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Immunohistochemical Localization of Estradiol, Progesterone, and Progesterone Receptor in Human Salivary Glands and Salivary Adenoid Cystic Carcinomas

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ABSTRACT. **Immunohistochemical analyses of estradiol, progesterone and progesterone receptor were carried out in human salivary gland and salivary adenoid cystic carcinoma. Immunoreactivity to estradiol and progesterone was found in cytoplasm of the cells of the excretory duct system within normal salivary glands, whereas the progesterone receptor was restricted to nuclei of the cells where both sex steroids were positive. In addition, we demonstrated the presence of both sex steroids and the receptor for progesterone in salivary adenoid cystic carcinomas. These data indicate that the human salivary gland is one of the target tissues of estrogen. This also suggests the good possibility that tumors which express progesterone receptors will respond to endocrine therapy.**

Human saliva contains the steroid hormones estrogen and progesterone, which provide a basis for radioimmunoassay for the different types of salivary steroids (9, 18, 20). However, there is no evidence presently available to indicate the direct action of sex steroid hormones on human salivary glands or salivary gland cancer. In addition, it is of great importance to know the exact localization of the two types of sex steroid hormones to understand the function and metabolic properties of normal and tumor cells; however, there has been no previous attempt to directly identify estradiol and progesterone in microscopic sections of normal and neoplastic salivary glands. Kurman *et al.* (8) have studied the steroid localization in sertoli-leydig tumors of the ovary and testis. Mercer *et al.* (10) also immunocytochemically detected the steroid hormones in breast cancer cells. Using these techniques, we recently demonstrated in rats that progesterone receptors are present in cell nuclei of the intralobular duct system within submandibular glands (14). These findings suggest that the immunohistochemical technique, using specific antibodies against estradiol and progesterone or these receptors, makes possible investigation of this subject in the hormone-dependent tissues and tumors.

Steroid hormone action on the hormone-dependent tissues and tumors requires the presence of the hormone receptor to mediate transcriptional activation (1). Using monoclonal antibodies, Perrot-Applanat *et al.* (15) and Hyde *et al.* (5) have devised immunohistochemical methods for studying the progesterone receptor in sections of paraffin-embedded tissue. Perrot-Applanat *et al.* (16) have further examined the immunocytochemical localization of progesterone receptors in frozen sections from human breast tumors with a monoclonal antibody to this receptor and demonstrated that the progesterone receptors are accumulated in tumor cell nuclei.

In this article we describe our results which demonstrate estradiol, progesterone and progesterone receptors in routine paraffin sections of human salivary glands and salivary adenoid cystic carcinomas, using an indirect immunoperoxidase technique.

MATERIALS AND METHODS

The following specimens were examined: 5 cases of primary adenoid cystic carcinoma and 6 specimens of normal salivary gland tissue (2 submandibular glands, 2 parotid glands and 2 minor salivary glands of the palate). The 5 patients included a 42-year-old man and 4 postmenopausal women, whose age at first diagnosis ranged from 61 to 82 (average 68) years (Table I).

All samples were fixed in 3.7% buffered formalin (in 0.1 M sodium phosphate buffer, pH 7.2) for 24 hrs, and embedded in paraffin for diagnostic surgical pathology. In addition to routine hematoxylin and eosin stains, sections from representative paraffin blocks of each tumor were deparaffinized and analyzed for the presence of estradiol, progesterone, and progesterone receptors using the indirect immunoperoxidase antibody method (11, 14). The primary antibodies used were polyclonal anti-estradiol antibody (Nichirei Co. Ltd., Tokyo),

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polyclonal anti-progesterone antibody (BioGenex Lab., San Ramon, California) and mPRI (Transbio, Paris), a monoclonal antibody to the human progesterone receptor.

For estradiol and progesterone localization, the deparaffinized sections were incubated with 3% H_2O_2 for 10 min, washed in three changes of 0.15 M (sodium chloride in 0.01 M phosphate buffer, pH 7.4) phosphate-buffered saline (PBS) for 5 min, and then were treated with 4% normal goat serum for 20 min at room temperature. Alternate sections were incubated with either anti-estradiol or anti-progesterone antiserum overnight at 4°C. Polyclonal antiserum to esteradiol and progesterone were used at the recommended dilutions. The sections were washed in PBS and then incubated for 30 min at room temperature with secondary antiserum (peroxidase-conjugated goat anti-rabbit immunoglobulins, IMT) diluted 1 : 100 in PBS.

Progesterone receptor localization was examined as described previously (14). Sections were deparaffinized and treated with 0.01 M sodium metaperiodate in 0.05 M Tris-buffered saline (TBS, pH 7.6) for 15 min and 1% sodium borohydride (in TBS) for 10 min, to remove residual aldehyde. The sections were then permeated with 1% Triton X-100 for 5 min, followed by 10 min in 5% dimethylsulfoxide (both in TBS). After washing with TBS, the preparation was incubated for 20 min in 1% hydrogen peroxide containing 20% normal goat serum and 1% bovine serum albumin (in TBS) to eliminate endogenous peroxidase activity and minimize nonspecific binding. They were then incubated overnight at 4°C with anti-progesterone receptor antibody, at a concentration of 15 μg/ml in TBS containing 0.5% Triton X-100, 0.1% gelatin and 0.02% sodium azide. After washing with TBS, sections were incubated for 30 min at room temperature with peroxidase-conjugated goat anti-mouse serum (Antibodies) diluted 1 : 25 in TBS.

Finally, all specimens were incubated for 10 min in diaminobenzidine (0.05% in TBS) with 0.01% hydrogen peroxide, counterstained lightly with Mayer's hematoxylin, dehydrated, and mounted in permount for light microscopic examination.

Negative control staining was also carried out by the following three methods: (i) omission of primary antiserum; (ii) substitution of primary antiserum with non-immune rabbit or non-immune mouse immunoglobulin; and (iii) use of preincubating the primary antiserum with a 10-fold excess of its respective antigenic peptide (estradiol-17, Sigma, St. Louis; progesterone, Wako, Osaka) for 12 hrs at 4°C. No immunopositive staining was noted.

Table I. RESULTS OF IMMUNOSTAINING IN 5 ADENOID CYSTIC CARCINOMAS.

Case no	Patient Age/Sex	Site of tumor	Antigen E_2	Antigen P	Antigen PR
1	44/male	Gingiva	++	+	+
2	61/female	Palate	+++	+	++
3	65/female	Palate	++	++	+++
4	65/female	Tongue	+	−	−
5	82/female	Gingiva	+	+	+

E_2, Estradiol; P, Progesterone; PR, Progesterone receptor. +++: more than half of tumor cells are positive. ++: less than half of tumor cells are positive. +: less than 10% of tumor cells are positive. −: no positive cells are visualized.

RESULTS

Normal salivary glands. Immunoperoxidase reaction product for estradiol was found uniformly throughout the cytoplasm of excretory, striated, and intercalated duct cells of normal salivary glands and was not observed in serous and mucinous acini (Fig. 1a). The myoepthelial cells surrounding acini and ducts were consistently negative.

The distribution pattern of immunoreactivity to progesterone closely paralleled that of estradiol (Fig. 1b). Immunoreactivity for progesterone receptors was restricted to the nuclei of some epithelial cells which correspond to the estradiol and progesterone positive cells (Fig. 1c). They were located chiefly in the distal portion of ducts, and occasionally, acinar cells were also positive. The myoepithelial cells were negative for progesterone receptor.

Adenoid cystic carcinomas. All five salivary neoplasmas were classical adenoid cystic carcinomas (Fig. 2a). The most typical diagnostic feature, the cribriform pattern, was found in every tumor, but the foci of tubular, trabecular and solid patterns were always associated with various proportions.

The results of immunohistochemical staining of the adenoid cystic carcinomas are summarized in Table 1. Similar to the normal salivary glands, estradiol (Figs. 2b and 2c) and progesterone immunoreactivity (Fig. 2d) in the tumors examined were found uniformly in the cytoplasm of the tumor cells, and the positive reaction for progesterone receptor was restricted to the nucleus of tumor epithelial cells (Figs. 3a–3d).

As shown in Table 1, estradiol was identified in all instances of the adenoid cystic carcinomas examined. In case 2 more than half of tumor cells were positive (+++), in cases 1 and 3 positive cells were found in 50–10% of cells (++), and in cases 4–5 less than 10% of cells (+) were positive. For progesterone, case 3 was two plus (++) and cases 1, 2 and 5 one plus (+). Immunoreactivity for progesterone receptors was present in four instances, showing three plus in one tumor (case 3), two plus in one (case 2) and one plus in two (cases 1 and 5). In case 4, the immunoreactivity to esteradiol was obvious in some tumor cells (one plus), but none of the cells was reactive for progesterone and progesterone receptor.

Some variation in distribution and intensity of staining among cells and different areas of the same section were a frequent occurrence, as seen in Figs. 2b–2d. The

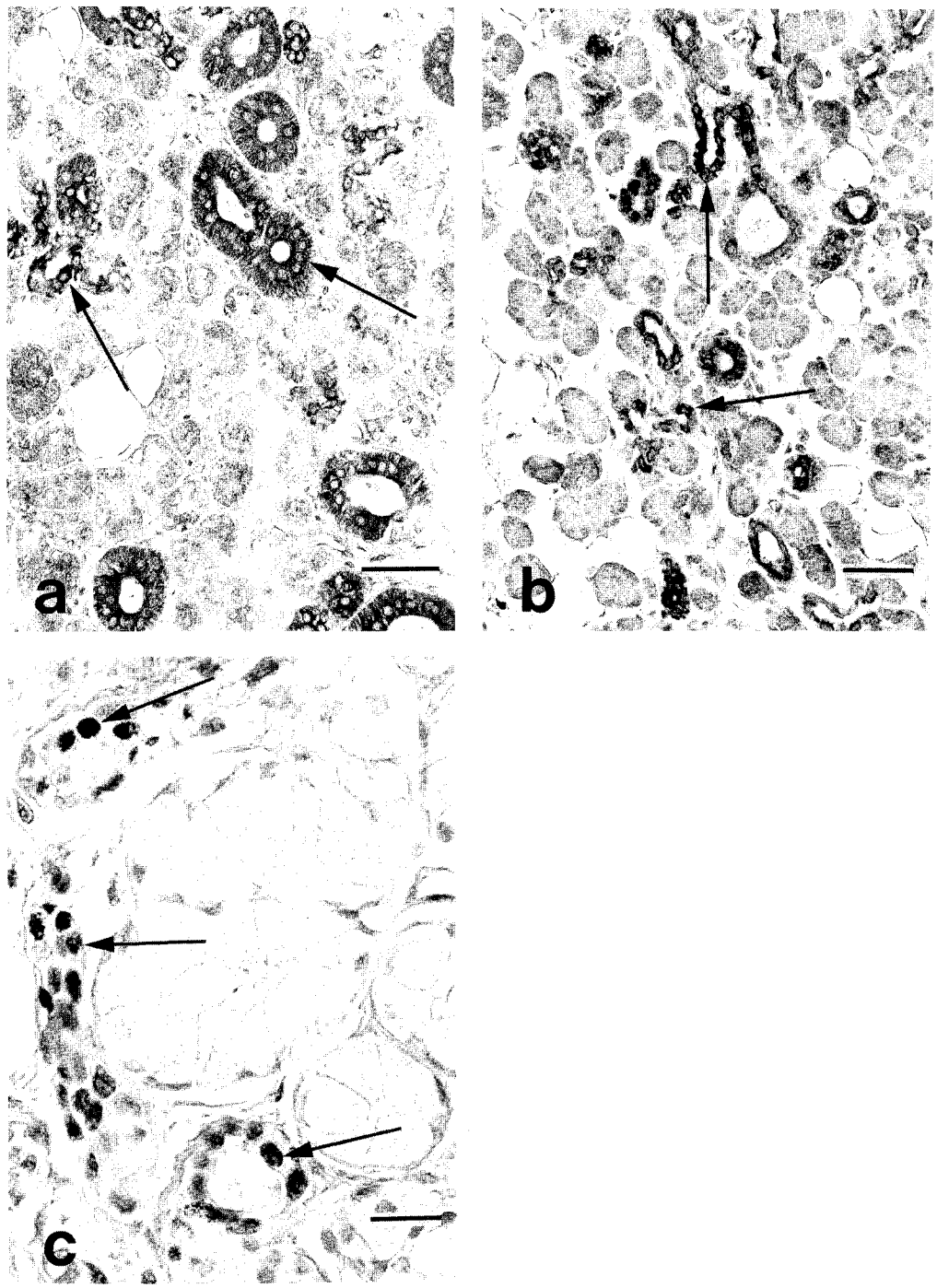

Fig. 1. Immunoperoxidase stain showing positive reaction (arrows) for estradiol (a), progesterone (b) and progesterone receptor (c) in the excretory duct system within normal salivary glands. a. A 21-year old man, submandibular gland. b. A 75-year-old man, parotid gland. c. A 68-year-old man, minor salivary gland of palate. Scale bar, 50 μm in a and b; 20 μm in c.

inner cells of the two layers which were arranged in a tubular pattern (Fig. 2b), were strongly positive for both estradiol and progesterone, whereas the outer tubular cells were negative. In the cribriform pattern, the staining for both estradiol and progesterone was absent or weak in the cyst-lining cells surrounding cylindrical spaces, but was more evident in the luminal cells forming small ducts within those nests (Figs. 2c and 2d).

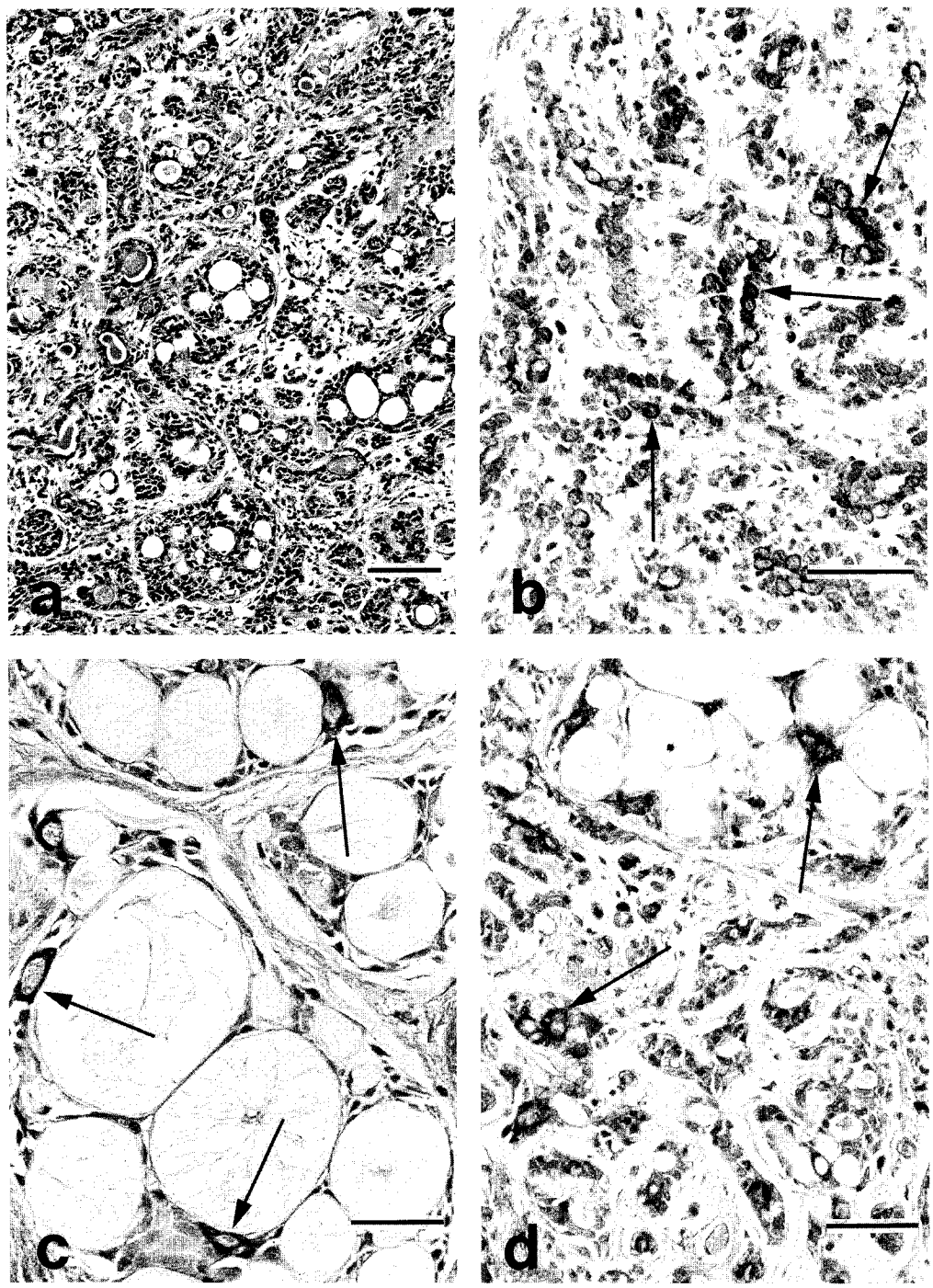

Fig. 2. Photomicrographs of adenoid cystic carcinomas. a. Cribriform pattern composed of small cells forming numerous cylindrical spaces (case 3, hematoxylin-eosin). b. Intracytoplasmic localization of estradiol in inner tubular cells (arrows, case 2). c. Immunostaining for estradiol in duct luminal cells within cribriform pattern (arrows, case 5). d. Immunostaining for progesterone in duct luminal cells (arrows, case 5). Scale bar, 100 μm in a and d; 50 μm in b and c.

Progesterone receptor was localized on tumor cells in areas where both estradiol and progesterone were positive. Without exception, immunopositive nuclei were detectable in all tumor patterns (Figs. 3a–3c); however, the number of progesterone receptor-positive cells varied greatly from case to case. In the cribriform pattern

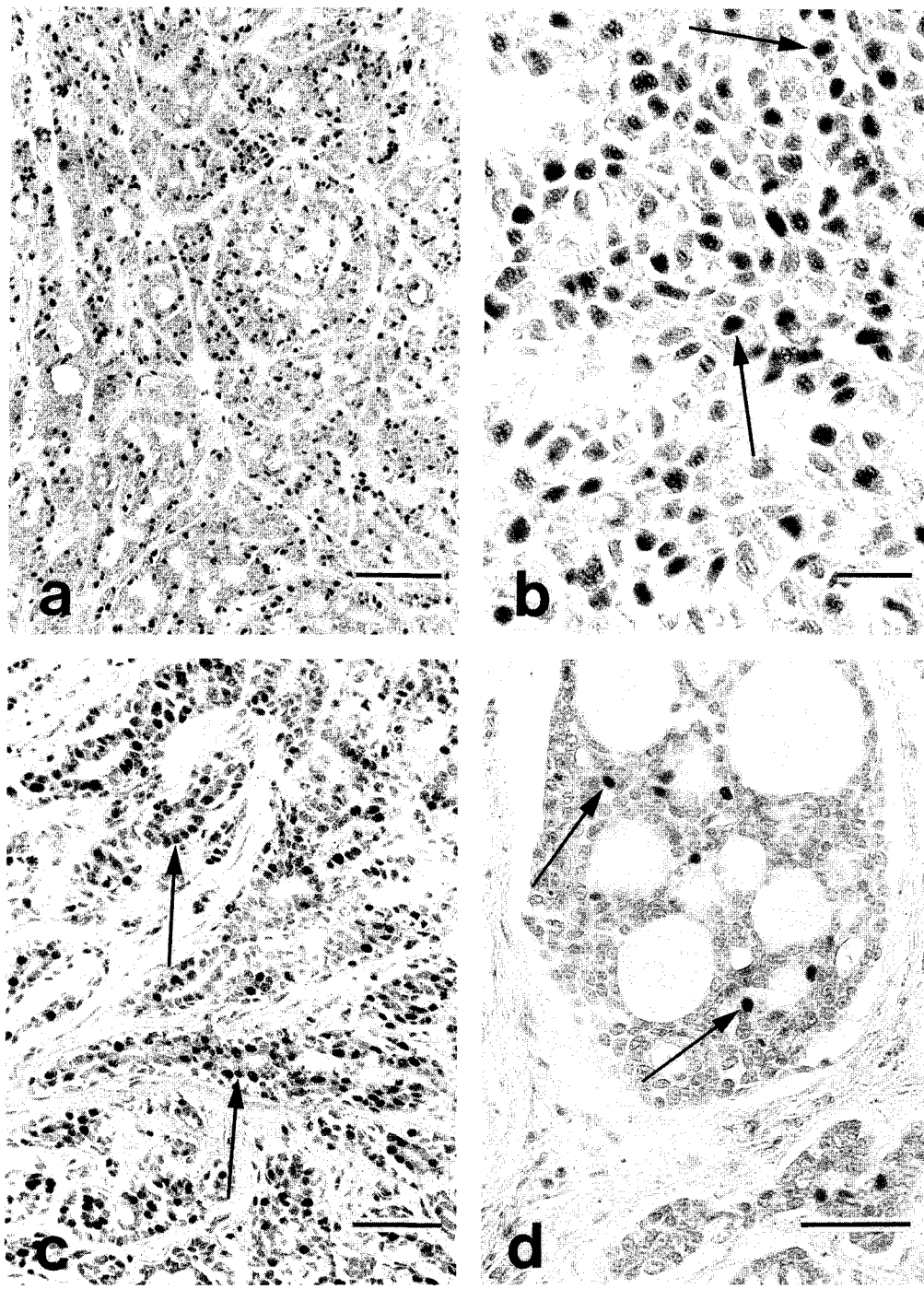

Fig. 3. Immunohistochemical staining of progesterone receptor in adenoid cystic carcinomas. a. Immunostaining is seen in most tumor cells (case 3). b. Tumor cells exhibit reaction products confined to the nucleus (arrows, case 3). c. Nuclear staining is seen in inner tubular cells (arrows, case 2). d. In the cribriform pattern, the immunoreactive products are seen in nuclei of the tumor cells located away from the cylindrical spaces (arrows, case 5). Scale bar, 100 μm in a; 25 μm in b; 50 μm in c and d.

areas, the staining for progesterone receptor was seen in the tumor cells located away from the cylindrical spaces, corresponding to duct luminal cells, and was observed solitarily among the cyst-lining cells (Figs. 3a and 3d).

DISCUSSION

In the present study, estradiol and progesterone were immunohistochemically demonstrated in cells of the excretory duct system within human salivary glands. In addition, this study demonstrated that progesterone receptors were distributed in nuclei of the cells which both estradiol and progesterone were positive. This immunolocalization pattern is in good agreement with nuclear localization of this protein in estrogen-sensitive tissues proved by the immunoelectron microscopic technique (15). Thus, our observations suggest that, as in estrogen-sensitive organs, the progesterone may modulate some functions of the excretory ducts of human salivary glands.

The induction of progesterone receptor by estrogen in the human endometrium and breast cancer has been well documented (2, 7). Horwitz *et al.* (3) have also shown that the presence of progesterone receptor is indicative of the estrogen receptor mechanism operating in those cells, and they deduced that the presence of estrogen receptors in the nucleus means to act as an effective stimulus. If this argument is correct, our present results suggest that the human salivary gland is one of the target tissues of sex steroid hormones, as well as breast, ovary, and endometium.

Immunohistochemical studies of 5 cases of salivary adenoid cystic carcinoma have confirmed that the tumor cells contain two antigenically different types of sex steroid hormones, estradiol and progesterone, and that in some cases progesterone receptor is present within most of them. No difference between the immunostaining patterns of salivary adenoid cystic carcinoma and normal salivary gland could be detected. The immunoreactivity of the three antibodies used in the present study was observed in tumors obtained from patients of postmenopausal status where the ovaries no longer produce significant amounts of steroid hormones. Moreover, these immunoreaction products were also found regardless of the sex of the patient. These data suggest that, in salivary adenoid cystic carcinoma, tumor cells are capable of synthesizing progesterone receptor proteins without distinction of age or sex, and that target cells may have retained the functional capacity for receptor binding.

It has been shown by light microscopic and electron microscopic studies that salivary adenoid cystic carcinoma is composed of neoplastic ductal cells and myoepithelial cells (6, 13). In these tumors, immunoreactivity for estradiol and progesterone was detected in both inner tubular cells of the two layers exhibiting a tubular pattern and duct luminal cells which were cribriform and solid patterns. It is reasonable to assume, on the basis of the normal distribution of sex steroid hormones in human salivary glands, that tumors arising from duct cells may contain estradiol and progesterone. The presence of progesterone receptor in salivary adenoid cystic carcinomas might be related to the differentiating potential of tumor cells. This, on the basis of the presence of progesterone receptor in the excretory duct system, seems to give additional evidence as to the origin of the duct cell tumor. Therefore, we propose that the tumor cells of ductal cell lineage are under the influence of progesterone, but the tumor cells of myoepithelial cells lineage are free from the influence.

Receptors for estradiol and progesterone have been detected in tumors of breast (6), endometrium (17) and ovary (4). It has been clearly established that the tumor cells of salivary adenoid cystic carcinoma contain estradiol and progesterone, but there is no convincing demonstration of which sex steroid hormones regulate the growth of this tumor. Thus, it is especially noteworthy that a high expression of progesterone receptor was recognized in this tumor, similar to that in hormone-dependent cancers.

In breast cancers, the presence of estrogen receptor is now widely accepted as an indicator of potential hormonal responsiveness and the efficacy of endocrine treatment of that tumor. However, the prognostic significance of progesterone receptor measurements in breast cancers is of increasing interest in relation to hormonal therapy. The validity of this finding has been confirmed; tumors lacking progesterone receptors rarely respond to hormonal therapy, whereas its receptor-positive tumors frequently regress with endocrine treatment (12, 19). Therefore, it is suggested that the presence of a progesterone receptor may be a good indication of tumor hormone dependence. On the basis of this information, our data also show that salivary adenoid cystic carcinoma appears to have the capability (presence of progesterone receptor) to respond to hormonal stimulation.

The question of whether the presence of hormone-specific staining in salivary adenoid cystic carcinoma is associated with the presence of estrogen receptor in these tumor cells and with tumor regression following hormonal therapy cannot be answered at present. From the results described here, and from other literature, it seems to be a good possibility that the tumor with a positive progesterone receptor should regress with endocrine manipulation. There have been no evaluable data on the use of endocrine manipulation for this tumor as an adjuvant. Further studies are needed before the clinical use of hormonal therapy for the management of this tumor can be recommended.

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