Iron-Binding Proteins in Adenoid Cystic Carcinoma of Salivary Glands : An Immunohistochemical Study

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TAKAHASHI, H., FUJITA, S., TSUDA, N., TEZUKA, F. and OKABE, H. *Iron-Binding Proteins in Adenoid Cystic Carcinoma of Salivary Glands : An Immunohistochemical Study*. Tohoku J. Exp. Med., 1991, 163 (1), 1-16 — An immunoperoxidase staining technique was used for detecting three major iron-binding proteins (transferrin, ferritin and lactoferrin) in 54 adenoid cystic carcinomas (ACCs) of major and minor salivary glands. Twenty-three normal salivary glands were investigated for comparison. Transferrin staining was detected mainly in the intercalated duct and serous acinar cells, and was found inconsistently in the myoepithelial cells surrounding normal ductules. Ferritin was always absent in the normal epithelial component of salivary gland. The presence of lactoferrin was demonstrated in the serous acinar cells and intercalated duct cells of normal salivary tissues. Five histological patterns were found in ACC, and for the cellular components of each, it was possible to establish a special immunohistochemical profile. Transferrin positivity was detected in the small angular cells of 25 cases (48%), in the duct luminal cells of 19 cases (37%) and in the hyalinized stroma of 4 cases (8%). Ferritin staining was positive in the small angular cells of 23 cases (44%) and in the duct luminal cells of 15 cases (29%). Lactoferrin was detected in only duct luminal cells of 38 ACCs (73%). The comparative immunohistochemical analysis between transferrin and ferritin showed a similar distribution in this carcinoma. On the basis of the immunohistochemical data, lactoferrin might be used as a marker of glandular differentiation. — adenoid cystic carcinoma ; salivary gland ; iron-binding proteins ; immunohistochemistry

Adenoid cystic carcinoma (ACC) is regarded as a specific variant of adenocarcinoma of salivary glands due to its characteristic histologic appearance. Histologically, cellular components of this neoplasm have been subdivided as follows : inner and outer tubular cells in the tubular pattern, duct luminal cells and

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layers against stroma in the trabecular pattern, duct luminal, small angular and "cyst"-lining cells in the cribriform pattern, and duct luminal and small angular cells in the solid pattern (Takahashi et al. 1990). Two main cell types are small angular cells and duct lining cells. The small angular cells are arranged in nests or sheets that are fenestrated by round or oval spaces, creating the characteristic "cribriform" fashion. Occasionally, the tumors have a predominantly solid cellular growth with a basaloid appearance that has few, if any fenestrations. Tubular structures with minimal stratification of the lining epithelium are often mixed with the classic cribriform and solid areas.

Transferrin, ferritin and lactoferrin are the three major iron-binding proteins in humans. By immunohistochemistry, the distribution of these iron-binding proteins has been extensively investigated in normal human tissues (Mason and Taylor 1978). In neoplastic conditions, the presence of transferrin, ferritin and lactoferrin has been studied in breast carcinomas (Rossiello et al. 1984) and thyroid tumors (Barresi and Tuccari 1987). Lactoferrin has been demonstrated in well differentiated carcinomas of the parotid gland (Caselitz et al. 1981a). Furthermore, transferrin and ferritin have been reported in benign salivary gland tumors (Sehested et al. 1985; Takahashi et al. 1988). More recently Caselitz et al. (1986b) have documented the presence of many markers including iron-binding proteins in ACC. However, detailed studies of their presence in ACC of salivary gland have not been undertaken.

The localization of iron-binding proteins in neoplastic cells appears to be very intriguing, particularly since transferrin is considered an obligate requirement for growing cells (Rossiello et al. 1984). In the current study, we aimed to define the immunohistochemical distribution of three iron-binding proteins in different cell types of a large number of ACCs. The results demonstrate that cellular components of ACC not only differ in morphology, but can also be distinguished by different immunohistochemical properties.

MATERIALS AND METHODS

Specimens from 54 cases of ACC were retrieved from the files of the Pathology Division, Central Diagnostic Laboratory, Nagasaki University Hospital, Nagasaki (26 cases) and Department of Pathology, Tohoku University Hospital, Miyagi (28 cases). These included tumors arising in parotid glands (11 cases), submandibular glands (10 cases), sublingual glands (2 cases) and minor salivary glands (31 cases). Twenty-three normal salivary glands (3 parotid and 20 submandibular glands) were obtained from radical neck dissections for non-salivary gland neoplasms. All tissues were fixed in 10% buffered formalin, and embedded in paraffin after dehydration. Specimens were cut at 3.5 μ m thickness for use in the detection of transferrin, ferritin and lactoferrin as well as for staining with hematoxylin-eosin (H & E), periodic acid-Schiff (PAS) and alcian blue.

The sections were treated in 0.3% hydrogen peroxide/methanol solution for 30 min to block the intrinsic peroxidase activity, and rinsed well in phosphate-buffered saline (PBS). They were then immersed in normal swine serum (1:20) for 30 min to prevent the non-specific adherence of serum proteins, incubated with rabbit anti-human transferrin (dilution 1:400), ferritin (dilution 1:200) or lactoferrin (dilution 1:200) (purchased from Dakopatts,

Copenhagen, Denmark) as first layer for 1 hr, and rinsed three times in PBS, 5 min each time. The sections were next incubated with swine anti-rabbit IgG (1 : 20) as second layer for 30 min, and rinsed three times with PBS. Sections were finally treated with peroxidase anti-rabbit peroxidase complexes (1 : 25) for 30 min, and rinsed with PBS. For the demonstration of peroxidase activity the sections were incubated in darkness for 10 min with 0.03% 3-3' diaminobenzidine hydrochloride (DAB) containing 0.005% hydrogen peroxide. Sections were counterstained with Mayer's hematoxylin and mounted in permount.

To test the specificity of transferrin, ferritin and lactoferrin stainings, each specific antiserum was replaced by either PBS, normal rabbit serum or absorbed with excess of purified human transferrin, human ferritin and human lactoferrin (Sigma Chemical Co., St. Louis, MO, USA). The results obtained were negative.

To evaluate the immunohistochemical profiles of each histologic variant, the 54 ACCs were categorized into five growth patterns: tubular, trabecular, cribriform, solid and hyalinized stromal patterns. In each growth pattern certain tumor cell types were examined: inner and outer tubular cells in the tubular pattern; duct luminal cells and layers against stroma in the trabecular pattern; duct luminal cells, "cyst"-lining cells and small angular cells in the cribriform pattern; and duct luminal cells and small angular cells in the solid pattern.

RESULTS

Light microscopic appearance of ACC

We recognized the following five histologic patterns in our series: tubular, trabecular, cribriform, solid and hyalinized stromal patterns. At least two of these components in variable proportions were present in each of our cases. The most characteristic pattern, the cribriform fashion, was found in 51 cases, but in varying amounts (Fig. 1a). In the area of this pattern, two types of lumen were distinguished those lined with "cyst"-lining cells and those lined with duct luminal cells. Tumor cells except before mentioned two kinds of cells were small angular cells. Occasionally, portions of an ACC showed a tubular growth pattern in which there are two distinct rows of tumor cells composed of inner and outer tubular cells (Fig. 1b). This pattern was found in 32 cases. The trabecular pattern was observed in 35 cases. This pattern is characterized by cords of polygonal cells forming central ducts and of short spindle cell layers against stroma (Fig. 1c). When cut longitudinally, tubular structures appear as long relatively thin tubules. Evans and Cruickshank (1970) have illustrated this pattern and use the term "trabecular" which we believe is also acceptable. In the solid pattern, the individual units were packed with small angular cells and only a few lumina were seen (Fig. 1d). Hyalinized stromal tissue was found in cribriform and trabecular patterns. In the cribriform pattern, some of the pseudocystic spaces contain eosinophilic material and may communicate with the surrounding connective tissue. The histological appearance of the trabecular pattern was characterized by broad hyaline ribbons of connective tissue between tumor parenchyma.

Immunohistochemical findings in normal salivary glands

The immunostaining results obtained with antibodies for transferrin, ferritin and lactoferrin in normal salivary gland are summarized in Table 1. Cytoplasmic immunostaining with transferrin was present in the flattened or triangular cells at

TABLE 1. *Distribution of transferrin, ferritin and lactoferrin in normal salivary gland*

Cellular component	Immunoperoxidase antibody		
	Transferrin	Ferritin	Lactoferrin
Myoepithelial cell	+	—	—
Acinar cell			
Mucous acinar cell	—	—	—
Serous acinar cell	+	—	+
Duct epithelial cell			
Intercalated duct cell	+	—	+
Striated duct cell	—	—	—
Interlobular duct cell	—	—	—

TABLE 2. *Distribution of transferrin, ferritin and lactoferrin in adenoid cystic carcinoma*

Component of ACC	Number of cases	Positive case of immunostainings		
		Transferrin	Ferritin	Lactoferrin
Tubular pattern	32			
Inner tubular cell		19 (59 %)	15 (47 %)	31 (97 %)
Outer tubular cell		16 (50 %)	13 (41 %)	0 (0 %)
Secretory product		7 (22 %)	5 (16 %)	20 (63 %)
Trabecular pattern	35			
Duct luminal cell		19 (54 %)	13 (37 %)	30 (86 %)
Layers against stroma		23 (66 %)	18 (51 %)	0 (0 %)
Secretory product		2 (6 %)	2 (6 %)	9 (26 %)
Cribriform pattern	51			
Duct luminal cell		19 (37 %)	10 (20 %)	38 (75 %)
Small angular cell		25 (49 %)	23 (45 %)	0 (0 %)
Cyst-lining cell		5 (10 %)	17 (33 %)	2 (4 %)
Secretory product		8 (16 %)	11 (22 %)	11 (22 %)
Solid pattern	13			
Duct luminal cell		1 (8 %)	1 (8 %)	3 (23 %)
Small angular cell		8 (62 %)	4 (31 %)	0 (0 %)
Hyalinized stromal pattern	27	4 (15 %)	0 (0 %)	0 (0 %)

the periphery of acini, which correspond to myoepithelial cells. In addition, strong transferrin positivity was observed in the intercalated duct and serous acinar cells (Fig. 2). Ferritin was demonstrated in only the fibroblasts and macrophages of the stroma. No staining for ferritin was noted in the acinar and ductal epithelial cells. Lactoferrin showed strong positivity in the serous acinar cells and moderately strong positivity in the intercalated duct cells (Fig. 3).

Immunohistochemical findings in ACC

Immunohistochemical results of ACC are summarized in Table 2. Five histologic patterns — tubular, trabecular, cribriform, solid and hyalinized stromal patterns — were observed.

Tubular pattern. Tubular patterns were found in 32 of the 54 cases. The immunohistochemical reactivity of inner and outer tubular cells was positive for transferrin (Fig. 4) and for ferritin (Fig. 5) in nearly half of 32 cases. Anti-lactoferrin antiserum strongly stained in inner tubular cells (Fig. 6) and luminal secretion.

Trabecular pattern. Trabecular patterns were found in 35 (65%) of the 54 cases. Duct luminal cells were positive for lactoferrin (Fig. 7) in 30 cases (86%), and those in 19 cases (54%) were positive for transferrin. In the layers against stroma, positive staining for transferrin (Fig. 8) was found in 23 (66%), and the reaction with ferritin (Fig. 9) was observed in 18 (51%). Lactoferrin was not found in this cell type.

Cribriform pattern. This histological pattern was found in 51 (94%) of the 54 ACCs. Small angular cells were positive for transferrin and ferritin in about one-half of these 51 cases. Lactoferrin was not identified in the small angular cells in areas showing the cribriform pattern. On the other hand, duct luminal cells were strongly positive for lactoferrin in 38 cases (75%). The frequency of positive staining for transferrin and ferritin in duct luminal cells and "cyst"-lining cells (Fig. 10) was lower than that in small angular cells.

Solid pattern. This pattern was found in 13 (24%) of the 54 cases and small angular cells predominated. Small angular cells were reactive to anti-transferrin antiserum in 8 (62%) of these 13 cases, and were stained with ferritin (Fig. 11) in 4 cases (31%). Duct luminal cells in the tubular structures were positive for transferrin, ferritin and lactoferrin only in a few cases.

Hyalinized stromal pattern. This pattern was recognized in 27 (50%) of the 54 cases. The hyalinized stromal matrix of these lesions were positive for transferrin in four (15%) of these 27 cases. Whereas ferritin and lactoferrin were always negative.

DISCUSSION

The "histogenesis" or cellular differentiation of ACC has been studied extensively by light and electron microscopy (Chomette et al. 1982; Orenstein et al.

1985; Chaudhry et al. 1986). With respect to histogenesis, two main cell types have been suggested: myoepithelial and undifferentiated duct cells (Eneroth et al. 1968; Hamperl 1970). The ultrastructural investigations, however, were limited in terms of helping to define cellular differentiation because it was difficult to separate various classes of intermediate filaments (Chaudhry et al. 1986). In order to supplement the ultrastructural findings, investigators made several attempts at immunohistochemical characterization of ACC and of normal salivary gland tissue by using antibodies against various intermediate filaments (Caselitz et al. 1981b; Seifert and Caselitz 1983; Caselitz et al. 1986a; Azumi and Battifora 1987), S-100 protein (Nakazato et al. 1985; Azumi and Battifora 1987), and other molecular markers (Caselitz et al. 1982; Seifert and Caselitz 1983; Nakazato et al. 1985; Caselitz et al. 1986a; d'Ardenne et al. 1986; Azumi and Battifora 1987).

In recent years, using immunoperoxidase procedures, the presence of three iron-binding proteins has been studied in normal salivary gland. Transferrin was found in the serous acinar cells, intercalated duct cells and periductular myoepithelial cells (Takahashi et al. 1988). Lactoferrin was seen in some acinar cells and intercalated duct cells (Caselitz et al. 1981a, b; Sehested et al. 1985). On the other hand, ferritin was not detected in all the epithelial component of normal salivary gland (Sehested et al. 1985). Metalloproteins, such as iron-binding proteins, have been considered as tumor markers (Seifert 1985). In the salivary gland neoplasia, lactoferrin has been detected in the glandular differentiated tumors (Caselitz et al. 1982). Transferrin has been studied in the adenolymphoma (Takahashi et al. 1988), and ferritin was found in both epithelial and mesenchymal components of pleomorphic adenoma (Sehested et al. 1985).

In the present study, secretory markers such as lactoferrin were found in ductal structures of the areas with cribriform and solid growth patterns. These structures appear to be true lumina previously defined ultrastructurally (Tandler 1971). Therefore, these findings indicate that some neoplastic cells in ACC undergo ductal differentiation. In areas showing the tubular pattern the inner tubular cells also expressed lactoferrin, and this finding is similar to duct luminal cells in areas with the cribriform and solid growth patterns. Orenstein and colleagues (1985) reported the fusion of pseudocysts and true lumina was occasionally observed. Most pseudocysts are larger than true lumina, but lumina may occasionally dilate to the size of small pseudocysts (Dardick 1985). Caselitz et al. (1986b) reported that anti-lactoferrin antiserum was not identified in the pseudocysts. Our study showed that the material in pseudocyst-like spaces stained with anti-lactoferrin antiserum. These findings can be explained by dilation of true lumina or by draining of true lumina into pseudocysts after the fusion of both spaces. In salivary gland tumors, lactoferrin has been considered the most important immunohistochemical marker of "glandular" differentiation (Caselitz et al. 1981a; Sehested et al. 1985; Seifert 1985). In fact, the absence of

lactoferrin is a constant finding in small angular cells of ACC, whereas a variously represented positivity is encountered in duct luminal cells as well as in their secretory products.

True lumina were stained not only by anti-lactoferrin antibody but also by anti-transferrin antibody. Caselitz and coworkers (1986b) also reported transferrin to be present in a layer of "cuboidal cells" and in pseudocystic lining cells but not at the periphery of tumor nests. The "cuboidal cells" in their study were duct luminal cells by our criteria. In the present study, transferrin was also found in small angular cells in 25 of the 51 cases and in "cyst"-lining cells in five of the 51 cases with a cribriform pattern. In the tubular pattern areas, transferrin was predominantly present in inner tubular cells. Outer tubular cells stained occasionally with anti-transferrin antiserum. Our results showed transferrin was present in small angular cells. However, this finding conflicts with prior observation that transferrin was negative in myoepithelial cells of tubular, trabecular and cribriform patterns (Caselitz et al. 1986b). The biological significance of the presence of transferrin in ACC remains unexplained. It is noteworthy that transferrin has a high affinity for iron (Birgens 1984). Neoplastic cells require increased amounts of iron for their replication (Gatter et al. 1983). However, it has been reported that iron is crucial for initiating and maintaining DNA synthesis (Robbins and Pederson 1970). Therefore, in our opinion, neoplastic cells of ACCs produce transferrin in order to have a greater availability of intracellular iron for their turnover.

Ferritin reacted with "cyst"-lining cells in 17 of the 51 cases with the cribriform pattern, and with small angular cells in 23 of the 51 tumors with a cribriform pattern. In addition, tubular pattern demonstrated ferritin positivity in the inner and outer tubular cells. Ferritin was always absent in normal salivary gland, whereas this antibody was observed in half of cases with ACCs. The positive immunohistochemical reaction for ferritin allows us to show iron storage in neoplastic cells (Arosio et al. 1976).

In summary, our immunohistochemical data suggest that iron-binding proteins such as transferrin, ferritin and lactoferrin is produced or stored in the neoplastic cells of ACC. Transferrin and ferritin have wide distribution, and the comparative immunohistochemical analysis between both proteins showed a similar distribution in duct luminal, "cyst"-lining and small angular cells. Lactoferrin may be useful as additional marker for localizing tubuloglandular element in neoplastic microfoci.

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[*Illustrations follow*]

Fig. 1. Histology of adenoid cystic carcinoma. a : The classic cribriform pattern of ACC, demonstrating the characteristic “swiss cheese” appearance. H & E, $\times 83$. b : The tubular pattern of ACC, demonstrating the tubular structures with inner and outer tubular cells. H & E, $\times 132$. c : The trabecular pattern of ACC, demonstrating the epithelial strands surrounded by hyaline stroma. H & E, $\times 132$. d : The solid pattern of ACC, demonstrating the packed proliferation of small angular cells. H & E, $\times 100$.

- Fig. 2. Transferrin in normal salivary gland. Positively stained intercalated duct cells, serous acinar cells and myoepithelial cell (arrow) are seen. PAP-hematoxylin, $\times 200$.
- Fig. 3. Lactoferrin in normal salivary gland. Positively stained acini and intercalated ducts are seen. PAP-hematoxylin, $\times 100$.
- Fig. 4. Transferrin in adenoid cystic carcinoma. Inner and outer tubular cells in an area showing a tubuloglandular component display positive staining. PAP-hematoxylin, $\times 100$.
- Fig. 5. Ferritin in adenoid cystic carcinoma. Outer tubular cells in an area showing a tubuloglandular pattern display marked staining. PAP-hematoxylin, $\times 132$.

- Fig. 6. Lactoferrin in adenoid cystic carcinoma. Inner tubular cells of tubuloglandular structure show intensive staining. PAP-hematoxylin, $\times 200$.
- Fig. 7. Lactoferrin in adenoid cystic carcinoma. Lactoferrin positive cells are present in duct luminal cells of trabecular structure. PAP-hematoxylin, $\times 66$.
- Fig. 8. Transferrin in adenoid cystic carcinoma. Strongly stained tumor cells are layered against stroma in the trabecular area. PAP-hematoxylin, $\times 60$.
- Fig. 9. Ferritin in adenoid cystic carcinoma. Ferritin positive cells are arranged at the periphery of trabecular structure. PAP-hematoxylin, $\times 135$.

Fig. 10. Ferritin in adenoid cystic carcinoma. Positively stained "cyst"-lining cells are seen in an area showing a cribriform pattern. PAP-hematoxylin, $\times 200$.

Fig. 11. Ferritin in adenoid cystic carcinoma. Cell layers bordering the stroma in an area with small solid pattern. PAP-hematoxylin, $\times 270$.