas. (**a–d, f**: ×270; **e**: ×216)

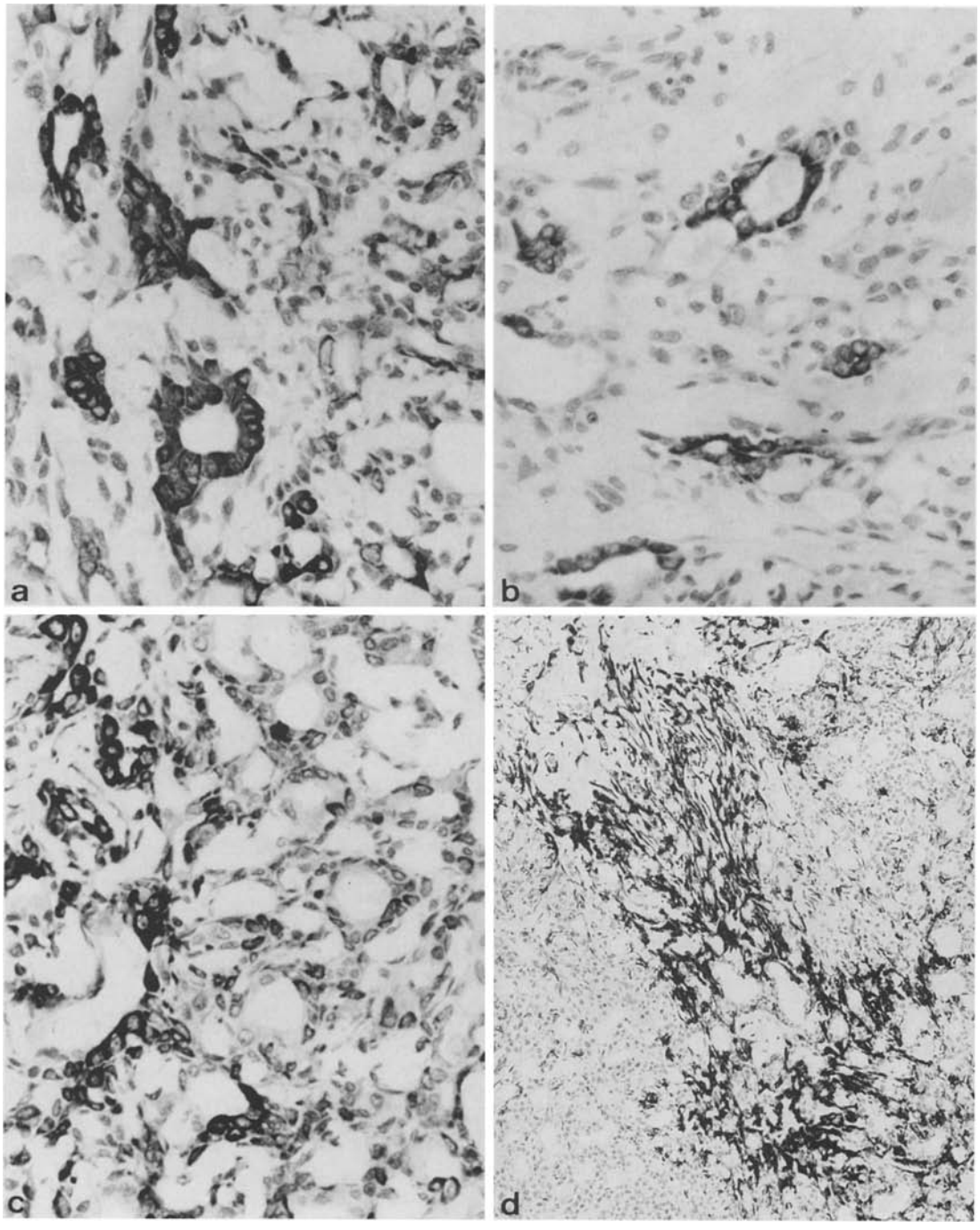

Fig. 5a–d. Immunoperoxidase staining of a carcinoma on a pleomorphic adenoma with SK56-23 (**a**), SK60-61 (**b**), SK2-27 (**c**) and anti-αsm-1 (**d**). SK56-23 stains the majority of tumoral cells (**a**). SK60-61 (**b**) and SK2-27 (**c**) stain isolated groups of cells without any recognizable specific pattern. Stromal cells are strongly stained with anti-αsm-1 (**d**). (**a–c**: ×270; **d**: ×90)

cells positive to SK60-61 (Fig. 4b) but several vimentin- (Fig. 4c) and S-100- (Fig. 4d) positive cells. In serial sections, we observed that many of these vimentin or S-100 positive cells were also positive for SK2-27 (data not shown). Ductular and pseudo-ductular structures displayed the highest immunoreactivity to SK60-61 (Fig. 4e) and PKK1. Cells positive for anti-αsm-1 (Fig. 4f), isolated or in clusters, were often contiguous to myxoid areas, without any relation to ductal-like structures. Their number was apparently related to the extension of myxoid areas. In several sections, we ob-

served that many of these cells were also positive for SK2-27 (data not shown).

In carcinomas, large spectrum cytokeratin antibodies were positive for a majority of tumour cell nests without showing any specific pattern (Fig. 5a); restricted specificity cytokeratin antibodies stained isolated groups of cells again without any specific pattern (Figs. 5b and c) contrary to what was observed in normal glands or adenomas. Only a small number of S-100-positive cells were found in stromal areas; however, strong positive staining for anti-αsm-1 in stromal cells was present in all cases (Fig. 5d). Double immunostaining with this antibody and SK56-23 excluded the possibility that α-smooth muscle actin positive cells contain intermediate filaments of the cytokeratin type. Desmin staining was always negative in neoplastic and stromal cells.

Discussion

Many studies have dealt with the cellular heterogeneity of pleomorphic adenoma of salivary and lacrimal glands (Krepler et al. 1982; Erlandson et al. 1984; Kahn et al. 1985; Caselitz et al. 1986a; Takata et al. 1987). In these tumours differentiated epithelial cells arranged in duct-like structures and isolated cells without epithelial-like coherence morphologically similar to mesenchymal cells, coexist (Krepler et al. 1982). In addition, areas of mesenchymal differentiation, with presence of myxoid, chondroid and smooth muscle-like structures are frequently found (Thackray and Sobin 1972). It is not clear whether this cellular heterogeneity reflects an origin of tumour cells from distinct cell types or is the result of the differentiation of a single original clone (Krepler et al. 1982).

Recently, it has been proposed that pleomorphic adenoma derives from the neoplastic transformation of a stem cell in the region of the intercalated ducts, capable of differentiating along ductal, acinar, squamous and myoepithelial pathways (Regezi and Batsakis 1977; Palmer et al. 1985a). Dardick et al. (1982) have rejected this hypothesis and suggested that pleomorphic adenoma may originate from the neoplastic growth of a complete ductal-acinar unit including ductal epithelium, acinar and myoepithelial cells.

It is largely accepted in the literature that myoepithelial cells play a key role in the histogenesis of pleomorphic adenoma. This hypothesis is supported by histochemical, ultrastructural and immunohistochemical studies (Hübner et al. 1971; Caselitz et al. 1981, 1982; Krepler et al. 1982; Erlandson et al. 1984; Crocker et al. 1985; Kahn et al. 1985; Palmer et al. 1985b). Myoepithelial cells have been reported to transform into squamous epithelial cells, mesenchymal-like cells and, occasionally, chondrocytes (Erlandson et al. 1984). Modified myoepithelial cells may be capable of synthesizing mucinous and chondroid substances in addition to basement membrane-like material and elastin (Erlandson et al. 1984). Myoepithelial cells have been held responsible for the myxoid appearance of the tumour (Thackray and Sobin 1972) and for the formation of hyalin cells (Lomax-Smith and Azzopardi 1978; Buchner et al. 1981). However, it appears unlikely that only myoepithelial cell differentiation accounts for the extreme variability of tumour cells in different cases and in different areas of the same case.

Our immunohistochemical data confirm the prevalence of cytokeratin-containing cells in pleomorphic adenoma, as revealed by the positive reaction with broad spectrum polyclonal and monoclonal cytokeratin antibodies. This finding is in accordance with previous reports in the literature (Caselitz et al. 1981; Krepler et al. 1982; Erlandson et al. 1984; Palmer et al. 1985a). Most of cytokeratin-positive cells are arranged in sheets, tubular, ductular and pseudo-ductular structures, but they are also found in solid, myxoid and chondroid areas. Moreover, the neoplastic constituents appear to be heterogeneous with regard to the cytokeratin content. Thus SK2-27, which is probably similar to the antibody CKB1 used by Caselitz et al. (1986a, b), a marker for myoepithelial cells in normal glands, shows high affinity for pleomorphic adenomas rich in solid and myxoid areas; in these areas, we have found a great number of S-100- and vimentin-positive cells, as well as colocalization of SK2-27 and vimentin immunostaining. The antibodies SK60-61 and PKK1, which selectively stain ductal luminal cells in normal glands, give a positive reaction mainly in tumours in which ductal and pseudo-ductular areas are largely represented.

Previous reports have demonstrated the presence of large amounts of actin in myoepithelial cells of pleomorphic adenoma using immunohistochemistry with general actin antibodies (Caselitz and Löning 1981; Caselitz et al. 1981). Moreover, typical coexpression of keratin and α-smooth muscle actin has been recently described in the myoepithelial cells of salivary and mammary glands (Skalli et al. 1986). Anti-αsm-1 staining was characteristically prevalent in myoepithelial cells of the normal glands and in neoplastic cells bordering the myxoid areas of adenomas; these cells were also positive for cytokeratins recognized by the

SK2-27 antibody. Co-expression of the myoepithelial type of cytokeratin (as seen by SK2-27 staining) and vimentin, another feature described in some normal myoepithelial cells and also present in some cells of adenomas (Caselitz et al. 1982) was prevalent in solid and trabecular areas. These findings suggest that myoepithelial features are present in at least two different types of tumour cells, one containing α-smooth muscle actin and cytokeratin, the other containing vimentin and cytokeratin. It is conceivable that the morphological variability of pleomorphic adenoma is due to the relative proportions of such subpopulations as epithelial cells, typical myoepithelial cells (containing α-smooth muscle actin) and modified myoepithelial cells (containing vimentin).

The most striking feature of malignant cells is the lack of specific distribution of immunochemical staining with restricted specificity cytokeratin antibodies. In malignant areas, we never found co-expression of cytokeratin and vimentin or of cytokeratin and α-smooth muscle actin; however, α-smooth muscle actin was detectable in considerable amounts in stromal cells. These data confirm the hypothesis that the malignant transformation of a pleomorphic adenoma may be linked to the malignant proliferation of a purely epithelial subclone (Caselitz et al. 1981; Caselitz et al. 1982).

The distribution pattern of α-smooth muscle actin in malignant tumours is similar to that previously observed in breast carcinoma (Skalli et al. 1986). This finding is not surprising if one considers the histological similarities between salivary, lacrimal and mammary glands. The presence of α-smooth muscle actin containing stromal cells may represent a useful additional criterion for differential diagnosis between benign and malignant salivary and lacrimal tumours of mixed type.

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