IMMUNOHISTOCHEMICAL STUDIES OF KERATIN DISTRIBUTION IN SALIVARY GLAND TUMORS

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Immunohistochemical staining with keratin antiserum was performed on formalin-fixed paraffin sections of 63 specimens of human salivary gland tumors including normal salivary glands. Strong keratin staining was evident in ductal segments of the parotid and submandibular glands. Significantly intense staining for keratin proteins was found in luminal epithelia of tubular, duct-like, and cystic dilated epithelial structures in pleomorphic adenoma, squamous metaplastic regions and squamous carcinomas. Myoepithelial cells in normal salivary glands were not clearly positive for keratin staining, nor were tumor cells which were possibly myoepithelium-derived, whereas, modified myoepithelial tumor cells in hyalinous and/or myxomatous regions displayed varying degrees of keratin staining. Epithelial cells of Warthin's tumor were strongly reactive. Mucoepidermoid carcinomas also showed positive keratin staining in proportion to the number of mixed epidermoid cells present. Adenoid cystic carcinomas showed slight staining for keratin in scattered tumor cells.

Keratin is a family of intermediate-sized filamentous proteins and is characteristically found in epithelial-derived tumor cells as well as in normal epithelial cells in humans and animals. The immunohistochemical detection of keratin polypeptides has been described in a number of human glandular tissues and their pathologic lesions (1, 5, 17-20). Many methods have been used for the demonstration of keratin in tissue sections. One method employed fluorescein isothiocyanate (FITC) staining of cryostat sections, because the proteins did not stain in formalin-fixed paraffin sections. Antisera for different types of keratin polypeptides have been made in various laboratories, but were not employed for wide research work in the field of pathology. An immunofluorescence method for detecting keratin polypeptides in ethanol-fixed materials has been described (1). Also, Sieinski *et al.* (19) reported the use of an indirect immunofluorescence technique for Bouin's-fixed paraffin sections of many types of human tumors. Gusterson *et al.* (12) have described that the alkaline phosphatase-conjugated antibody method used for formalin-fixed paraffin sections gave significantly positive keratin staining in human lung carcinomas. Histochemical detection of intermediate-sized filamentous proteins in parotid glands and their tumors has been reported by Caselitz *et al.* (4, 5), who compared the cellular distribution of prekeratin, vimentin, and desmin in these materials. The present study reports the distribution of keratin in salivary gland

neoplasms by the use of an indirect immunohistochemical technique involving peroxidase-antiperoxidase complexes (PAP) in formalin-fixed paraffin sections.

MATERIALS AND METHODS

Materials: The tumor specimens (63) were obtained from biopsy or surgery of major and minor salivary gland tumors. All the tumor tissues were fixed in 10% formalin solution for 12 hr, dehydrated with ethyl alcohol, and embedded in paraffin. Ten serial sections cut at 4 μ m were made from each specimen to detect keratin proteins as well as for routine histologic examination using hematoxylin-eosin, PAS, and toluidine blue stainings. The locations of the tumors and histopathologic classification are shown in Table 1.

Immunohistochemical Detection of Keratin Protein: Deparaffinized sections were rinsed with 0.05 M Tris buffer (pH 7.6) and immersed in 0.3% H_2O_2 /methanol solution for 30 min to inactivate endogenous peroxidase. The sections were rinsed with 0.05 M Tris buffer and then immersed in normal swine serum (1 : 20 dilution) for 30 min. They were then reacted with rabbit anti-human keratin antiserum (1 : 40) for 60 min, and were rinsed with 0.05 M Tris buffer and reacted with swine anti-rabbit IgG antiserum (1 : 20) for 30 min. The sections were rinsed with 0.05 M Tris buffer and reacted with rabbit peroxidase antiperoxidase complexes (PAP) (1 : 50) for 30 min. Finally, they were rinsed well and reacted with 0.05 M Tris buffer containing 0.3% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.05% H_2O_2 for 15 min. All reactions were done at room temperature (20°C). Control sections, with anti-human keratin antiserum subjected to these procedures, gave negative results. Rabbit anti-human keratin antiserum was purchased from Dakopatts, Santa Barbara Ca. USA. Swine normal serum, swine anti-rabbit serum IgG antiserum and PAP were purchased from Dakopatts, Copenhagen, Denmark. According to documentation of Dakopatts, keratin proteins were purified from human stratum corneum (foot pat), and antikeratin serum will stain 68 K, 58 K, and 56.5 K keratin polypeptides in formalin-fixed sections.

RESULTS

Normal Salivary Glands

The duct system of the parotid and submandibular glands was characterized by the presence of keratin proteins. The highest level of keratin staining was found in the striated and secretory ducts of the parotid gland, and varying staining in-

TABLE 1. *Classification of salivary gland lesions*

Pleomorphic adenoma	59
Adenoid cystic carcinoma	2
Mucoepidermoid carcinoma	1
Warthin's tumor	1
Total	63

tensities were found in intercalated duct cells. Acinar components were devoid of detectable keratin. The intercalated ducts lined by flattened or cuboidal epithelial cells displayed an irregular distribution of keratin, some cells being strongly positive and others negative (Fig. 1A).

Pleomorphic Adenoma

Pleomorphic adenomas of salivary glands were composed of many types of proliferating epithelial cells—tubular, ductal, and cystic as solid sheets or cords—which grew as a network of strands into the connective tissue stroma. Hyalinous or myxomatous stromas were occasionally observed. Large tumor foci were generally mixed with different types of epithelial cells and also included transforming epithelial cells of ductal and/or myoepithelial origin to varying modification of tumor epithelia and squamous metaplasia. Distribution profiles of keratin proteins in pleomorphic adenomas were classified into the following histologic patterns; 1) tubular and duct-like structures, 2) cystic structures, 3) solid structures, 4) network or diffuse proliferation of spindle cells, and 5) squamous-metaplastic epithelia.

1) Tubular and duct-like structures

The tubular and duct-like structure pattern was the most prominent one in this type of salivary gland tumor. It consisted of two cellular types, i.e., inner-lining cells originating from ductal epithelium and outer epithelial cells of myoepithelial origin which proliferated on the outside of the duct-like structures. Keratin was particularly prominent in the inner-lining epithelia which occurred as single, double, or pseudostratified layer of cells; on the contrary, the outer epithelia showed flattened or spindle-shaped cells which were generally devoid of keratin staining (Figs. 1B, 1C, 1D). In the case of the large tumor cell type of pleomorphic adenomas, the luminal side consisting of a columnar or cuboidal lining of cells disclosed positive keratin staining, whereas those columnar cells which were arranged in a radial pattern contained almost no keratin protein (Fig. 1C). In some cases of ductal structures in large tumor masses, the inner-lining epithelial cells displayed a very intense keratin deposition, and adjacent outer epithelial cells which were spindle-shaped or elongated showed a wide variety of patterns indicating negative to strong staining (Fig. 1B). Double-layered ductal structures were characterized by prominent keratin staining in the inner-lining epithelial cells. Large duct-like epithelial compartments showing an axletree arrangement of single-layered high columnar cell indicated positive keratin staining in all the epithelial structures including fusiform-shaped interlacing cells located outside of the columnar cell layer. Ductal structures with wide lumens, and having a single layer of cuboidal or columnar epithelial cells, showed strong keratin staining, particularly in the apical cytoplasm (Fig. 2A).

2) Cystic structures

Cystic epithelial components and dilated duct-like structures were composed generally of single flattened or rounded lining cells which displayed a prominent staining for keratin proteins (Figs. 2B, 2C, 2D). Microcystic and irregular luminal structures located in solid epithelial masses also indicated a strong keratin staining

in their inner epithelial cells, whereas adjacent outer tumor cells were devoid of keratin deposits.

3) Solid structures

There was wide variation in the histologic pattern of this type. There were foci of loosely connected spindle cells, scattered islands of small cells, and stratified or pseudostratified epithelial cell masses without keratinization including central luminal aspects. Occasional spindle cell tumor masses disclosed the presence of keratin; however, there were no ductal structures within them. Large solid foci with a central lumen and consisting of pseudostratified epithelial cells indicated varying intensities of keratin staining, but innerlining epithelial cells always showed a strong keratin deposition (Fig. 4A). Solid glandular structures showed moderate keratin staining, and tumor cells lining small lumens disclosed rather strong staining (Fig. 4B).

4) Network or diffuse proliferation of spindle cells

These cellular structures were generally composed of spindle and stellate cells arranged as a network of cellular strands. Elongated stellate cells growing into myxomatous stroma displayed an intense keratin staining (Figs. 3A, 3C), and scattered epithelioid cells distributed among hyalinous structures also showed a weak keratin reaction (Fig. 3B). Hydrotic clear tumor cells arranged in a radial pattern indicated the presence of very little keratin. Epithelial cells loosely connected with each other showed varying degrees, from negative to the highest, of keratin staining in their cytoplasm.

5) Squamous metaplastic tumor cells

There were wide variations within the squamous metaplastic type. There were solid tumor masses without any keratinization, those showing parakeratosis with microcystic structures, and fully orthokeratinized ones in individual neoplastic foci. Non-keratinized epithelial structures showed irregular low levels of keratin staining (Fig. 3D). Hyperparakeratinized tumor masses indicated strong keratin staining proportional to the extent of the parakeratinized area (Fig. 4C). Peripheral tumor cells corresponding to the basal layer cells of squamous epithelia were lacking in keratin deposits (Fig. 4C). Well keratinized area and hyperorthokeratinized cells were devoid of keratin staining, whereas adjacent granular cells displayed a strong keratin reaction (Fig. 4D).

FIG. 1A. Normal parotid gland. Note intense keratin staining in the striated duct and intercalated duct cells. Epithelial cells of intercalated duct consist of strongly keratin stainable cells and negative cells. $\times 75$

FIG. 1B. Pleomorphic adenoma of the parotid gland. Inner side of epithelial cells in tubular structure shows strong keratin staining. $\times 75$

FIG. 1C. Pleomorphic adenoma of the parotid gland. Luminal aspects of columnar epithelial cells in ductal structure show stronger staining to keratin proteins, whereas outer side of epithelia show no staining. $\times 150$

FIG. 1D. Pleomorphic adenoma of the parotid gland. Single lining epithelia of tubular or duct-like structure indicates marked keratin staining, whereas outer side cells of ductal epithelia show no keratin staining. $\times 75$

FIG. 2A. Duct-like arranged tumor cells show positive keratin staining in luminal side of cells. Submandibular gland. $\times 150$

FIG. 2B. Dilated ductal epithelial cells display marked keratin staining. Parotid gland. $\times 75$

FIG. 2C. Microcystic structure of parotid pleomorphic adenoma. Flattened cystic epithelial cells show marked keratin staining. $\times 75$

FIG. 2D. Same tumor as Fig. 2C. Epithelial cells of microcystic structure indicate positive keratin staining, whereas outer side of inner epithelia shows no keratin staining. $\times 75$

FIG. 3A. Spindle shaped interconnecting tumor cells distribute in myxomatous stroma. Note positive keratin deposition in tumor cells. Submandibular gland. $\times 75$
 FIG. 3B. Note tumor epithelial cells in hyalinous stroma, with slight keratin staining. Palatal gland. $\times 75$
 FIG. 3C. Solid tumor epithelial structure shows intermingled cellularity with strongly keratin staining cells and low or negative cells. Parotid gland. $\times 150$
 FIG. 3D. Squamous metaplastic tumor structure without keratinization shows moderate keratin staining. Parotid gland. $\times 75$

FIG. 4A. Solid epithelial structure of the tumor displays marked keratin staining in inner side (luminal side) of the epithelia. Parotid gland. $\times 75$

FIG. 4B. Single lining epithelia of inner side of the tumor foci show more marked keratin staining as compared to outer neoplastic cells. Parotid gland. $\times 75$

FIG. 4C. Marked squamous metaplasia area of pleomorphic adenoma shows most intense keratin staining. Parotid gland. $\times 75$

FIG. 4D. Highly keratinized type of pleomorphic adenoma shows keratin staining is granular cells, and fully keratinized area are devoid of keratin reaction. Parotid gland. $\times 75$

5A

5B

5C

FIG. 5A. Mucoepidermoid carcinoma. Epidermoid cells contain higher amounts of keratin. $\times 75$

FIG. 5B. Adenoid cystic carcinoma. Some tumor cells intermingled into tumor structure show strong keratin staining. $\times 75$

FIG. 5C. Warthin's tumor. Epithelial cells of the tumor are characterized by existence of high keratin staining. $\times 75$

Mucoepidermoid Carcinomas

Keratin staining was proportional to the number of epidermoid cells which showed intense staining, whereas mucous-secreting cells displayed low or negative keratin (Fig. 5A).

Adenoid Cystic Carcinomas

Neoplastic cells in these tumors usually showed a light keratin distribution intermingled with cells giving a strong keratin reaction (Fig. 5B).

Warthin's Tumor

Eosinophilic epithelial cells indicated a positive keratin distribution (Fig. 5C).

DISCUSSION

The immunohistochemical observation of cytokeratin has been made in various epithelial structures including salivary glands. In these glands, ductal segments—epithelial cells of intercalated, striated, and excretory ducts—have been shown to contain keratin proteins (4, 5, 9). In the present study, the results of keratin staining in ductal epithelia of the parotid and submandibular salivary glands are similar to those from previous reports. It has also been accepted that keratin proteins in the cells of squamous, intestinal, respiratory and genitourinary epithelia, as well as in ductal cells of salivary glands, correspond to the abundant tonofilaments seen at the ultrastructural level.

The histogenesis of salivary gland tumors, either malignant or benign, has been suggested to involve two types of cells—ductal (6, 7) and myoepithelial cells (8). Inner-lining epithelial cells of tubular and duct-like structures of variable staining patterns may be transformed from a ductal origin, as intercalated and striated ducts give a high degree of keratin staining. Such patterns of keratin distribution have been found in pleomorphic adenomas of salivary gland and breast carcinomas. Also Gusterson *et al.* (13) have reported that salivary gland membrane antigen was distributed in normal ductal epithelial cells and in ductal lumen cells of salivary gland tumors. This paper relating to experimental carcinomas of mouse submandibular glands showed that keratin protein could be detected in degranulated tubules and its concentration gradually increased in metaplastic to transforming neoplastic epithelia. Tumor cells of epidermoid and keratinized types of experimental carcinomas also indicated marked keratin staining, as well as squamous epithelium-derived tumors (21).

The second cell type involved in the development of pleomorphic adenomas is the myoepithelial cell which is located surrounding acini and intercalated ducts as the basket cells (6, 11). Histologically, neoplastic cells of myoepithelial origin are spindle-shaped with variant morphology and are distributed on the outer side of ductal or tubular compartments. They grow in striated patterns and interconnecting fibrillar structures. The pattern of keratin localization in these cells of probable myoepithelial origin was highly irregular. Caselitz *et al.* (4, 5) have demonstrated keratin proteins in salivary gland tumor cells derived from myoepithelial cells. In the present study, modified neoplastic-changed myoepithelial cells located in myxomatous, hyalinous, and degenerated stromas displayed positive

keratin staining; however, their intensities varied from negative to moderate and there were very few instances of intense staining. Furthermore, in the common pattern of the pleomorphic adenoma, tumor cells growing on the outer side of tubular and duct-like structures or microcystic structures generally lacked keratin proteins; however, some scattered spindle cells stained strongly for keratin. Such keratin containing tumor cells may be derived or transformed from cells of either inner ductal epithelial or myoepithelial origin, but identification of their homologous cells could not be made by the present method.

Positive keratin distributions in normal myoepithelial cells have been described in salivary glands (5, 10), breast tissue (1), and sweat glands (10). In the present study, immunohistochemically detectable keratin in myoepithelial cells of normal salivary glands was usually absent; that is, most of the myoepithelial cells did not stain for keratin peptides; whereas some rarely gave a positive reaction for keratin. In this regard, Altmannsberger *et al.* (1) have reported that keratin could not be detected in myoepithelial cells of the human breast. No complete positive keratin staining in myoepithelial cells in salivary glands and neoplastic lesions would be used to differentiate types of anti-keratin serum as the comparison to the use of previous works (4, 5, 10). On the other hand, myoepithelial contractile cells in breast, salivary glands, and skin were positive for myosin and actomyosin contained immunologically detectable smooth muscle proteins (2). Line and Archer (16) have pointed out that myoepithelial cells in the rat submandibular gland also reacted with human actomyosin antisera.

Warthin's tumor was characterized by the presence of eosinophilic epithelial cells and lymphocytic infiltration. These eosinophilic cells histochemically resembled mitochondria-rich cells, as did striated duct cells (22). Striated duct cells in the parotid gland revealed the most prominent staining for keratin protein, and epithelial cells of Warthin's tumor also gave high keratin staining, which may indicate that the tumor arises from striated duct cells containing high oxidative enzyme activities (3, 14, 15).

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