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Characterization of cytoskeletal proteins in basal cells of human parotid salivary gland ducts*

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Summary. From previous immunofluorescent, immunohistochemical and ultrastructural studies, myoepithelial cells have been reported to be absent from the striated and excretory ducts of human salivary gland. Yet recently, certain anti-cytokeratin monoclonal antibodies which specifically label the myoepithelium of salivary gland acini and intercalated ducts have also been found to stain basally situated cells in both striated and excretory ducts. In this study, we have used eight samples of normal human parotid gland (methacarn-fixed and frozen sections) to establish if basal cells of striated and excretory ducts have the cytoskeletal protein complement (actin and cytokeratins) of myoepithelial cells. Using a muscle-specific actin, HHF35, not only is the myoepithelium of acini and intercalated ducts stained in all cases, but stellate and spindle shaped cells are also detected all along the inter- and intralobular striated ducts in four of the eight examples. With double-labeled frozen sections and fluorescent microscopy, the actin-specific probe, phalloidin, and the myoepithelial-selective anti-cytokeratin 14 antibody, 312C8-1, confirm that the striated duct does have a population of basal cells with the cytoskeletal protein make-up of myoepithelium. The monoclonal antibody 8.12 (specific for cytokeratin 13 and 16) also stains some basal cells of striated and excretory ducts, as well as luminal cells of ducts at all levels, but does not label the myoepithelium of acini and intercalated ducts. Both the anti-cytokeratin antibodies and the actin-detecting mechanisms reveal that the basal cell population of striated and excretory ducts is more heterogeneous, and likely functionally more complex, than has been realized previously. Such findings are not in agreement with certain aspects of current theories of the histogenesis of salivary gland tumours.

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Introduction

The basal cells of the excretory ducts and luminal cells of the intercalated ducts are thought to have major roles in the histogenesis of salivary gland tumours. In 1971, Eversole formulated the "semipleuripotential" concept that certain stem (reserve) cells associated with both intercalated and excretory ducts were involved in the induction of salivary gland tumours. The reserve cell basis for neoplasia in these organs was developed further by Regezi and Batsakis (1977) and maintains a central role in views about the morphogenesis of such tumours (Batsakis 1980; Batsakis 1985; Chaudhry et al. 1986a, b). However, the existence of specific reserve cells for the regenerative capacity of salivary ducts remains hypothetical; these cells have neither been identified morphologically nor have their functional capacities been established. Based on Eversole's (1971) original observations, the implication has been that at least some reserve cells should occupy a basal position in differentiated salivary ducts.

In the salivary gland excretory duct in particular there is morphological evidence for an almost continuous layer of cuboidal to triangular shaped cells below the luminal epithelium (Tamarin and Sreebny 1965; Shackleford and Schneyer 1971; Testa-Riva et al. 1981). Recently, such cells have been shown to certain cytokeratin polypeptides by a variety of monoclonal antibodies (Palmer et al. 1985; Knight et al. 1985; Palmer 1986; Caselitz et al. 1986a, b; Dairkee et al. 1985, 1986; Dardick et al. 1987). In some cases, these antibodies also stain the myoepithelial cells of acini and intercalated ducts (Dairkee et al. 1985; Caselitz et al.

1986b; Dardick et al. 1987). Generally, myoepithelial cells are considered to be confined to acini and intercalated ducts (Tandler 1965; Tandler 1977; Cutler et al. 1977) but on the basis of immunohistochemical studies, Caselitz et al. (1981) have indicated that myoepithelium may extend proximally beyond the intercalated ducts. Since myoepithelium also occupies a basal position in relation to acinar and duct luminal epithelium, both the location of this cell-type and its relationship to basal cells at other levels of the duct system are critical issues for the histogenesis and morphogenesis of salivary gland tumours.

Using a variety of techniques, this study attempts to characterize further the nature of basal cells associated with the striated and excretory ducts of normal human parotid gland.

Materials and methods

Normal salivary gland parenchyma was obtained from eight superficial parotidectomy specimens resected for pleomorphic adenoma. In all cases, the tissue was fixed in methacarn (60% methanol, 30% chloroform, and 10% glacial acetic acid), prior to paraffin embedding, to take advantage of the superior immunohistochemical staining with this procedure (Mitchell et al. 1985). Deparaffinized 4 to 5 micron thick tissue sections were immunostained with monoclonal antibodies 312C8-1 (diluted 1:300), 8.12 (diluted 1:50), and HHF35 (diluted 1:2000) using an indirect peroxidase technique (Unistain Kit, Becton Dickinson, Mississauga, Ontario). Antibody 312C8-1 (kindly supplied by S.H. Dairkee, Oakland, CA, USA) is an anti-cytokeratin that recognizes cytokeratin 14 (using the catalogue of Moll et al. 1982) and in salivary gland selectively stains myoepithelial and basal cells (Dairkee et al. 1985, 1986), while antibody 8.12 (ICN ImmunoBiologicals, Lisle, IL, USA), also an anti-cytokeratin, recognizes polypeptides 13 and 16. Antibody HHF35 (Enzo Biochemical Inc., New York, USA) recognizes actins that are specific to muscle cells (smooth, skeletal, and cardiac) but also decorates myoepithelial cells of salivary gland (Tsukada et al. 1987a, b). Appropriate positive and negative controls were used.

Tissue from four of the eight superficial parotidectomy specimens was snap-frozen in isopentane-cooled liquid nitrogen and stored at −70 degrees Celsius until required. Sections, 4 to 5 microns thick, were mounted on glass slides, fixed for 10 min in cold absolute acetone, and air-dried. Following rehydration in phosphate buffered saline (PBS), these sections were then sequentially stained with the anti-cytokeratin monoclonal antibody 312C8-1 (Dairkee et al. 1985; 1986) and rhodamine-conjugated phalloidin (Molecular Probes Inc., Junction City, Oregon, USA), which detects filamentous actin in the cell cytoplasm (Wulf et al. 1979; Geiger et al. 1987). A 1:700 dilution of antibody 312C8-1 in PBS was applied for 45 min, the sections washed with PBS, and a goat anti-mouse IgM (μ) conjugated to fluorescein isothiocyanate (1:100; Kirkegaard and Perry Lab. Inc., Gaithersburg, MA, USA) applied for 45 min. Following washing with PBS, rhodamine-phalloidin (supplied as a 3.3 μM solution in methanol), diluted 1:10 in PBS was applied to the sections for 20 min. After washing with PBS, the stained sections were coverslipped using a 50% aqueous glycerol-PBS solution to which was added *p*-phenylenediamine (0.1%) to retard fading of the fluorescence. A Zeiss epifluorescence microscope equipped with both an xenon and a mercury lamp and appropriate filters for both rhodamine and fluorescein was used for photography. Appropriate areas of the sections were sequentially photographed (Ilford XP1 400 film) using both fluorescent markers.

Results

For orientation purposes in describing the findings related to the basal cells of human salivary gland ducts, the intercalated duct/acinar segments will be referred to as the distal portions of the duct system, while the main excretory duct will be considered the proximal part. Similarly, for the striated duct, intralobular portions adjacent to the intercalated ducts will be positioned distally and interlobular segments merging with the excretory duct will be referred to as proximal portions.

Using monoclonal antibody 312C8-1 on methacarn-fixed parotid gland tissue, either the entire or at least the greater portion of the circumference of the major interlobular excretory ducts displayed basal cells that were strongly stained, while luminal epithelial cells were unstained (Fig. 1a, b). In the case of antibody 8.12, staining (moderate to strong) of basal cells in this portion of the duct system was usually more discontinuous, and there was weak to moderate staining of the luminal cells (Fig. 1c). In serial sections, it was apparent that some basal cells stained with both 312C8-1 and 8.12, while others only stained with antibody 312C8-1 (Fig. 1b, c).

Intralobular and the distal portions of interlobular striated ducts also displayed moderately to strongly stained basal cells using both monoclonal antibodies 312C8-1 and 8.12 (Fig. 2a, b). However, the pattern of distribution of basal cells differed from that of the proximal interlobular striated ducts and the excretory ducts; single, or at most groups of two to three, basal cells were irregularly distributed both longitudinally along the duct and around the perimeter (Fig. 2a, b). From case to case, there was considerable variation in the number and distribution of stained basal cells in striated ducts. Some cross-sections displayed none, the majority had one to four, and some had as many as 10 to 12 basal cells. Particularly in the intralobular striated ducts, some segments either had very infrequently stained basal cells or appeared to be entirely devoid of these cells (Fig. 2a). In glancing sections of the outer aspect of striated ducts, basal cells were often irregular in shape and possessed elongated cytoplasmic extensions (Fig. 2a). Again, luminal cells were un-

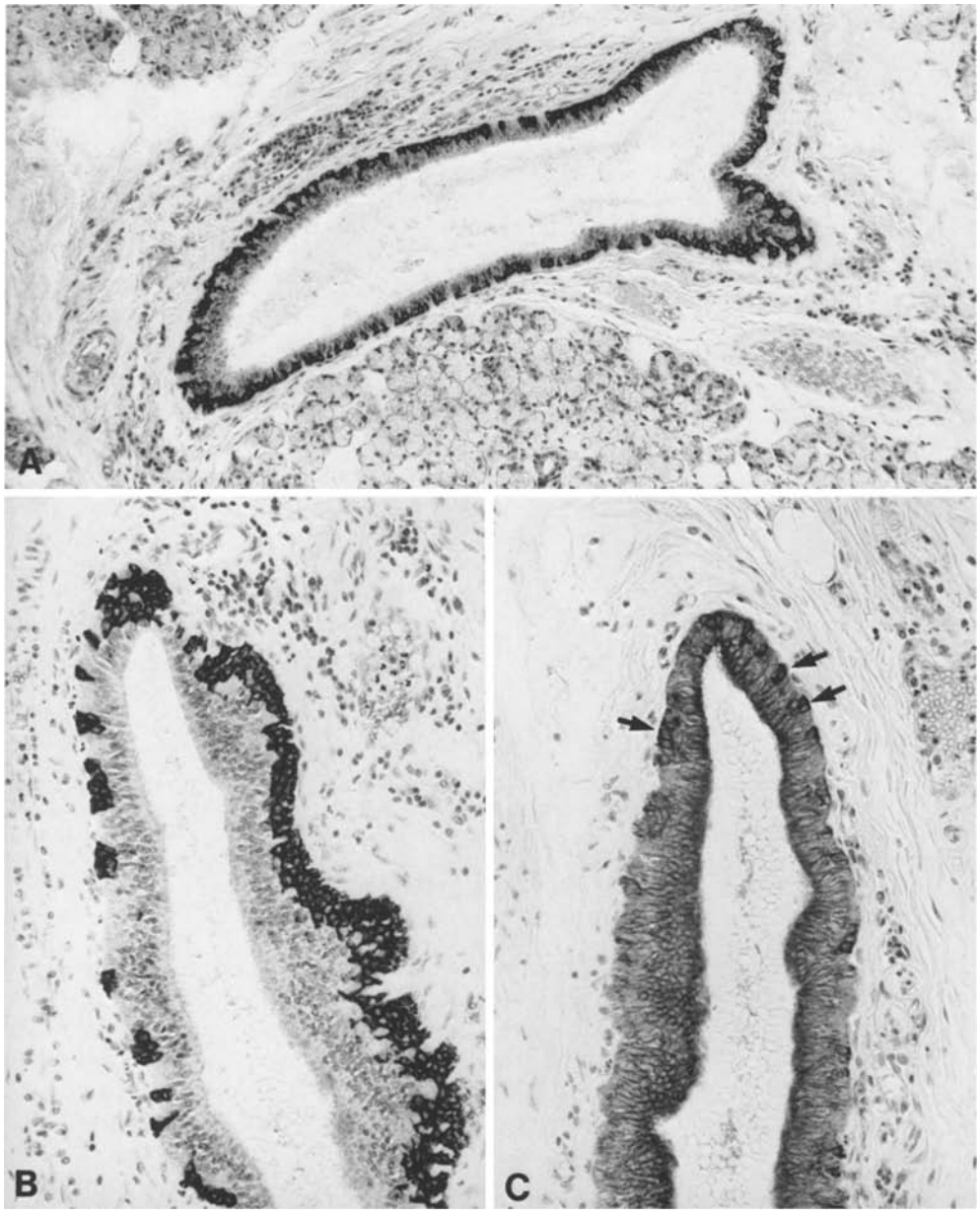

Fig. 1. Interlobular excretory duct. **A** Anti-cytokeratin antibody 312C8-1 stains an almost continuous row of variably shaped basilar cells, while luminal cells are unstained. **B** Anti-cytokeratin antibody 312C8-1. In this segment, positively stained basal cells are continuous on one side and intermittently spaced on the other. **C** Anti-cytokeratin antibody 8.12. In a serial section of the excretory duct in (**B**), both luminal and basal cells are stained, but the positively stained basal cells (*examples are arrowed*) are interrupted on both sides of the duct. Comparing (**B**) and (**C**) reveals that some single and groups of basal cells are stained with both antibodies. Immunoperoxidase with haematoxylin counterstain. **A,** ×120; **B** and **C,** ×240

stained by antibody 312C8-1 (Fig. 2a) and there was weak to moderate staining of these cells by antibody 8.12 (Fig. 2b). In contrast to antibody 312C8-1, where myoepithelial cells of acini and intercalated ducts were strongly stained, antibody 8.12 produced no staining of myoepithelium in these locations in all eight of the parotid glands. Generally, in both excretory and striated ducts, a larger number of basal cells were stained by the anti-cytokeratin 312C8-1 than the anticytokeratin 8.12.

In all of the examples of parotid gland stained with monoclonal antibody HHF35, crescent- or spindle-shaped myoepithelial cells bordered portions of some acini, the majority of intercalated ducts and some cross-sections of intralobular

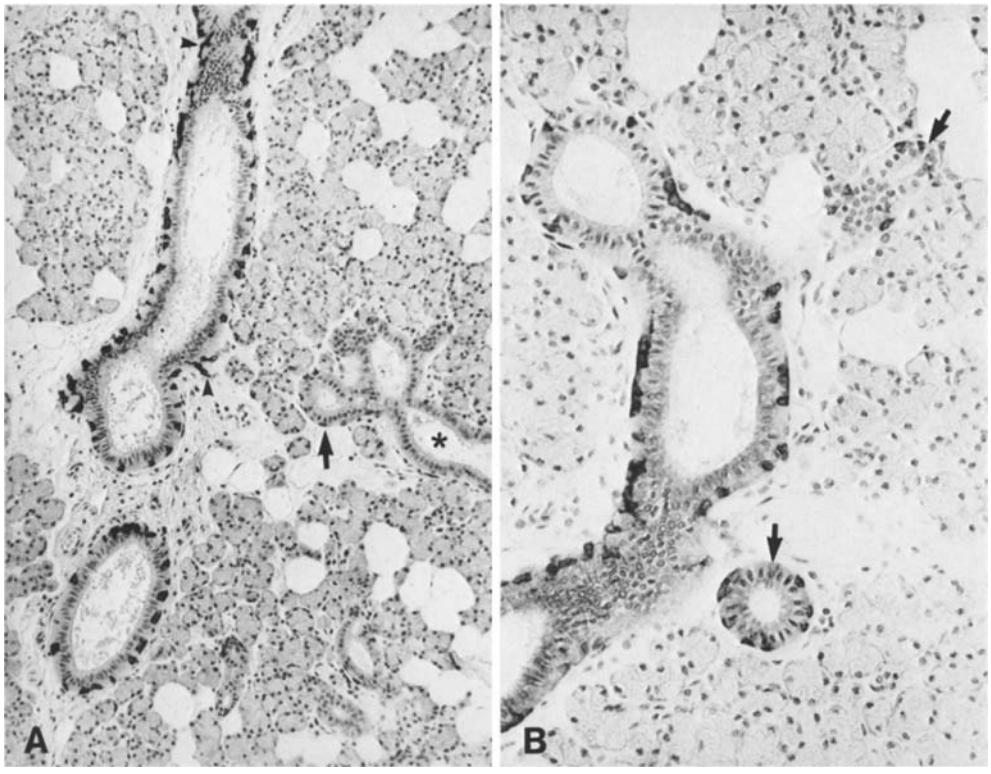

Fig. 2. Inter- and intralobular striated duct. **A**. Anti-cytokeratin antibody 312C8-1. Interlobular ducts have fairly evenly distributed strongly stained basal cells that in a glancing section through the duct wall (*arrowhead*) have elongated cytoplasmic processes. Intralobular striated ducts have fewer stained basal cells (*arrows*) and some segments (*asterisk*) have few, if any, stained cells. **B**. Anti-cytokeratin 8.12. Both intralobular (*arrows*) and interlobular ducts have strongly stained basal cells, either singly or in small, linear groups. Luminal cells are weakly stained. Immunoperoxidase with haematoxylin counterstain. **A** ×100; **B** ×200

striated ducts (Fig. 3a). This antibody also showed that many segments of proximal intralobular and distal interlobular striated ducts also had strongly stained, elongated and triangularly shaped cells scattered along the outer aspect of the duct epithelium (Fig. 3b). Where the ducts were tangentially cut at the periphery, some basilar cells had very elongated, narrow cytoplasmic processes splayed out over the outer surface of the ducts (Fig. 3b). As distal interlobular striated ducts joined larger, more proximally situated interlobular striated ducts, stained cells were infrequent or, more often, absent (Fig. 3b). This type of staining pattern of striated ducts was particularly evident in four out of eight parotid glands. In the remainder, HHF35-positive basal cells associated with striated ducts were infrequent or absent at all levels. In all of the cases, no HHF35-positive basal cells were noted in the main excretory ducts, even though a continuous row of basal cells was apparent (Fig. 3c). Smooth muscle cells of both intra- and interlobular blood vessels were strongly stained (Fig. 3b, c).

Using frozen sections double-labeled with rhodamine-phalloidin (for actin) and the monoclonal antibody 312C8-1 (for cytokeratin 14) complexed with a fluorescein-conjugated secondary antibody, all of the four parotid gland tissues produced the same results. At the periphery of both acini and intercalated ducts, the cell bodies and the many, elongated cytoplasmic processes of myoepithelial cells were identically identified by the anti-cytokeratin antibody (Fig. 4a, inset) and rhodamine-phalloidin (Fig. 4b, inset).

Using the monoclonal antibody 312C8-1, intralobular striated ducts had a population of polygonal-, triangular-, and somewhat spindle-shaped basal cells that were intensely stained (Fig. 4a). In the comparable field using rhodamine-phalloidin, the staining pattern of these basal cells was more complex (Fig. 4b). Some basal cells of striated ducts, particularly the spindle-shaped ones, were intensely fluorescent. Other basal cells staining for cytokeratin (Fig. 4a) displayed only a narrow band of fluorescence at the base of the cell, while still others were apparently not labelled by the rhodamine-phalloidin probe (Fig. 4b). Luminal epithelial cells of striated ducts, unstained by the cytokeratin antibody (Fig. 4a), displayed strong staining

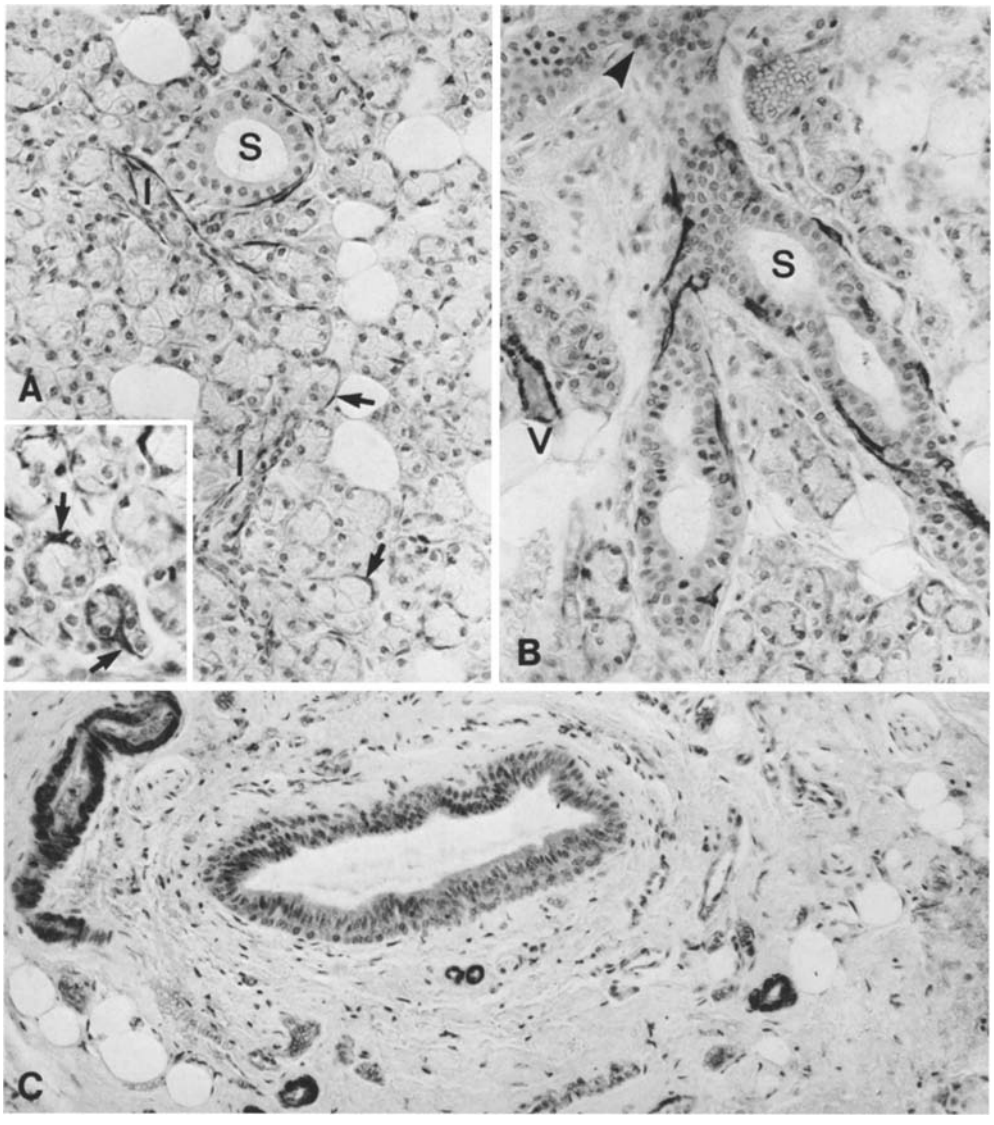

Fig. 3. Anti-actin antibody HHF35. **A** Myoepithelial cells at the bases of acini (*arrows*) and intercalated ducts (*I*) are intensely stained. Similar appearing stained cells are present at the base of an intralobular striated duct (*S*). The inset details the darkly staining myoepithelial cells and their cytoplasmic processes (*arrows*) associated with acini. **B** Attenuated, actin-containing basal cells are also present along proximal segments of interlobular striated ducts (*S*), but are absent from more distal segments (*arrowhead*) at the junction with larger interlobular ducts. Circular smooth muscle cells of a small blood vessel (*V*) are also strongly positive. **C** Basal cells of an excretory duct are unstained, but the blood vessel walls are positive with antibody HHF35. Immunoperoxidase with haematoxylin counterstain. **A** ×300 (inset, ×320); **B** ×240; **C** ×160

of the subapical regions as well as moderate staining of basal areas (Fig. 4b).

Discussion

One of the main criteria for the identification of myoepithelial cells in salivary gland is the presence of larger than the usual amounts of actin and myosin in the cytoplasm of epithelial cells. Previous identification of these proteins in myoepithelium has used antibodies to actin and/or myosin from a variety of sources in either fluorescent microscopic (Archer and Kao 1968; Archer et al. 1971; Drenckhahn et al. 1977; Caselitz et al. 1981) or immunoperoxidase (Saku et al. 1984; Palmer 1986) studies. In the majority of these studies, the myoepithelium identified has been confined to the acini and intercalated ducts, but one such study (Caselitz et al. 1981) suggested that myoepithelial cells might extend to the more proximal segments of the duct system. Recent reports that certain anti-cytokeratin antibodies selectively decorate myoepithelium

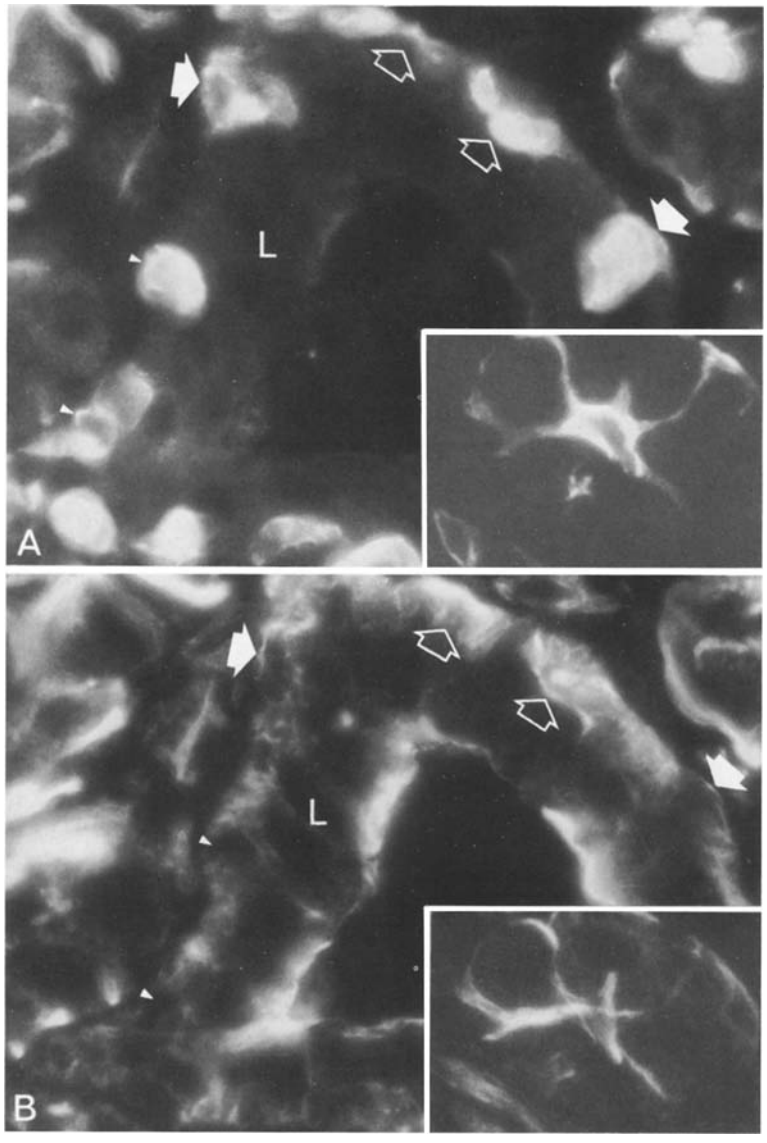

Fig. 4. Rhodamine-phalloidin and anti-cytokeratin 312C8-1. **A** Anti-cytokeratin 312C8-1 strongly decorates the basal cells of a striated duct while luminal cells (*L*) are unlabeled. **B** Using rhodamine phalloidin, some basal cells are brightly fluorescent (*open arrows*), others only have a narrow band of fluorescence at the base (*closed arrows*), and still others have little or no fluorescence (*arrowheads*). Luminal (*L*) cells have moderate fluorescence associated with "striated" regions at the base and brighter fluorescence in the subapical regions of the cytoplasm. Inset on **A** and **B** shows a myoepithelial cell spreading over the surface of an acinus. Note the similarity in the distribution of actin and cytokeratin filaments, both in the main, central portion of the cell and its many cytoplasmic processes. Immunofluorescence. **A, B,** and insets ×1080

in salivary gland and breast tissue, as well as the basal cells of salivary gland ducts (Nathrath et al. 1982; Nagle et al. 1985; Geiger et al. 1987; Dairkee et al. 1985, 1986; Caselitz et al. 1986a, b; Dardick et al. 1987, 1988), provide additional tools to study the latter cell-type. It is of some significance that antibodies to cytokeratin 14 (Dairkee et al. 1985, 1986; Caselitz et al. 1986a, b; Dardick et al. 1987, 1988) stain both the myoepithelial cells of acini and intercalated ducts and the basal cells of striated and excretory ducts, while other anti-cytokeratin antibodies such as 16a recognizing cytokeratins 17 and 18 (Palmer 1986; Knight et al. 1986), the 8.12 product used in this study, and antibody KS 8.58 (Geiger et al. 1977) (the latter two are antibodies to cytokeratins 13 and 16), only decorate the basal cells of the striated and excretory ducts. Moreover, as the current study shows, the basal cells of striated and excretory ducts vary in the expression of certain cytokeratins, so that while most of the basal cells seem to contain cytokeratin 14, a smaller proportion also contain cytokeratins 13 and 16 (Fig. 1b and c). Currently, no information is available to explain such variation in cytoskeletal proteins in this particular cell-type.

Histological descriptions of the striated ducts in salivary glands do not indicate the presence of basal or myoepithelial cells (Young and van Lennep 1978; Regezi and Batsakis 1977; Cutler et al. 1977). The ultrastructural survey of salivary gland ducts by Cutler and associates (1977) suggested that cytoplasmic processes of myoepithelial cells situated on intercalated ducts can extend onto proximal segments of striated ducts, but indicated

that a specific complement of myoepithelial cells was absent both from striated and excretory ducts. Recent ultrastructural findings reveal that myoepithelial cells are indeed present at least on intra- and interlobular striated ducts (Dardick et al. 1988) a finding that is supported by the detection of actin-containing, basally situated cells in the same location both by the anti-actin antibody HHF35 (Takasuda et al. 1987a, b) and the actin-specific probe phalloidin (Wulf et al. 1979; Geiger et al. 1987). Equally important is the current observation that at least some cytokeratin polypeptide-containing basal cells of striated ducts out to the junction with the excretory duct system have a complement of actin filaments similar in amount and distribution to typical myoepithelium. These ductal-type myoepithelial cells also display variation in the cytoplasmic content of actin using the rhodamine-phalloidin probe.

The anti-actin antibody HHF35 allowed a more extensive survey for positively stained basal cells along striated and excretory ducts. A few observations using this antibody require emphasis: first, excretory ducts in all eight of the samples surveyed were devoid of basal cells containing actin in the amounts usually evident in myoepithelial cells; second, in four of the eight samples of parotid gland, positively stained cells were infrequent or absent even along striated ducts, despite the detection of cytokeratin 14-containing basal cells at least in some segments of these ducts; and third, in those samples with actin-containing basal cells along striated ducts, longitudinal sections revealed that some segments appeared devoid of stained cells. The current studies reveal that the cellular population of ductal epithelium, particularly of the striated and excretory duct, is more variable than previously realized. Undoubtedly, the structural diversity of basilar cells is reflected in functional differences.

There are two important conclusions from this characterization. First, myoepithelial cells can extend from acini to striated ducts, though the distribution along the latter is variable from duct to duct and even between normal parotid salivary glands; and second, the population of basal cells along the striated and excretory ducts is heterogeneous with regard to both cytokeratin polypeptides and actin content. Such features suggest that all basal cells are not likely to be stem (reserve) cells. Geiger et al. (1987) suggest that as basal cells (expressing cytokeratin 13, 14, and 16) differentiate to duct luminal cells there is a loss of cytokeratins 13 and 16. If this interpretation is correct, one might expect at least a small number of luminal cells, or some form of intermediate or transitional cell, in which cytokeratins 13 and 16, or even cytokeratin 14, are still present. No such differentiating luminal cells were identified by Geiger et al. (1987) or in this report. In view of current histogenetic theories of salivary gland tumours (Regezi and Batsakis 1977; Batsakis 1980), functional studies of basilar epithelium are essential. Given the presence of myoepithelial cells in striated ducts, and the central role of neoplastic myoepithelial cells in many salivary gland tumours (Batsakis et al. 1983; Dardick et al. 1985; Dardick and van Nostrand 1987), the tumours could as readily derive from the striated duct as from the intercalated duct, as current theory dictates (Eversole 1971; Regezi and Batsakis 1977).

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