Immunohistochemical Localization of Estrogen Receptors in the Submandibular Gland Tumors of Female Rats

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ABSTRACT. The localization of estrogen receptors (EsR) in the tumor tissues of submandibular glands was examined in female rats, using the indirect immunoperoxidase method in combination with the *in situ* hybridization technique. Tumors were experimentally produced by 9,10-dimethyl-1,2-benzanthracene (DMBA), and the tumor tissues were fixed with formalin or paraformaldehyde and then embedded in paraffin. In the DMBA-induced submandibular gland tumors, immunoreactivity to EsR-peroxidase conjugate was found in nuclei of the tumor cells which occupied the peripheral rim of the tumor cell nests. In contrast, the reactivity in the normal submandibular glands without tumor was mostly confined to nuclei of the duct cells. When EsR mRNA expression was analyzed in the tumor tissue by *in situ* hybridization with a cDNA probe, its distribution was identical with that of immunoreactivity to EsR. These data suggest that the ductal cells of the submandibular gland are responsive to ovarian steroids, and that estrogens may play an important role in the maintenance of growth of the submandibular gland tumors.

Since the immunohistochemical techniques using the monoclonal antibody to the estrogen receptor (EsR) have been developed (4, 7, 11), there exists a surfeit of evidence on the cellular localization of these receptors within target tissues. As an example, recent studies have shown that the immunoreactive EsRs are confined to the nuclei of both the epithelial and stromal cells of the primate female reproductive tract (8, 10, 15, 16). In addition, the intense labelling of this receptor is present in nuclei of various cells of the hypothalamus whose neuronal structures are capable of sensing estrogens (7).

Recently, we found in rats that the normal submandibular glands contain the receptor protein which specifically binds to progesterone (12). More recently, we also demonstrated immunohistochemically that progesterone receptors are localized in the cell nuclei of human salivary glands and salivary adenoid cystic carcinomas (13). There is, however, as yet no direct evidence for the presence of EsR in the submandibular glands.

With the immunohistochemical technique, several workers have studied the localization of EsRs (4, 24) and progesterone receptors (10) in reproductive tissues and have shown that both receptors are recognized as nuclear proteins. Horwitz *et al.* (3) further reported that progesterone receptors in human breast tumors always coexist. It is, therefore, suggested that EsR may be pre-

sent in the submandibular gland. In the present study, this possibility was tested immunohistochemically in the tumor tissues of rat submandibular glands. We also examined the expression of EsR mRNA using the *in situ* hybridization method.

MATERIALS AND METHODS

Tissue preparations of the submandibular gland tumor. Fifty Fischer strain rats, seven-week-old females, were used as experimental animals. The animal was anesthetized by an intraperitoneal injection of pentobarbital sodium (35 mg Kg⁻¹). For production of the submandibular gland tumor, a pellet of 9,10-dimethyl-1,2-benzanthracene (DMBA, approximately 10 mg), a chemical carcinogen, was implanted into the surgically exposed left side of the gland. After 20 weeks, these animals were sacrificed and underwent a complete autopsy. The left submandibular mass was removed and divided into two portions: one portion was fixed in a 10% neutral-buffered formalin (in 0.1 M sodium phosphate buffer, pH 7.2) for 16 hrs and the other portion (about 3 mm in size) was fixed with 4% paraformaldehyde at 4°C for 2–3 hrs. These were dehydrated, embedded in paraffin and then sectioned into 5 µm thickness.

Histopathological examinations. Histopathological examinations were carried out by staining sections of the formalin-fixed paraffin-embedded tissue with hematoxylin and eosin.

Immunohistochemical staining. The immunohistochemi-

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cal determination of EsR in the submandibular glands was carried out according to our previous method with the indirect immunoperoxidase technique (12, 13). Sections (5 μm in thickness) 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. After 5 min of permeation with 1% Triton X-100 (in TBS), the sections were incubated for 10 min in 5% dimethylsulfoxide in the same buffer saline. These were washed in TBS and incubated for 20 min in 1% hydrogen peroxide diluted with TBS containing 20% normal sheep serum (NSS) and 1% bovine serum albumin (BSA), to eliminate endogenous peroxidase activity and minimize nonspecific binding. Sections were then incubated for 60 min at room temperature with a mouse monoclonal antibody against EsR (Novocastra, Newcastle, UK) diluted 1 : 500 in NSS solution (which was diluted 1 : 5 in TBS). The samples were washed in TBS, and then incubated for 30 min at room temperature with secondary antiserum (peroxidase-conjugated goat anti-mouse serum, DACO, Copenhagen, Denmark) diluted 1 : 40 in a NSS solution. Following the wash with TBS, sections were incubated for 15 min in diaminobenzidine (0.05% in TBS) in the presence of hydrogen peroxide (0.01%), followed by a wash with distilled water. Sections were then counterstained lightly with Mayer's hematoxylin to help identify immunoreactive cells. After dehydration, they were mounted in permount for light microscopic examination. Control immunohistochemical analysis was performed either by the substitution of normal mouse serum for the primary antibody or by simply omitting the primary antibody. Some rat uterine and normal submandibular gland sections were also stained for EsR using the same immunoperoxidase procedure.

In situ hybridization. *In situ* hybridization was performed on 5 μm sections of tumor tissue fixed in 4% paraformaldehyde, utilizing a digoxigenin-labeled DNA probe prepared from the rat EsR cDNA which was subcloned into a pUC118 plasmid (5, 6). This cDNA was kindly provided by Dr. M. Muramatsu (Department of Biochemistry, Faculty of Medicine, University of Tokyo, Tokyo).

Sections were deparaffinized, treated with 0.05 M HCl for 10 min and then digested with proteinase K (8 $\mu\text{g ml}^{-1}$) for 15 min at 37°C. They were preincubated in hybridization buffer [which contained 50% formamide, 5 $\times$ Denhardt's solution, 2 $\times$ standard saline citrate (SSC) and 2 $\mu\text{g ml}^{-1}$ denature salmon sperm DNA] for 30 min at 37°C before adding 250 ng ml^{-1} denature cDNA probe. After overnight hybridization at 42°C, sections were washed twice in 2 $\times$ SSC for 30 min, twice in 1 $\times$ SSC for 30 min, twice in 0.5 $\times$ SSC for 30 min and three times in TBS for 15 min, respectively. These samples were further incubated for 1 hr with a blocking TBS solution containing 5% BSA, 5% NSS and 0.1% Tween 20 at room temperature. Detection of the cDNA probe was carried out by incubation with peroxidase-conjugated mouse anti-digoxigenin antibody (Sigma Chemicals, St. Louis, MO, U.S.A.), followed by addition of diaminobenzidine in the pre-

sence of 0.02% hydrogen peroxide. The resultant sections were counterstained with Mayer's hematoxylin. Negative controls consisted of omission of the digoxigenin probe or antibody.

RESULTS

Pathological observations. Thirty-five of fifty animals developed a swelling on the left side of the submandibular gland 12 to 16 weeks after the implantation of DMBA. At 20 weeks when these animals were sacrificed, they exhibited a swelling with larger and more advanced lesions, and in some of them large ulcerating masses were developed in the neck. In these animals, the tumors were fairly well circumscribed and the cystic

Fig. 1. Typical examples of experimental carcinogenesis in submandibular glands in rats. a, Well-differentiated epidermoid carcinoma. Sections were stained with hematoxylin and eosin. b, Epidermoid cyst having a large amount of keratin often found in the tissues studied. Each scale bar, 50 μm .

Fig. 2. Immunostaining of EsR in the normal uterus and submandibular gland of rats. a, EsR in uterus. Note that the immunoreactivity to EsR is localized in cell nuclei of both epithelial (arrows) and stromal cells (arrowheads). b, The immunoreactivity to EsR in submandibular gland. Nuclear staining is observed mainly in ductal cells (arrows) and in few acinar cells (arrowhead). Each scale bar, 50 μ m.

cavity within their nests appeared to be filled with keratin.

Among the 35 tumors produced 17 were diagnosed

Fig. 3. Immunohistochemical localization of EsR in the well-differentiated epidermoid carcinoma (a), epidermoid cyst (b) and duct-like structures (c) of the submandibular gland of rats. a, The immunoreactivity to EsR localized in the tumor cells occupying the peripheral rim of tumor cell nests. b, EsR-positive cells appear mainly in the basal cell layers of cyst epithelium. c, The immunoreactivity is present in the epithelial cells which form duct-like structures between the intact gland and a peripheral area of the tumor. The immunoreactivity to EsR seen in a, b and c is confined only to cell nuclei. Arrows indicate the EsR-immunopositive cells. Each scale bar, 50 μ m.

Fig. 3.

Fig. 4.

histologically as well-differentiated epidermoid carcinomas, arising from the epithelium of the cyst wall (Fig. 1a), whereas the other 18 cases were epidermoid cysts with or without early malignant alterations in the cystic epithelium (Fig. 1b). Duct-like structures were proliferated in connective tissue present at the periphery of the remaining gland. No cases of adenocarcinoma developed, nor was there any instance of undifferentiated carcinoma. Neither distant metastasis nor localized spread to lymph nodes was observed.

In the submandibular glands from non-tumor-bearing animals, a small mass of dense hyaline-like connective tissue was identifiable adjacent to the space occupied by carcinogen.

Immunohistochemical observations. In order to evaluate the antiserum against EsR, we first examined the distribution of immunoreactivity to this receptor in sections of rat uterus which were formalin-fixed and paraffin-embedded. Immunoreactivity to EsR was, in agreement with the previous findings (8, 11), localized to the nuclei of both stromal (Fig. 2a, arrowheads) and epithelial cells (Fig. 2a, arrows). Thus, immunohistochemical staining of the sections prepared from the submandibular glands was carried out using this antiserum.

In the normal submandibular glands without any tumors, like the observations of the uterus, a specific nuclear staining was observed in epithelial cells as shown Fig. 2b. These immunoreactive cells were confined mainly to the duct system (arrows in Fig. 2b), and occasionally, acinar cells were also positive (arrowhead in Fig. 2b). However, the myoepithelial cells surrounding the acini and intercalated ducts were consistently negative.

When similar immunohistochemical experiments were done in the epidermoid carcinoma (17 cases), the immunoreactivity to EsR was found in all cases. In 9 cases of them, immunoreactive cells occupied 10–30% of the total tumor cells and in the other 8 cases less than 10% were immunopositive. The immunoreactivity to EsR was localized only in nuclei of the tumor cells which were present at the peripheral rim of tumor cell nests (Fig. 3a, arrows). In contrast, in the epidermoid cysts (18 cases), EsR-containing cells were observed in 5 cases and located in the basal layer of the cystic epithelium (Fig. 3b, arrows). The other regions displaying immunopositive staining were non-malignant duct epithelium (Fig. 3c, arrows). Evidence for a cytoplasmic locali-

Fig. 4. *In situ* detection of EsR mRNA of the submandibular gland tumors. a, Well-differentiated epidermoid carcinoma. Strong cytoplasmic staining is seen in the tumor cells occupying the peripheral rim of tumor cell nests. b, Peripheral area of the well-differentiated epidermoid carcinoma. Epithelial cells forming duct-like structures show positive staining of the cytoplasm. c, Epidermoid cyst. The majority of cells in basal layers show positive staining of the cytoplasm. Arrows indicate the EsR mRNA-containing cells. Each scale bar, 50 μ m.

zation of EsR was not found in any cases. No reaction product in cell nuclei was observed in the specimen treated with normal mouse serum or in the absence of the primary antibody (data not shown).

Observations of the *in situ* hybridization. Analysis using the *in situ* hybridization technique revealed a strong cytoplasmic, but non-nuclear staining of EsR mRNA in the DMBA-induced tumor cells of the submandibular glands which occupied the peripheral rim of tumor cell nests (Fig. 4a). Tumor tissues, which had contained EsR immunoreactivity, also showed strong *in situ* hybridization positivity of its mRNA. In the peripheral area of this tumor, a similar staining was detected in the epithelial cells forming duct-like structures (Fig. 4b). In contrast, positive cells in the epidermoid cyst were observed mainly in basal layers (Fig. 4c). The normal submandibular gland showed a positive staining in the duct cells, although the reaction was extremely weak (data not shown). Negative control gave no signal.

DISCUSSION

Steroid hormones, including estrogen or progesterone, are diffused through the plasma membrane of the target cell and bind reversibly to specific steroid hormone-receptor proteins in the cytosol (2, 9, 22). By complexing to the hormone, the receptor protein acquires an affinity for DNA, and accumulates in the cell nucleus. There the hormone-receptor complex binds to chromatin and regulates the transcription of genes. Thus, immunohistochemical analysis using the monoclonal antibody against EsR protein is useful for the study of cellular localization of this receptor or mechanism(s) underlying estrogen actions within the target cells. The present experiments of the uterus using the monoclonal antibody to this receptor resulted in the distribution of EsR which was similar to the previous findings obtained in rhesus uterus (11) and mouse genital tracts (23). In addition, no reaction product was observed in the control experiments. Therefore, we can say that the monoclonal antibody used was EsR-specific.

Our data show that the EsR is identified in the normal submandibular glands of rats and located only in cell nuclei of the duct system. In our earlier study, the progesterone receptor is present in the duct cell nuclei of this gland (12), thus implying the ductal cells in the submandibular gland not only possess both EsR and progesterone receptor, but also may be direct targets for these hormones. Using the autoradiographic technique, Stumpf and Sar (22) have shown that estrogens stimulate the expression of progesterone receptors and modulate the target cell functions by an increase in their affinity for DNA and subsequent regulation of the transcription of genes. Taken together, it is probable that these ovarian steroids may cause a cell proliferation and cyto-

differentiation of duct cells in the submandibular glands.

In the present experiments, all of the DMBA-induced tumors, which originated from the submandibular gland parenchyma, were of an epidermoid carcinoma type. This agrees with the previous finding that the neoplastic process is preceded by squamous metaplasia of the duct, with a subsequent epidermoid cyst formation, and is finally linked to the malignance of the metaplastic cyst epithelium (17, 18, 21). In these tumor cells, the immunoreactivity to EsR was confined to the cell nuclei, similar to the subcellular localization of this receptor in the uterine or normal submandibular glands. Furthermore, the immunohistochemical localization of EsR in the submandibular gland tumors did correspond to the expression of mRNA of this receptor when *in situ* hybridization was applied. From these observations, it seems likely that the EsR proteins are synthesized in the tumor cells originating from the malignantly transformed submandibular ducts. Furthermore, because the steroid hormones are involved in the development of the target tissue (19) and in the maintenance of growth of the hormone-dependent tumors (1, 9, 14), the presence of EsR in the neoplastic lesions suggests a steroid hormone-regulated mechanism for growth of the tumors in the submandibular gland.

After the implantation of DMBA into the submandibular gland parenchyma, the squamous metaplasia developed in the duct epithelium. Chemical carcinogens in rats have been shown to elicit metaplasia and malignant neoplastic transformation of the submandibular gland duct epithelium (17, 18, 21). In human salivary glands, various degrees of the squamous differentiation are constantly observed under the reactive and neoplastic conditions. However, an adequate explanation concerning the functional significance of EsR in the development of the squamous metaplasia is still lacking.

The development of the epidermoid carcinoma is extremely rare in human salivary glands. It is also very difficult to produce experimental salivary gland adenocarcinoma in animals. However, the induction of EsR in the submandibular gland tumor cells suggests that this receptor is one of the most helpful tools to predict the response to endocrine therapy in advanced human cancers in this gland. This assumption is strongly supported by our recent finding that progesterone receptors are immunohistochemically detectable in human adenoid cystic carcinoma originating from the salivary gland (13).

We propose that estrogens may be involved not only in duct cell functions, but also in the development or growth of submandibular gland tumors.

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