Salivary Gland Components Involved in the Formation of Squamous Metaplasia

IRVING DARDICK, MD, M. T. DIANE JEANS, ART, NANCY M. SINNOTT, MSc, JOHN F. WITTKUHN, RT, HARRIETTE J. KAHN, MD, and REUBEN BAUMAL, MD

From the Canadian Tumour Reference Centre, Ottawa, Ontario, and Departments of Pathology, The Hospital for Sick Children and Women's College Hospital, Toronto, Ontario, Canada

Squamous metaplasia is not an uncommon feature of a number of salivary gland lesions. Arterial ligation of rat submandibular and sublingual salivary glands was used for study of the processes and cell types involved in the development of the squamous metaplasia that occurs in ischemic and infarcted portions of gland parenchyma 6 to 8 days following vessel ligation. Light and electron micrographs show that the principal portion of salivary gland tissue undergoing squamous metaplasia is the acinar–intercalated duct cell complex. Early stages of this process involve a gradual dedifferentiation of acinar cells and hyperplasia of acinar, duct luminal cells, and myoepithelium. Subsequently, both luminal and myoepithelial cells have increasing accumulation of tonofilaments and formation of desmosomes, and centrally located cells may undergo keratinization. Immunohistochemical staining of ischemic salivary gland tissue with developing squamous metaplasia was performed with the use of rabbit antisera to human epidermal and Mallory body cytokeratins. The two antisera gave complementary patterns in normal acini and ducts, with antibody to epidermal cytokeratin (ECK) staining only myoepithelial cells and antibody to Mallory body cytokeratin (MBCK) staining mainly luminal epithelial cells. In early phases of squamous metaplasia (6 days after ligation), antibody to ECK stained central and peripheral (myoepithelial) cells, but by 8 days after ligation only central cells were stained. At 6 days after ligation, a proportion of central cells in squamoid clusters stained with antibody to MBCK, and myoepithelial cells were unstained. By 8 days after arterial ligation, cell clusters exhibiting squamous metaplasia were completely unstained with antibody to MBCK, despite the presence ultrastructurally of numerous tonofilament bundles in both types of cells forming these clusters. The propensity for squamous alteration of acinar–intercalated duct complexes has important connotations for salivary gland tumors such as pleomorphic adenoma and mucoepidermoid carcinoma. (Am J Pathol 1985, 119:33–43)

THE DEVELOPMENT of squamoid or frankly squamous epithelium is a common feature of a number of reactive and neoplastic conditions of salivary gland. Chronic sialadenitis,[1] necrotizing sialometaplasia,[2,3] and radiation of salivary glands[4] can all be associated with squamous metaplasia. In addition, varying degrees of squamous differentiation are evident in salivary gland neoplasms such as mucoepidermoid carcinoma, pleomorphic adenoma, basal cell adenoma, and Warthin's tumor.[1]

The observation that squamous metaplasia occurs in excretory ducts in chronic sialadenitis has suggested that the origin of mucoepidermoid carcinoma may lie in this portion of salivary gland parenchyma.[5] However, two types of investigations suggest other portions of the gland parenchyma. First, ultrastructural observations of pleomorphic adenoma and mucoepidermoid carcinoma indicate that squamous metaplasia occurs in the counterpart of the more terminal portions of the duct system and in acini and involves both luminal epithelial and myoepithelial cells.[6–8] Secondly, immunohistochemical investigations have established that the intermediate filament cytokeratin is present as part of the cytoskeleton of duct epithelial, acinar, and myoepithelial cells of normal salivary glands.[9–11] Because these filaments are present in squamous epithelium, a

Supported by a grant from the National Cancer Institute of Canada.

Accepted for publication November 21, 1984.

Address reprint requests to Dr. I. Dardick, Canadian Tumour Reference Centre, Clinical Studies Unit Building, Ottawa Civic Hospital, 60 Ruskin Avenue, Ottawa, Ontario, Canada K1Y 4M9.

potential mechanism for squamous metaplasia exists in all components of salivary gland.

Experimental induction of squamous metaplasia in salivary gland has been noted following arterial ligation,[12-14] radiation,[4] and implantation of carcinogens.[13,15] Of these methods, arterial ligation provides a rapid and reproducible induction of squamous metaplasia which can be analyzed by light and electron microscopy and immunohistochemical investigations. In the present study, we show by ultrastructural means that the duct-acinus-myoepithelium complex is involved in squamous metaplasia of the salivary gland following arterial ligation. In addition, immunohistochemical analysis of the induced squamous metaplasia, using cytokeratin antibodies, has shown modifications in the cytokeratin complement of the newly formed squamous cells.

Methods and Materials

Experimental System

Six-month-old Sprague-Dawley albino rats (250-300 g) were anesthetized with halothane, and an incision was made in the ventral midline of the neck. The left submaxillary and sublingual salivary glands were freed from the overlying fascia and retracted laterally for exposure of the pedicle containing their ducts and artery. By blunt dissection, the ducts were separated from the artery, and the latter were ligated with a silk suture at the point prior to its division into separate branches for the submaxillary and sublingual glands. The opposite side was left unligated to serve as a control.

The animals were sacrificed 1, 2, 3, 4, 6, 8, and 10 days after the operative procedure. Control and arterially ligated submaxillary and sublingual salivary glands were excised as a unit and bisected longitudinally. One-half of each gland complex was fixed in 10% buffered formalin for routine light microscopy and immunohistochemical examination, while the other half was fixed in Karnovsky's solution for ultrastructural studies. To ensure that specific areas were available for electron microscopy, review of paraffin-embedded tissues was followed by the cutting of selected regions from the Karnovsky-fixed gland tissue and their mincing into small cubes. After postfixation in 1% osmium tetroxide, the tissue portions were dehydrated in graded alcohols and embedded in Epon-Araldite. One-micron-thick, toluidine-blue-stained plastic sections were used for preselection of areas for thin sections. Uranyl acetate and lead citrate stained grids were screened and photographed with a Philips EM 301 electron microscope at 60 kV.

Immunocytochemical Staining

Deparaffinized sections of control and arterially ligated salivary glands were stained with two antibodies, produced as previously described,[16,17] by the use of an immunoperoxidase technique.[18] The antisera used were: 1) epidermal cytokeratin (ECK) antibodies produced by immunization of rabbits with purified epidermal cytokeratin[19,20] (this antibody stained normal epidermis and sweat glands of skin and bile ducts in the liver, but not hepatocytes) and 2) Mallory body cytokeratin (MBCK) antibodies produced by immunization of rabbits with purified Mallory bodies isolated from human autopsy liver[21] (this antibody did not stain epidermis but did react with sweat glands of skin, bile ducts in the liver, and hepatocytes). Substitution of normal rabbit serum for the cytokeratin antibody containing sera served as a negative control. Following immunohistochemical staining, the tissue sections were counterstained with hematoxylin.

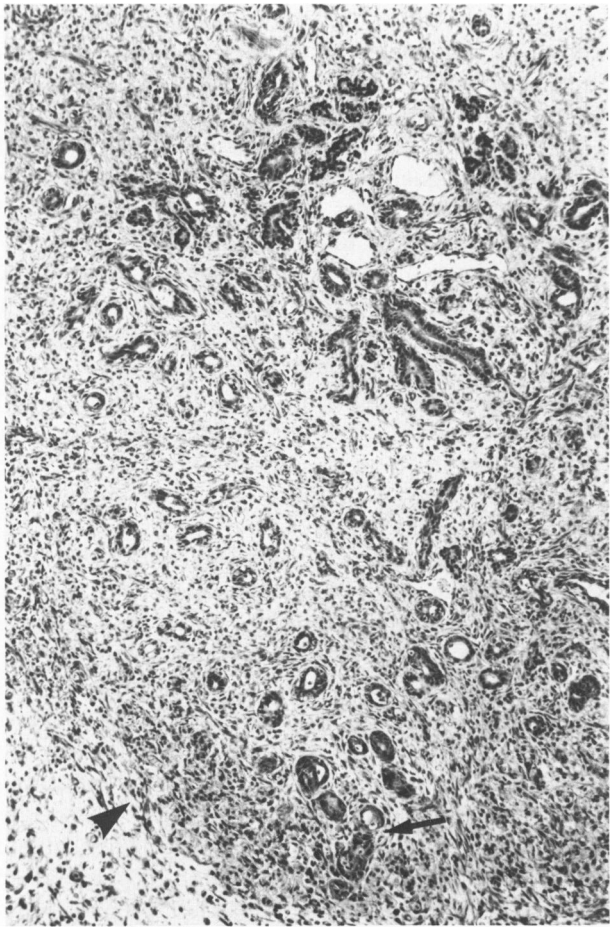
Immunoblotting was performed by the method of Towbin et al[21] with the use of antibodies to ECK and MBCK and cytokeratins extracted from skin and colon. The antibody to ECK reacted with molecular weight keratins in the range of 55 to 60 kilodaltons (kd), which correspond to cytokeratins 4, 5, and 6 of Moll et al.[22] The antibodies to MBCK reacted with molecular weight keratins of 45 and 53 kd, which correspond to cytokeratins 8 and 18.[22]

Results

Microscopy

Examination of paraffin sections revealed that the degree of infarction produced by ligation of the principal arterial supply to rat submandibular and sublingual salivary glands varied from virtually complete to partial. In the latter circumstance, distal portions of the submandibular gland were most severely altered. However, in both situations, early phases of squamous differentiation were evident at 3-6 days after ligation, and fully developed squamous metaplasia was evident by 8 days.

With the severest degrees of infarction, only a few scattered tubular structures persisted in a narrow band adjacent to the periphery of the gland, and it was this residual salivary gland tissue that showed the most obvious squamous differentiation (Figure 1). Glands that showed partial degrees of infarction and parenchymal atrophy 6-8 days following arterial ligation (Figures 2 and 3) proved the most useful in assessing the tissue and cellular modifications culminating in squamous metaplasia. Such modifications had a lobular pattern,



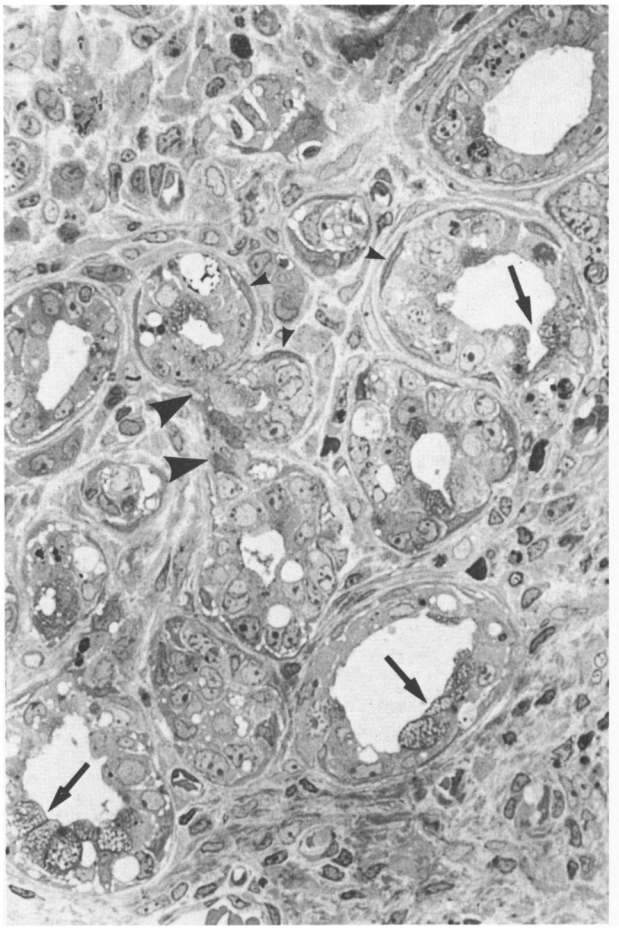


Figure 1—Tubuloglandular structures, a few of which show early stages of squamous metaplasia (*arrow*), persist in a region of severe ischemia in submandibular rat salivary gland 6 days after arterial ligation. The region of the original gland capsule is marked by an *arrowhead*. (×110) **Figure 2**—Rat submandibular gland at 6 days after arterial ligation. A region of ischemic lobular parenchyma composed of modified acini, some of which are interconnected by intercalated ducts (*large arrowheads*). Many cuboidal lining cells have apical secretory granules (*arrows*) and portions of flattened myoepithelial cells on their basal aspects (*small arrowheads*). (Toluidine-blue–stained Epon section, ×550)

with evidence of residual intra- and interlobular ducts (Figure 3).

The ultrastructural cellular alterations occurring in ischemic salivary gland parenchyma should be assessed in relation to normal tissue, particularly the terminal portions of the secretory apparatus. Figure 4 shows a portion of proximal intercalated duct and the zone in which the duct cells were in apposition to acinar cells. The sparse number of electron-dense apical granules and limited amounts of rough endoplasmic reticulum (RER) in intercalated duct cells contrasted with the larger, numerous, electron-lucent secretory granules and prominent basally situated RER of mucinous acinar cells (Figure 4).

In an animal sacrificed 6 days after arterial ligation, by light microscopy (LM), portions of parenchyma from ischemic regions of the submandibular gland consisted of groups of closely associated, glandlike structures, some of which were interconnected (Figure 2). Such structures were clearly derived from acini, since cuboidal to pyramid-shaped cells lined these glands, and the apical regions of some cells retained varying numbers of secretory granules. Narrow portions of cell cytoplasm were evident at the periphery, partially encircling the glands (Figure 2). With the use of transmission electron microscopy (TEM) (Figure 5), the acinar cell composition of these glands were confirmed. Lining cells showed considerable modification with decreased numbers of apical secretory granules and reduced prominence of basal RER when compared with normal acinar cells in nonligated salivary glands (Figure 4). Additional effects of ischemia were evident as widened intercellular spaces and phagocytozed materials within lysosomal vacuoles (Figure 5). Adjacent glands contained lining cells that often showed a complete absence of mucinous or seromucinous droplets and minimal

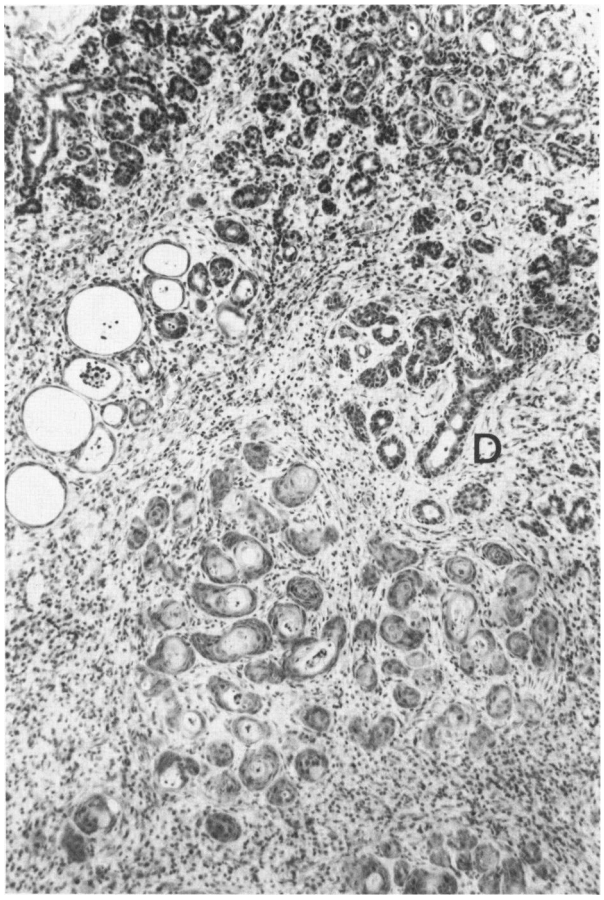


Figure 3—Rat submandibular gland 8 days after arterial ligation. Altered lobules contain portions of dilated and hyperplastic intercalated ducts (*D*) and numerous smaller ducts and tubules showing a gradual transition to zones of frank squamous differentiation. (×110)

cytoplasmic organelles. With both LM and TEM, many such acini had a hyperplastic appearance due to cell crowding and multilayering with resulting obliteration or marked reduction in the size of the lumen (Figure 2).

Light-microscopic examination of a submandibular gland, 8 days after arterial ligation, showed lobular regions, some of which contained numerous small ductlike structures, in addition to obvious squamous metaplasia (Figure 3). Occasional metaplastic cell clusters contained mitotic figures. In a region with a gradual transition to squamous metaplasia, small ductlike structures were evident ultrastructurally as acinar–intercalated duct cell complexes surrounded by hyperplastic myoepitheliumlike cells and infiltrated by inflammatory cells (Figure 6). Acinar cells showed decreased numbers of secretory granules, and persisting granules were more electron-dense than those from control glands; RER was also reduced to varying degrees (Figure 6). When compared with normal intercalated duct cells by TEM (Figure 4), some intercalated duct cells in ischemic glands showed little or no morphologic alteration (Figure 6). Acinar–intercalated duct cell complexes were partially or completely surrounded by myoepithelial cells, many of which had decreased numbers of myofilaments (Figure 6). In the complex illustrated, there was little evidence of squamous differentiation, but other acini and intercalated ducts had occasional cells with increased numbers of tonofilament bundles.

In the zones of developing squamous metaplasia illustrated in Figure 3, acinar cell characteristics were no longer identifiable by TEM, and most of the cell clusters resembled hyperplastic intercalated ducts (Figures 7 and 8). Central, more electron-lucent duct epithelial cells (Figures 7 and 8), sometimes surrounding a persisting lumen (Figure 8), were partially (Figure 7), or completely (Figure 8) surrounded by darker staining myoepithelium. These latter cells often retained certain cytoplasmic features such as filaments (6–9 nm in diameter), some of which were associated with fusiform densities, plasma membrane-associated dense plaques, pinocytotic vesicles, and hemidesmosomes (Figure 8), indicating their myoepithelial cell origin. The cytoplasmic alterations associated with squamous metaplasia were evident in both duct luminal epithelial and myoepithelial cells. Figure 7 shows the increasing accumulation of tonofilaments and cell degeneration associated with keratinization occurring in luminal epithelial cells, while Figure 8 shows the acquisition of tonofilament bundles principally by hyperplastic myoepithelium.

Squamous metaplasia was also observed to occur in residual striated and interlobular excretory ducts as early as 3 days post-arterial ligation. However, squamous metaplasia never developed in these ducts to the extent observed in modified acini and intercalated ducts.

Immunohistochemistry

In normal salivary gland, antibodies to ECK and MBCK demonstrated complementary patterns (Table 1). The ECK antibody stained myoepithelial cells of acini and intercalated ducts, and some basally situated cells of interlobular ducts of normal salivary glands, while the MBCK antibody stained the luminal cells of acini and all ducts, but not the myoepithelial cells of acini. With antibodies to ECK and MBCK, the staining patterns of residual acini and ducts were basically unaltered in salivary glands following arterial ligation. The aggregates of small ducts developing in ischemic regions (Figure 3) retained the staining pattern seen in the normal tissue with both antibodies.

With the alteration to squamous metaplasia in ischemic and infarcted glandular tissue at 6 and 8 days following vascular ligation, both antisera produced staining patterns different from normal tissue (Table 1).

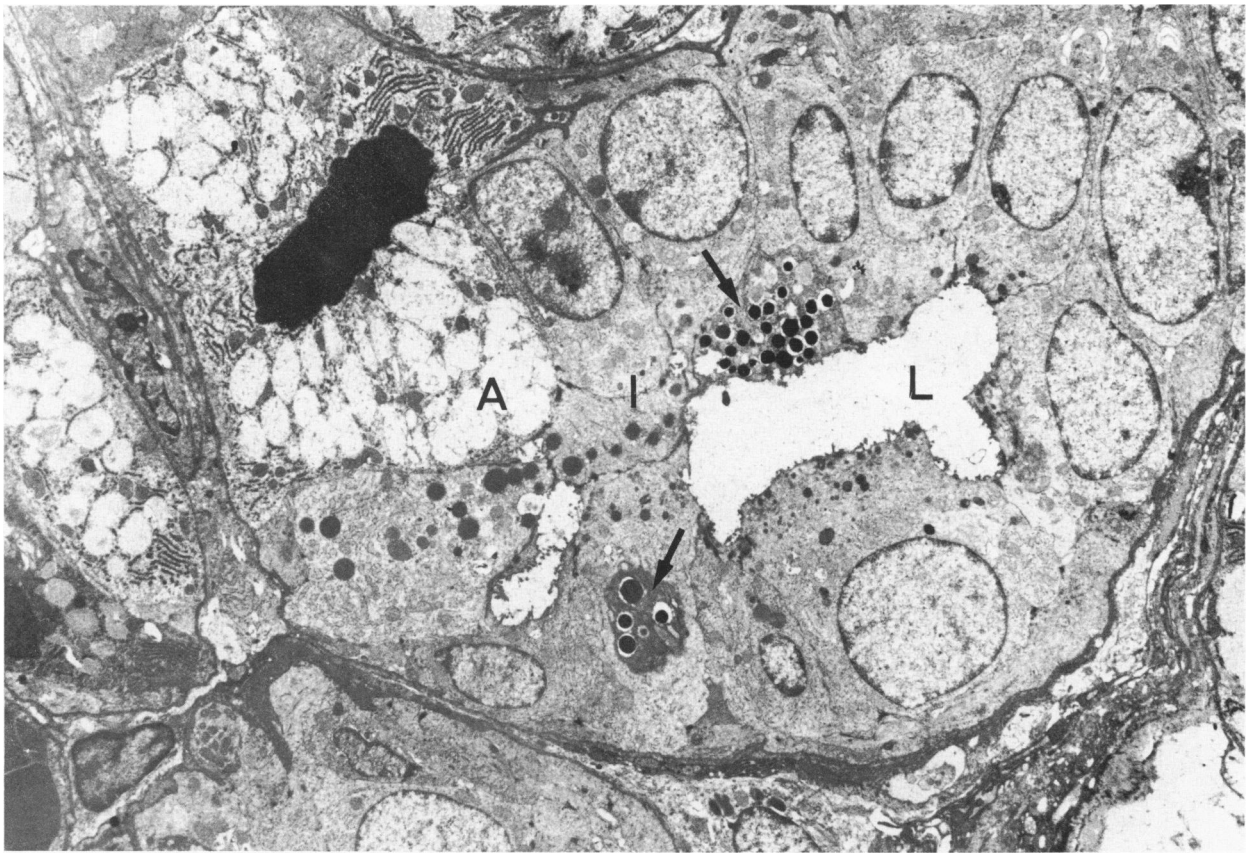


Figure 4—Ultrastructural features of normal rat submandibular gland at the interface of acinar (*A*) and intercalated duct cells (*I*). Intercalated duct cells have scanty, small, densely staining apical granules and limited RER. The *arrows* denote apical regions of a granular cell recently identified in this region.[36] Intercalated duct lumen (*L*). (×3700)

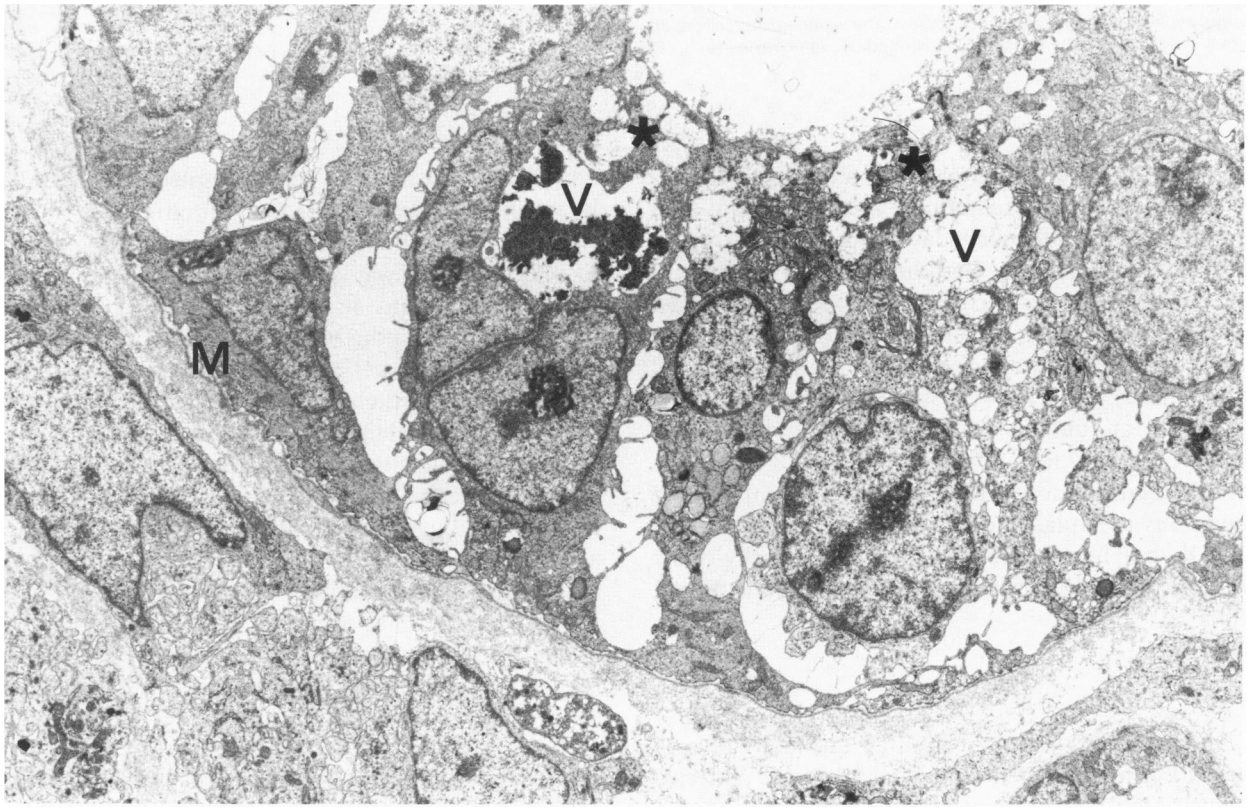


Figure 5—Electron micrograph of a portion of the tissue represented in Figure 2. Modified mucinous acinar cells have markedly reduced apical secretory granules (*asterisks*), depleted RER, and prominent lysosomal vacuoles (*V*). Acinar cells are separated by widened intercellular spaces but retain an association with a myoepithelial cell (*M*). (×4600)

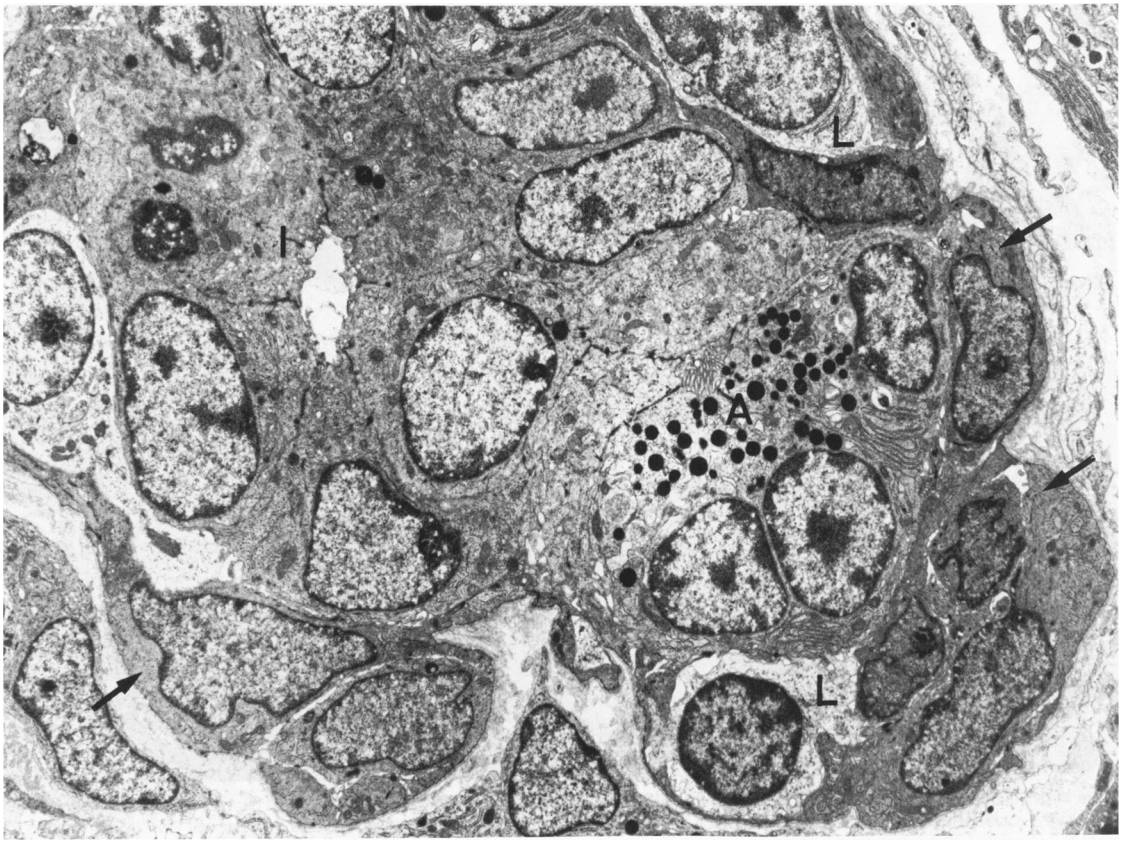


Figure 6—Electron micrograph of a small ductlike structure in Figure 3 reveals that it is composed of modified acinar cells (*A*), intercalated duct cells (*I*), and hyperplastic myoepithelium (*arrows*). The widened intercellular spaces evident in the 6-day sample (Figure 5), presumably due to ischemia, have largely resolved, but the complex is infiltrated by lymphocytes (*L*). (×3900)

Antibody to ECK stained both centrally and peripherally oriented cells in squamous nests at 6 days but only luminal and central cells 8 days after-arterial ligation (Figure 9 and Table 1). Peripheral cells in squamoid nests, identified ultrastructurally as modified hyperplastic myoepithelial cells, were unstained (Figure 9). With antibody to MBCK, only a proportion of central cells in squamous cell nests were stained at 6 days after arterial ligation, but as in normal acini and ducts, cells on the periphery remained unstained. At 8 days after arterial ligation, none of the aggregates composed of squamous metaplastic cells stained with antibody to MBCK, but luminal epithelial cells of nearby ducts continued to stain with this antibody (Figure 10 and Table 1).

Discussion

Salivary-gland tumors exhibit an unusually diverse range of cellular differentiation and histologic patterns. The variability of these neoplasms provides a unique opportunity for research into the molecular and cellular mechanisms responsible for their ultimate form and tumor differentiation in general. The experimental protocol of this study was designed for investigation of cellular modifications in salivary gland parenchyma, resulting in squamous metaplasia.

A number of experimental studies have shown the efficiency with which squamous metaplasia can be induced by arterial ligation[13,14] and carcinogenic compounds[15] in rat salivary gland. After arterial ligation, squamous metaplasia was consistently evident 5–6 days later,[13,14] and in one case was even apparent at 3 days after ligation.[15] After the implantation of dimethylbenzanthracene into salivary gland parenchyma, squamous metaplasia has occurred as early as 4 days.[31] In human minor salivary glands, squamous metaplasia has been noted 1 week after completion of a fractionated dose of irradiation administered over a 23–28-day period.[4]

In terms of the histogenesis and the development of certain histologic features of human salivary gland

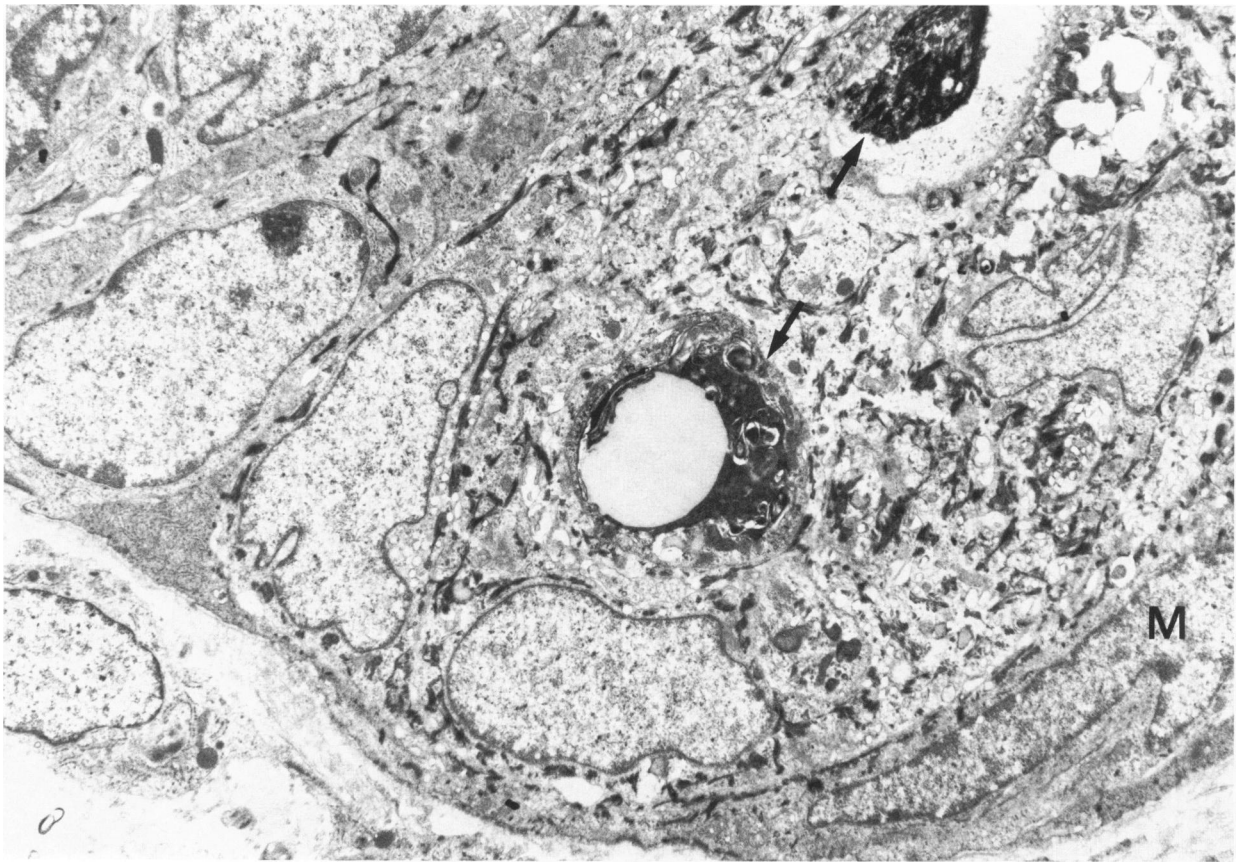


Figure 7—Some cell clusters undergoing squamous metaplasia in the 8-day sample (Figure 3) have modified luminal cells (containing numerous tonofilament bundles) and degenerating (keratinized) cells (*arrows*), partially enclosed by myoepithelial cells (*M*). (×6000)

tumors, it is important to determine the glandular structures responsible for squamous metaplasia. Standish and Shafer[14] stated that metaplasia and hyperplasia following salivary gland ischemia occurred in ductal epithelium, but the specific region of the ductal system involved was not identified. Ductal epithelium was also implicated in the development of squamous metaplasia associated with experimental carcinogenesis.[13,15] In two studies, one utilizing coal tar derivatives[24] and the other radiation,[4] metaplasia was observed originating in acini as well as ducts of salivary gland. In the development of squamous metaplasia, it is the possible role of the acinus and/or its associated intercalated duct that requires verification, particularly in view of suggestions that neoplastic alterations in the excretory duct are central in the histogenesis of mucoepidermoid carcinoma.[5,25]

It is significant that the rapid atrophy of acini associated with salivary duct ligation is accompanied by an equally prompt increase in the number of ductlike structures present in the residual glandular tissue.[14] Experimentally induced adenocarcinomas of rat pancreas have been noted to involve initial stages of acinar cell dedifferentiation and a subsequent modification of acinar units to tubular and ductulelike structures.[26] Resulting histologic features in these pancreatic tumors closely resemble the "small" ductlike complexes induced by ischemia in the current study (Figures 1–3) and as reported by others.[13,14] The number of "small" ducts and tubules in ischemic regions of salivary glands would seem to be too numerous to have resulted simply from persisting intra- and interlobular ducts. Furthermore, the peripheral location of squamous metaplasia in infarcted glands (Figure 1) and the lobular arrangement in ischemic glands (Figure 3) mitigate against the metaplastic changes occurring only in the intralobular striated or interlobular excretory ducts. Ultrastructurally, most tubular complexes in ischemic salivary glands are composed of acinar and intercalated duct cells (Figures 5 and 6). This is further evidence that residual striated and interlobular ducts are not the sole source for such tubular structures and that the modified acinus–intercalated duct unit cannot be dismissed as the portion of the gland tissue developing squamous meta-

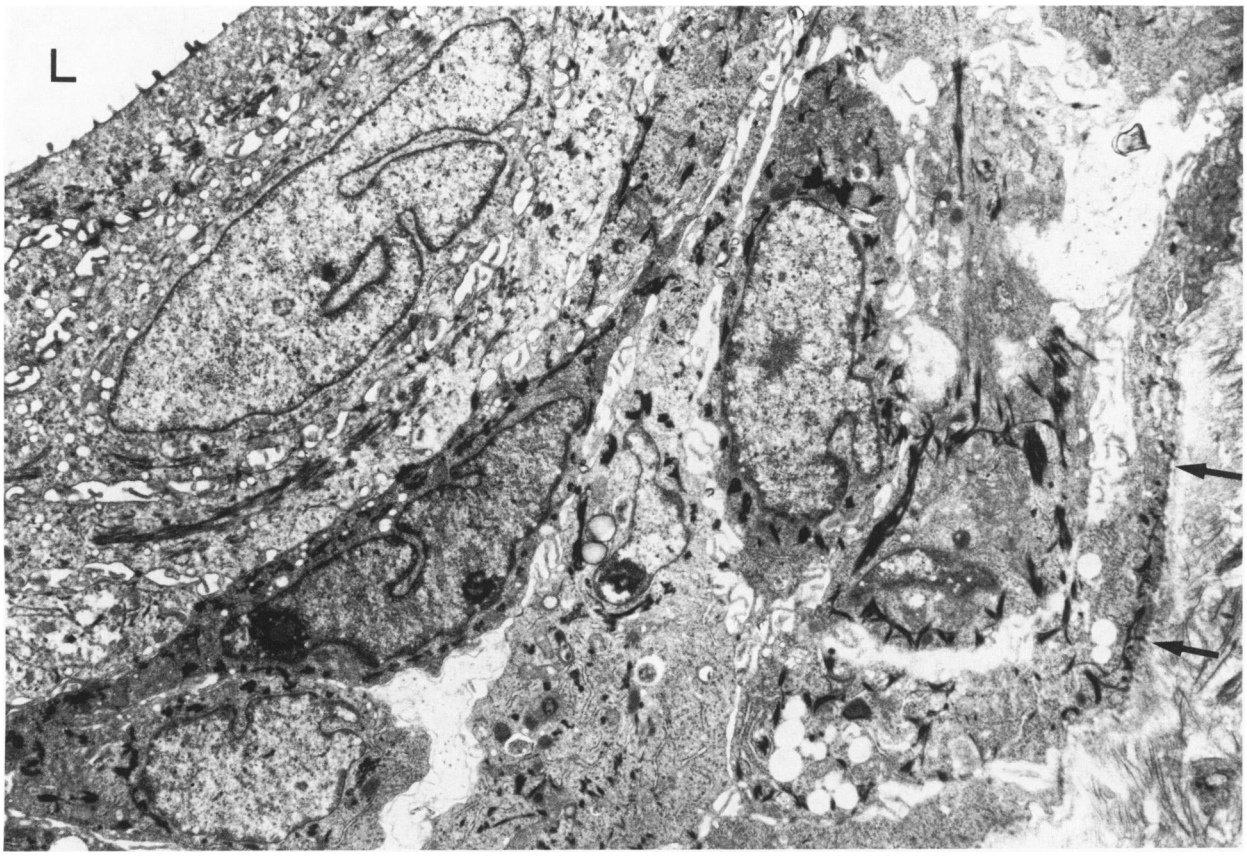


Figure 8—Another cell group from the region of squamous metaplasia in Figure 3 is composed of centrally oriented, flattened, lighter-staining cells, some of which have infrequent short microvilli on the luminal surface (*L*), and peripherally located angular and polygonal electron-dense cells. Both cell types contain tonofilament bundles and are joined by well-formed desmosomes. Basally situated cells are more loosely arranged and have hemidesmosomes (*arrows*) associated with the enclosing basal lamina. (×6700)

plasia. Gradual acinar and myoepithelial cell dedifferentiation is the principal early phase of this modification.

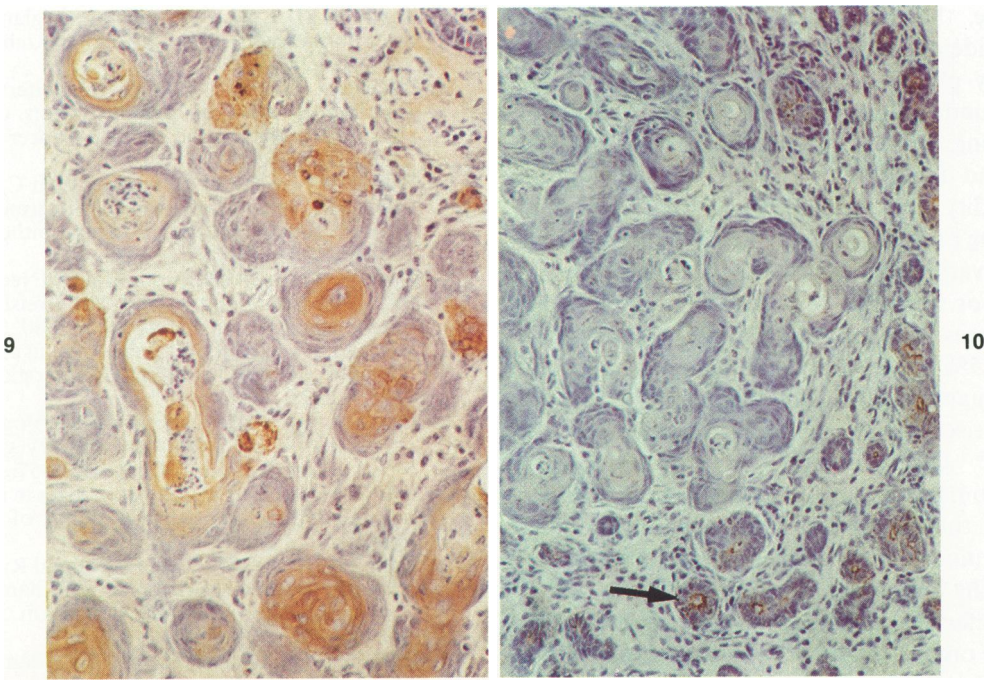
Immunostaining of ischemic salivary gland with antibodies to ECK and MBCK reveals staining patterns of the ductal and tubular structures involved in the early development of squamous metaplasia reflecting that of normal salivary parenchyma, whether acinar or ductular. Similar immunostaining patterns of acini and ducts in rat salivary gland using antibodies to ECK have been reported.[9,10] Intermediate filaments of epidermal cytokeratin-type have been noted in normal salivary gland and squamous cell carcinoma arising in this organ,[9,10] and this finding has been interpreted[9] as support for the contention that mucoepidermoid and squamous cell carcinomas arise from interlobular excretory ducts.[5,25] The current results suggest that no level of the secretory and excretory system can be dismissed as a source for salivary gland tumors with an extensive development of squamous differentiation.

Table 1—Rat Salivary Gland Immunoperoxidase Staining

	ECK	MBCK
Normal gland		
Acini		
Luminal cells	–	+
Myoepithelial cells	+	–
Ducts		
Luminal cells	–	+
Myoepithelial cells	+	+/–
Ligated gland—6 days		
Squamous metaplasia		
Luminal or central cells	+	+/–
Outer cells	+	–
Ligated gland—8 days		
Squamous metaplasia		
Luminal or central cells	+	–
Outer cells	–	–

Note: +/– indicates a proportion of a particular cell type was unstained.

The complexity of the process involved in the acquisition of increased numbers of cytoplasmic cytokeratin intermediate filaments is evident from the immunohistochemical results. In nests of cells exhibiting squamous metaplasia, centrally located cells that show ultrastructural features of a derivation from acinar or duct luminal cells gradually lose their complement of intermediate filaments reactive with antibody to MBCK



9 10

Figure 9—Immunoperoxidase staining of a squamous metaplastic region of rat submandibular gland at 8 days after arterial ligation with anti-ECK antibodies. Central cells are stained, but a ring of peripherally situated cells, corresponding to the myoepitheliumlike cells in Figures 7 and 8, remain unstained. (PAP, counterstained with hematoxylin, ×200) **Figure 10**—Immunoperoxidase staining of same region as in Figure 9 with anti-MBCK. Neither central nor peripheral cells of the squamous metaplastic region are stained. There is positive staining of adjacent ducts (*arrow*). (PAP, counterstained with hematoxylin, ×200)

but acquire those reactive with antibody to ECK. Similarly, ECK antibody staining present in the modified myoepithelial cells of the outer layer of cell groups with squamous metaplasia at 6 days after arterial ligation is absent at 8 days after ligation in similar cells in zones of squamous metaplasia (Table 1). However, it is obvious from electron micrographs (Figures 8 and 9) that modified myoepithelial cells at the periphery of cellular clusters exhibiting squamous metaplasia have extensive accumulations of tonofilament bundles, desmosomes, and intermediate filaments in the 8-day sample. Such a result implies a lack of reactivity of these intermediate filaments with either of the antibodies used in this study.

Biochemical and immunologic studies have revealed the multiplicity and complexity of the cytokeratin polypeptides, their varying expression during epithelial differentiation, and the tissue or cell specificity of the various combinations of polypeptides of the cytokeratin family.[27-31] Particularly relevant to the findings in the present study are the differing set of intermediate filament polypeptides in ductal, myoepithelial, and alveolar secretory cells of normal breast tissue evident with the use of antibodies to human epidermal cytokeratins and mouse liver cytokeratin D[32] or antibody to human epidermal cytokeratin raised in two different animals.[33] Using antibodies to human epidermal and mouse liver cytokeratins, Krepler et al[32] found complementary staining of myoepithelial and proliferating ductal cells in human intraductal carcinoma and heterogeneity of the intraductal tumor cell population with zonal differences in expression of cytokeratin filaments. In the present study, with antibodies to ECK and MBCK, reciprocal staining of luminal and myoepithelial cells is evident in normal salivary gland, and zonal differences are seen in squamous metaplastic salivary gland tissue.

Although formalin fixation may be a reason for differences observed between the staining patterns for the two cytokeratin antibodies,[30] other factors have to be considered. First, failure to demonstrate MBCK in the squamous metaplastic epithelium is unlikely to be due to masking by formalin fixation, because nonmetaplastic epithelium of adjacent ducts in the same sections stained positively (Figure 10). Second, in formalin-fixed material some cytokeratin subtypes can be demonstrated in sections of benign and malignant glandular epithelia but not in squamous epithelium.[17] Antibody to MBCK reacts with cytokeratins of 45 and 53 kd molecular weight, which occur mainly in glandular, but not squamous, epithelium. On this basis, it is not surprising that when squamous differentiation occurs in the salivary gland, antibodies to MBCK do not react with the morphologically altered epithelium.[22]

Furthermore, the ultrastructural and immunohistochemical evidence in the current report shows that as the centrally placed cells derived from luminal epithelial cells undergo squamous metaplasia keratin filaments reacting with ECK antibody become expressed (Figure 9 and Table 1).

The rapidity and ease of the switch in genetic programming of cytokeratin filaments induced by ischemia in salivary gland appears to be reflected in the propensity for some salivary gland tumors to exhibit varying degrees of squamous differentiation. We have noted that 25% of pleomorphic adenomas have some degree of squamous metaplasia[34] and that this process appears to arise largely in relation to modified myoepithelial cells.[6,7] Ultrastructural studies in mucoepidermoid carcinomas have suggested that squamous differentiation in this lesion can occur in either luminal epithelial or modified myoepithelial cells.[8] We have proposed that the development of both of these tumors involves proliferation of luminal and myoepithelial cells, and that the organization of these two types of cell in normal acini and intercalated ducts, though modified and exaggerated, is reflected in the tumors.[6-8,34,35] The marked ability of acinar units in rat salivary gland to undergo squamous metaplasia lends further support to this premise and provides new evidence for common histogenetic pathways in human salivary gland tumors such as pleomorphic adenoma and mucoepidermoid carcinoma.

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Acknowledgments

We thank Dr. H. Yeger of the Hospital for Sick Children, Toronto, Ontario, for performing the immunoblotting and Jean Mattinson for preparation of the manuscript.