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Histochemical studies of intercellular components of salivary gland tumors with special reference to glycosaminoglycan, laminin and vascular elements

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Summary. In 41 salivary gland tumors, the characteristics of the intercellular components and vascular endothelial cells were surveyed by immunohistochmical staining for laminin and factor VIII-related antigen (VIII R:Ag), and by mucopolysaccharidase-digestion for glycosaminoglycan (GAG). In myxomatous areas of pleomorphic adenomas, small vessels (diameter 6.5 ± 0.11 μm) were frequent and found to be negative or weakly positive by VIIIR:Ag staining although endothelial cells were clearly positive for VIIIR:Ag in capsule surrounding the tumor tissues. Alcian blue stainability was diminished by treatment with both Streptomyces hyaluronidase and chondroitinase. By laminin staining, a vascular pattern was clearly detected, but the majority of tumor cells were not stained. In adenomatous areas, the basement membrane-like linear laminin-staining reaction was observed to be weak and inconsistent around some tumor cell nests. However, in adenoid cystic carcinomas, laminin-positivity was much more intense than in other tumors such as pleomorphic adenoma, mucoepidermoid tumor and adenocarcinoma. In cylindromatous areas, the inner luminal surface in the pseudocysts was markedly positive for laminin, and there was weak positivity around tumor cell nests having a trabecular pattern. By immunoelectron microscopy, a juxtacellular network of replicated basal lamina of tumor cells which lined the inner surface of pseudocysts was positive for laminin. Alcian blue-positivity in the pseudocyst was abolished with heparitinase and chondroitinase, but not with hyaluronidase.

Key words: Pleomorphic adenoma – Adenoid cystic carcinoma – Laminin – Factor VIII-related antigen – Glycosaminoglycan

The histogenesis of pleomorphic adenoma, the most common type of salivary gland tumor, has been discussed frequently in the literature. The tumor

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has been considered to consist of epithelial and mesenchymal components, since it is characterized by the appearance of "mesenchymal" mucin. Recent studies showed that this mesenchymal mucin was produced by the epithelial tumor cells, both of ductal and myoepithelial cell origin. Myoepithelial cells have been reported to produce a significant amount of glycosaminoglycan (GAG), and play a role in pleomorphic adenoma (Azzopardi and Smith 1959; Mylius 1960; Hübner et al. 1969, 1971; Takeuchi et al. 1975). Thus, since the intercellular matrix is considered to be mostly derived from the epithelial tumor cells, the purely non-epithelial element is composed of vascular tissues with a few fibroblasts and collagen fibers. Adenoid cystic carcinoma has also been reported to have a ductal cell and myoepithelial cell origin by Hoshino and Yamamoto (1970).

Recently, laminin, one of the glycoproteins serving as an important structural constituent of the basal lamina, has been isolated (Timpl et al. 1979). Immunohistochemically, laminin was demonstrated in the basement membrane of epithelium and in vascular endothelium, peripheral nerves, fat cells and muscles (Foidart et al. 1980). Vascular endothelial cells synthesize and contain factor VIII-related antigen (VIIIR:Ag) (Bloom et al. 1973; Hoyer et al. 1973; Jaffe et al. 1973; Shearn et al. 1977) which can be demonstrated in vascular endothelium by an immunoperoxidase method (Mukai et al. 1980).

In the present study, in order to elucidate the morphological and biological characteristics of the intercellular components of various kinds of salivary gland tumors, we observed the distribution and localization of laminin and VIIIR:Ag by immunoperoxidase histochemistry, and the distribution of GAG-components by mucopolysaccharidase treatment. In some cases, immunoelectron microscopical methods were used. Laminin was most obvious in adenoid cystic carcinoma, and some relationships were observed between GAG-components and laminin-positivity and between GAG-components and VIIIR:Ag-positivity.

Materials and methods

Forty-one salivary gland tumors (22 pleomorphic adenomas, 9 adenoid cystic carcinomas, 5 mucoepidermoid tumors, 4 Warthin's tumors and 1 adenocarcinoma) were examined. Each tumor was fixed in 10% buffered formalin, 80% ethanol or 95% ethanol:glacial acetic acid (99:1, v/v), and embedded in paraffin and sectioned. Histologically, each section was stained by the following dyes: H-E. periodic acid Schiff's reagent, orcein or Alcian blue. In order to detect the glycosaminoglycan components, a digestion test was performed with chondroitinase ABC (Yamagata et al. 1968) (pH 8.0, 10 units/ml, 37° C, 1 h), Streptomyces hyaluronidase (Ohya and Kaneko 1970) (pH 5.0, 100 turbidity reducing units/ml, 37° C, 1 h) or heparitinase (Cifonelli and Dorfman 1970) (pH 8.0, 0.3 units/ml, 37° C, 1 h).

Immunoperoxidase staining for laminin and VIIIR:Ag. Tumor tissue was fixed at 4° C in 95% ethanol:glacial acetic acid (99:1, v/v) (Sainte-Marie 1962), embedded in paraffin and sectioned. The sections 4 μm in thickness were deparaffinized, washed with phosphate buffered saline (PBS, pH 7.6), and depleted of endogenous peroxidase by incubation for 20 min with 3% hydrogen peroxide in methanol at room temperature. The sections were pretreated with crystalline trypsin (Worthington Diagnostic Systems Inc., Cat. No. 3707) at a concentration of 5 μg/ml in PBS for 10 min at 37° C (Albrechtsen et al. 1981), and then processed for indirect

immunoperoxidase staining for laminin. After washing the sections were exposed sequentially to a 1:100 dilution of rabbit antiserum to laminin overnight at 4° C, and to a 1:200 dilution of horseradish peroxidase-labeled IgG-fraction purified from goat antiserum to rabbit IgG (Cappel Lab., Cat. No. 3212-0081) for 40 min at room temperature. The staining was developed by a solution of 0.05% diaminobenzidine tetrahydrochloride (Sigma Chem. Co.) and 0.01% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6. In some cases, the nuclei were counterstained with haematoxylin or methyl green. As a control normal rabbit serum or anti-laminin serum which had been absorbed with mouse laminin was used instead of the primary antiserum. In some cases, tissue sections processed after formalin fixation were also stained for laminin after pretreatment with a 0.4% solution of pepsin (Sigma Chem. Co., Cat. No. P-7012) (Ekblom et al. 1982) in 0.01 N HCl for 2 h at 37° C, but the background staining was often high.

Immunoperoxidase staining for VIIIR:Ag was performed with HISTOSET, immunoperoxidase staining kit (Immulok Inc.) and based on the peroxidase-antiperoxidase (PAP) method (Sternberger 1979). The sections from formalin- or ethanol:glacial acetic acid-fixed tissues were processed for this immunoperoxidase staining. In some cases, the nuclei were counterstained with Mayer's haematoxylin. Some sections were pretreated with 1 mg/ml or 5 μg/ml trypsin for 35 min at 37° C. The prior trypsinization produced a marked improvement in the sensitivity of VIIIR:Ag detection in the tissues as described by Sehested and Hou-Jensen (1981) and McComb et al. (1982).

Some of the fixed tissues were cut on a cryostat (6 μm), and stained for laminin and VIIIR:Ag by the same procedure as above.

Immunoelectron microscopy for laminin. Fresh samples were fixed in periodate-lysine-paraformaldehyde fixative for 16–18 h at 4° C on a shaker, washed overnight with 3 changes of 10% sucrose in PBS, and embedded in O.C.T. (Ames Co.) and quickly frozen in dry-ice acetone. Six μm thick sections cut on a cryostat were mounted on albumin-coated glass slides and stained by the indirect immunoperoxidase technique. After washing with PBS, the sections were incubated for 12 h with a 1:100 dilution of rabbit antiserum to laminin at 4° C. After washing with PBS, the sections were incubated for 40 min with a 1:200 dilution of horseradish peroxidase-labeled IgG-fraction purified from goat antiserum to rabbit IgG at room temperature, and then washed in PBS. The sections were incubated for 30 min in Karnovsky's diaminobenzidine solution without H_2O_2 and then incubated for 5 min in complete Karnovsky's solution containing H_2O_2 (0.01%) (Graham and Karnovsky 1966). The sections were washed in PBS and post-fixed with 2% OsO_4 for 1 h. After dehydration in graded ethanol solutions, the sections were embedded in EPON 812 by the inverted capsule method. Ultrathin sections were cut with LKB ultramicrotome, and unstained sections were examined with a Hitachi H600 electron microscope.

Preparation of antiserum to laminin. An anti-mouse laminin antiserum prepared by immunizing a rabbit against the commercial laminin (BRL, Cat. No. 6260 LA) isolated from an EHS sarcoma and commercial rabbit antiserum to laminin (EY Lab. Inc., Cat. No. AT-2404) were used. The purity of laminin was evaluated by SDS-polyacrylamide gel electrophoresis, and both of the antisera gave a single band on Ouchterlony diffusion against laminin and the same staining reaction. Albrechtsen et al. (1981) demonstrated that either anti-rat or anti-mouse laminin antiserum had a cross-reactivity with human laminin.

Results

Non-neoplastic salivary gland tissues

By immunoperoxidase staining for laminin, basement membranes outlining the ducts and acini were delineated. Blood vessels, fat cells, peripheral nerves and muscles were also positive for laminin. By immunoperoxidase staining for VIIIR:Ag, vascular endothelial cells were positive in salivary gland tissues.

Pleomorphic adenoma

Pleomorphic adenomas consist of many areas showing the following patterns: 1) myxomatous area: the tumors are composed of islands and strands of stellate and spindle cells in a loose myxoid matrix which contains a large amount of GAG, 2) chondromatous area: a pseudocartilaginous element is observed within the tumor stroma, 3) solid cellular area: the tumor is composed mainly of a massive growth of myoepithel-like cells which are elongated and spindle-shaped with eosinophilic fibrillar cytoplasm, 4) adenomatous area: the tumor shows a distinct glandular pattern, 5) hyalinized area: hyaline material stained with eosin is observed in the intercellular mucous matrices, and 6) highly hyalinized area: the tumor is mainly composed of hyaline material which are intensely stained with eosin.

Myxomatous areas. Intercellular matrices stained with Alcian blue were abundant and the vascular pattern was clearly detected by laminin staining (Fig. 1), but the majority of the tumor cells which were sparsely distributed in this area were not stained. In some areas, basement membranes outlining the small nest of the tumor cells were stained, but the stainability was weak and discontinuous (Fig. 2). The vessels located in this area had a narrow lumen measuring about 6.5 μm on average as shown in Table 1. The diameter of vessels in this area was much smaller than in other hyalinized or highly hyalinized areas. By VIIIR:Ag staining, the capillary endothelial cells in this area were weakly positive or negative, though the vessels in the capsule surrounding the myxomatous components were clearly positive as shown in Fig. 3. The stainability for laminin and VIIIR:Ag was observed to be relatively stronger in the frozen sections than in the embedded ones, but the endothelial cells in the myxomatous areas were weakly positive or negative for VIIIR:Ag even in the frozen sections. The stromal components were intensely stained with Alcian blue, and the stainability of matrices in which the msall vessels were distributed was abolished by treatment with hyaluronidase and chondroitinase ABC.

Chondromatous areas. Stromal matrices were intensely stained with Alcian blue, which were abolished with chondroitinase ABC, but not with hyaluronidase. By staining for laminin, this area was negative and vessels were very few.

Solid cellular areas (myoepitheliomatous areas). Myoepithel-like cells which were elongated and spindle-shaped with an eosinophilic fibrillar cytoplasm showed a solid pattern. In some portions, hyaline and fibrillary materials were observed among the spindle cells showing a palisading pattern, where orcein staining was strongly positive. In these neurinoma-like areas, laminin was hardly detected, though perivascular areas were weakly positive for laminin. The blood vessels located in this area were weakly positive for VIIIR:Ag.

Adenomatous areas. Basement membrane-like linear laminin staining around the tumor cell nests was weak. Vessel walls were laminin and VIIIR:Ag-positive.

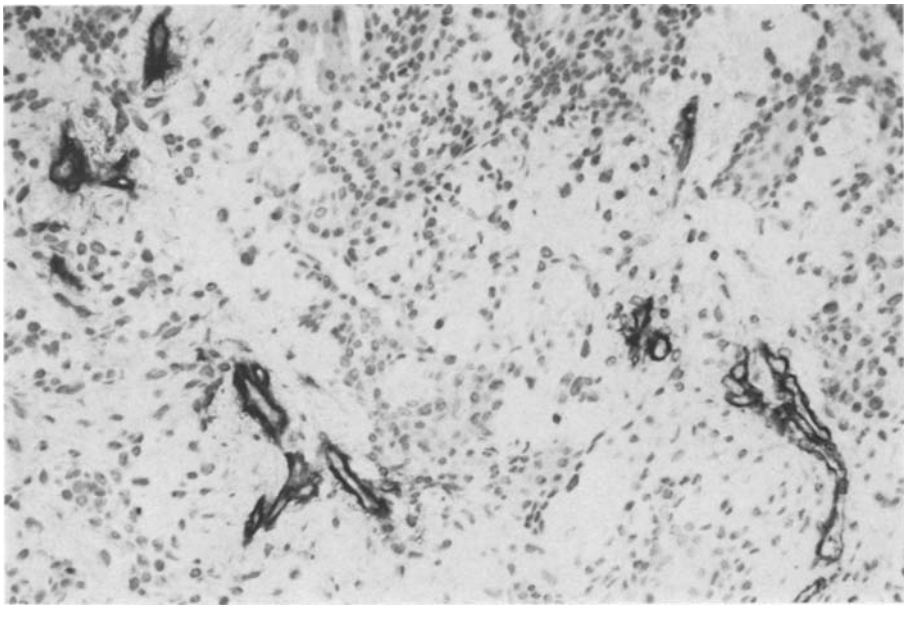


Fig. 1. Microscopic section of a myxomatous area in the pleomorphic adenoma tissue. A vascular pattern is clearly detected. (Immunoperoxidase staining for laminin with haematoxylin counterstaining, 150:1)

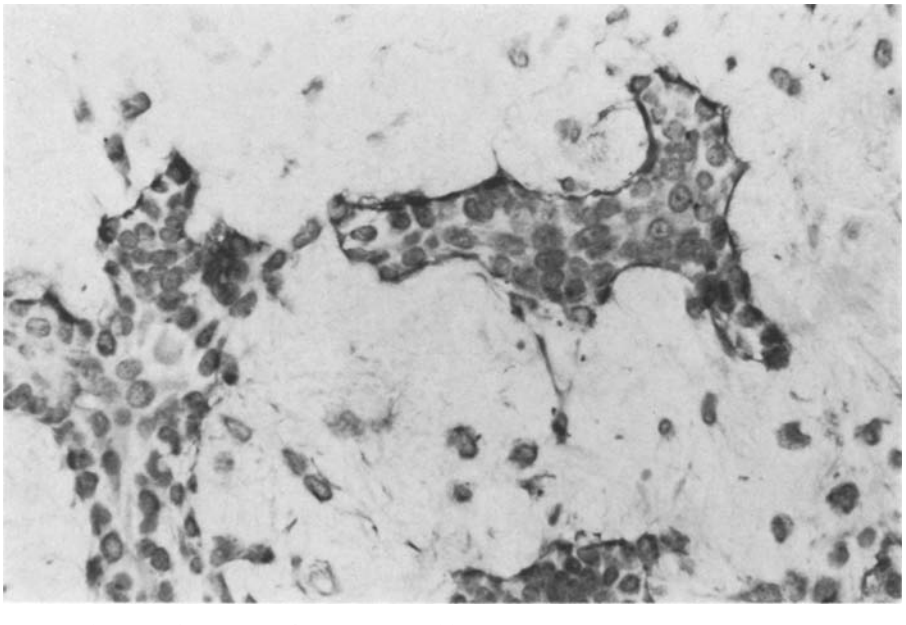


Fig. 2. Microscopic section of the pleomorphic adenoma tissue, showing a discontinuous and weak basement membrane-like linear positivity for laminin around the small nest of tumor cells in myxomatous area (Immunoperoxidase technique with haematoxylin counterstaining, 300:1)

Table 1. Diameter of blood vessel lumen in salivary gland tumors

	No. of vessels measured	Diameter (μm) Mean ± SE	P*
Pleomorphic adenoma			
myxomatous area	1069	6.5 ± 0.11	$P<0.001$
hyalinized area	1066	15.0 ± 0.58	$P<0.001$
highly hyalinized area	280	19.0 ± 1.12	$P<0.001$
chondromatous area	105	21.7 ± 1.69	$P<0.001$
Adenoid cystic carcinoma			
cylindromatous area	221	10.0 ± 0.67	$P<0.001$
trabecular area	160	18.8 ± 0.69	$P<0.001$

P* Statistical significance of difference in size of diameter of vessels between myxomatous areas and other areas. The evaluation was based on Student's t-test

Micrographs of histological sections were taken, and the maximal transverse diameter of blood vessel was measured in each area of tumor tissues

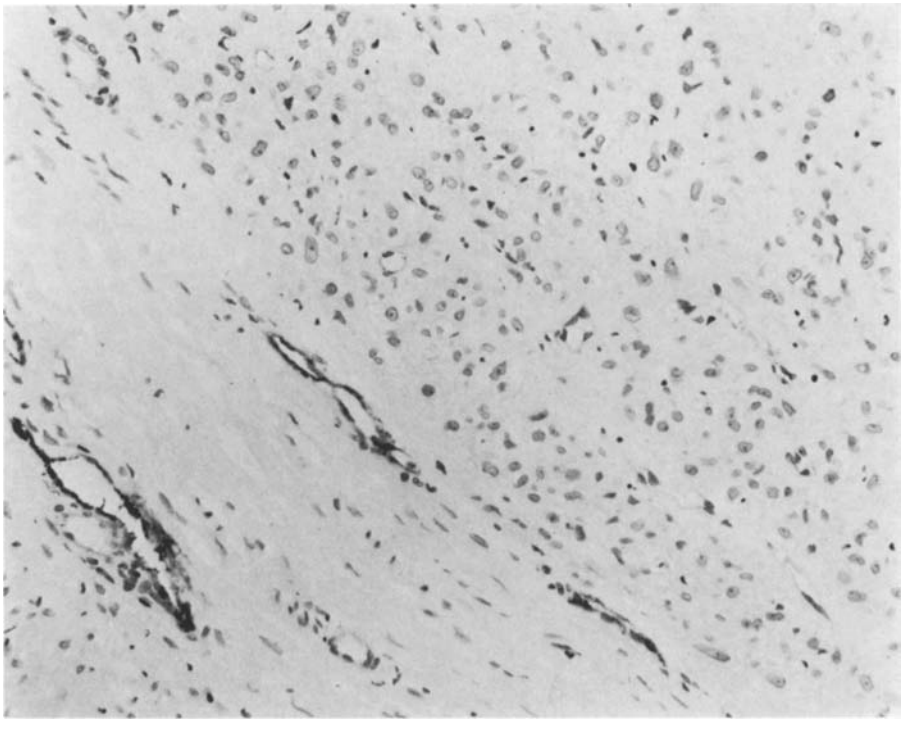
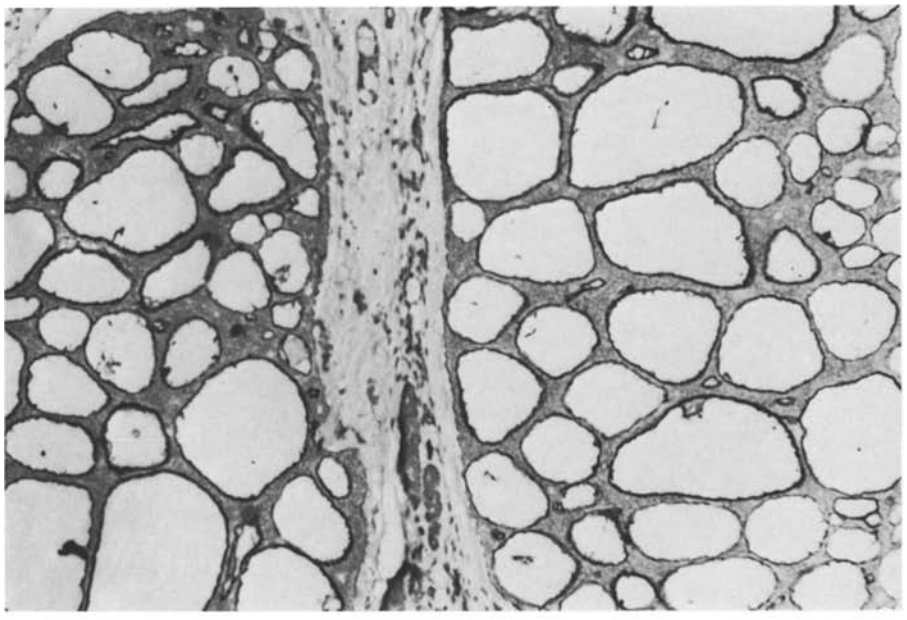


Fig. 3. Immunoperoxidase staining for VIIIR:Ag in the pleomorphic adenoma. The capillary endothelial cells in myxomatous area are negative, but the vascular endothelial cells in capsule are clearly positive. (Counterstained with haematoxylin, 150:1)



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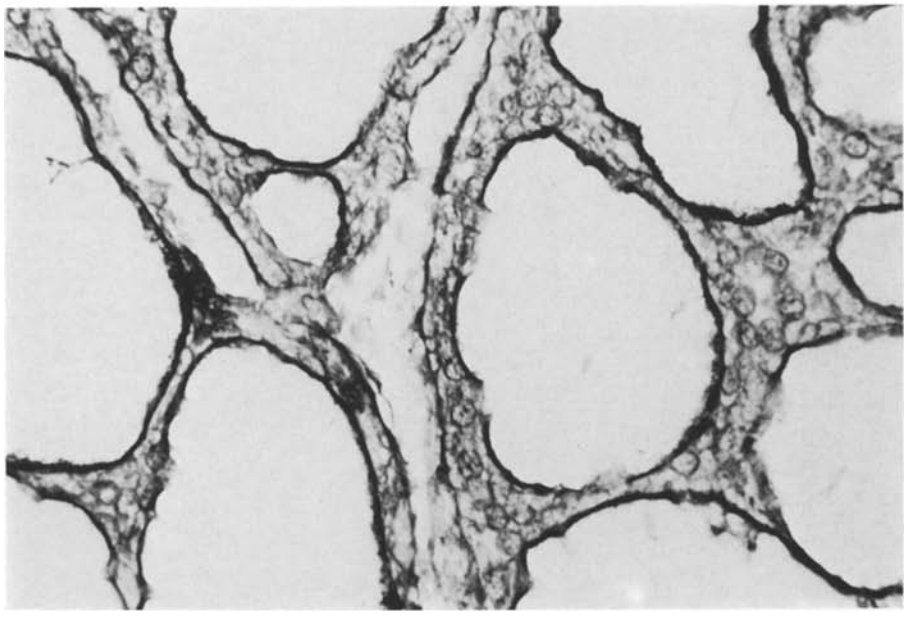

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Figs. 4 and 5. Microscopic section of the adenoid cystic carcinoma tissue, showing an intense linear positivity for laminin at the inner lumen surface in pseudocyst. A basement membrane-like linear positivity outlining the tumor cell nest is also observed in Fig. 5, but it is weak. (Immunoperoxidase technique with haematoxylin counterstaining, Fig. 4. 75:1, Fig. 5. 300:1)

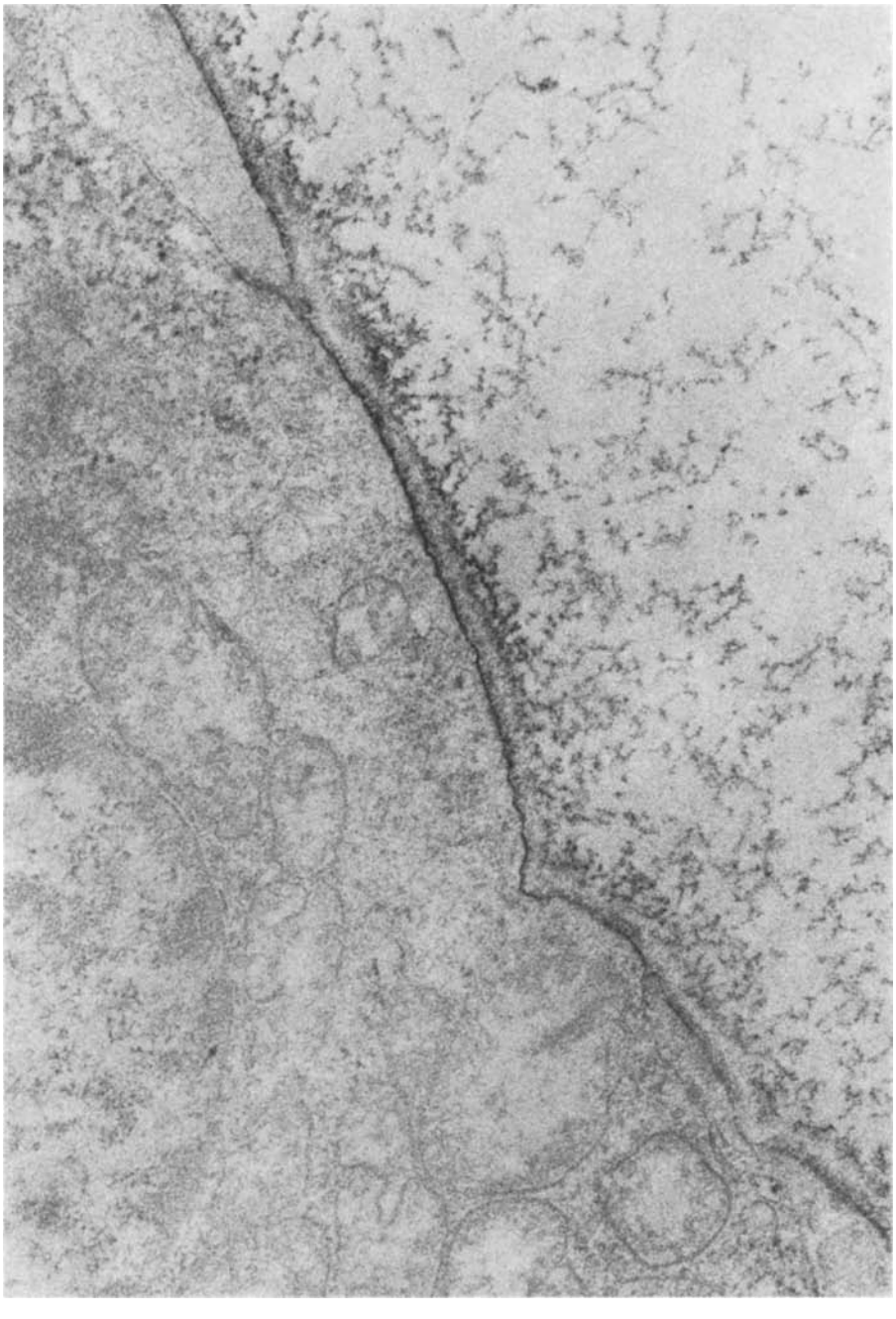

Fig. 6. Immunoelectron micrograph for laminin at the luminal surface of the tumor cells lining a pseudocyst in cylindromatous area of adenoid cystic carcinoma. A juxtacellular zone of a network of replicated basal lamina is clearly positive. (5,000:1)

Adenoid cystic carcinoma

In the tumor tissues, a cylindromatous area and an area showing trabecular pattern are observable.

In cylindromatous areas, the inner surface of lumen in pseudocyst was markedly positive for laminin as shown in Figs. 4 and 5. By immunoelectron microscopy, a clearly positive stain for laminin was observed in the juxtacellular zone of the tumor cells lining the inner surface of the pseudocysts (Fig. 6). In the lumen of pseudocysts, Alcian blue-positive material was abundant, and it was removed with chondroitinase ABC and heparitinase, but not with hyaluronidase. Basement membrane-like linear staining for laminin was also observed around the tumor cell nests having a cribriform or trabecular pattern, but it was weak and discontinuous. Vascular endothelial cells distributed in the interstitial element were positive for VIIIR:Ag in any areas of adenoid cystic carcinomas.

In one case of adenoid cystic carcinoma, many oncocytic cells were observed. Around the oncocytoma-like cell nests and at the luminal surface of the lining oncocytic cells, basement membrane-like linear staining for laminin was clearly observed. This positive staining was more intense than in the adenoid cystic carcinoma.

Other tumors

Warthin's tumor. Beneath the double-layered epithelium, a distinct basement membrane-like linear stain for laminin was observed. In the lymphoid element, many vessels showed laminin-positivity, and all these endothelial cells were positive for VIIIR:Ag.

Mucoepidermoid tumor. The stromal component was composed of a large amount of fibrous or hyalinized connective tissue. The basement membrane-like linear positivity for laminin was observed to be inconsistent around the tumor cell nests, though the vessel walls and peripheral nerves were clearly positive. VIIIR:Ag was hardly detected in the vascular endothelial cells surrounding the tumor cell nests.

Adenocarcinoma. Tumor cells showed a solid pattern. The center of the tumor cell nest sometimes exhibited small pseudotubular or rosette-like formations. The stroma was narrow and fibrous. Around the tumor cell nest, basement membrane-like linear staining for laminin was observed, though it was weak. Vascular endothelial cells showed VIIIR:Ag-positivity.

Discussion

Recently, the morphological and biological characteristics of salivary gland tumor cells have been studied by immunohistochemical methods. Caselitz et al. (1980, 1981, 1981, 1982 and 1982) reported the localization of actin, keratin, prekeratin, vimentin, desmin, carcinoembryonic antigen (CEA) and lactoferrin. McDicken (1981) reported the distribution of CEA in tumors

of human minor salivary glands. Hara et al. (1983) and Nakajima et al. (1982) noted the presence of S-100 protein in the various kinds of salivary gland tumors. The present study showed the localization and distribution of laminin and VIIIR:Ag in the neoplastic and non-neoplastic salivary gland tissues by an immunohistochemical methods. The present results indicated that laminin-positivity was most clearly observed in adenoid cystic carcinomas. The inner surface of pseudocysts in cylindromatous areas were intensely positive, and basement membrane-like linear positivity was also noted around the tumor cell nests showing a cribriform or solid pattern, however weak and inconsistent. Tandler (1971) found by electron microscopical studies of adenoid cystic carcinoma that the cyst-like formations in the acellular areas of the tumor are not glandular lumina; instead, they represent an extracellular compartment lined by highly replicated basal laminae, which correspond to the hyaline-like material seen by light microscopy. He also indicated that foci of concentrically arrayed microfibrils are frequently observable within interstices of the multilaminar basement membrane, precipitated mucin being abundant in the extensive intercellular spaces, in the lumina, and in the cyst-like formations. Chen (1976) showed electron microscopically three readily recognizable zones in the cystic spaces of adenoid cystic carcinomas: a juxtacellular zone of a network of replicated basal lamina, an intermediate zone of stellate granules of mucoid material, and a central core of densely packed periodic filaments. Electron microscopy in the present study revealed laminin-positivity in the juxtacellular zone. The present result also showed that Alcian blue-positive material within the lumen of pseudocyst was abolished with heparitinase and chondroitinase ABC, but not with hyaluronidase. This suggests that the mucinous material within the lumen of pseudocyst consists mainly of heparan sulfate and chondroitin sulfate. Laminin is known to be cross linked by disulfid bonds, and to form a heavily cross-linked structural framework within basement membranes (Chung et al. 1979). Sakashita et al. (1980) found that laminin binds to heparan sulfate and heparin. It was quite conceivable that the mucinous material containing heparitinase-sensitive substance has an intimate relation with the localization of laminin which was clearly detected in the present study. It is not clear whether synthesis and secretion of laminin are enhanced by a heparan sulfate-rich matrix or whether laminin synthesized by the tumor cells is linked by heparan sulfate in matrices and thereby remains there.

The present results also indicated that, in myxomatous areas of pleomorphic adenomas, endothelial cells had much narrower vascular lumina than in the other areas and/or in other types of tumors. They were surrounded by hyaluronate-rich matrices, which were abolished with hyaluronidase. By staining for VIIIR:Ag, they were weakly positive or negative even in the frozen sections. It was noted that VIIIR:Ag is a reliable marker for well-differentiated endothelial cells (McComb et al. 1982). Sehested and Hou-Jensen (1981) suggested that endothelial cells might lose their ability to produce VIIIR:Ag with an increasing level of dedifferentiation. It is known that a hyaluronate-rich extracellular matrix is suitable for cell migration and proliferation, preventing differentiation (Tool 1982). In the myxo-

matous areas containing mainly hyaluronic acid, the endothelial cells of small vessels may have a proliferating tendency. Takeuchi et al. (1976) demonstrated a very large amount of GAG consisting mainly of hyaluronic acid in the intercellular matrices of intracanalicular fibroadenoma of breast where capillary endothelial cells were distributed. Prodi and Romeo (1967) observed histochemically that hyaluronate was the main component of intercellular matrix in the early proliferative stage of granulomatous tissue. Thus, the finding of the capillary endothelial cells in myxomatous area showing weakly positive or negative for VIIIR:Ag may reflect their unidfferentiated state. We tried to perform the pretreatment with mucopolysaccharidase, but no enhancement of stainability for VIIIR:Ag was observed in any myxomatous areas as well as in the other areas. Therefore, the diminished stainability for VIIIR:Ag of the endothelial cells in the myxomatous area may not be due to masking of the antigenic determinants of VIIIR:Ag by a large amount of GAG in the intercellular matrices.

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