Identification, Subcellular Localization, and Estrogen Regulation of Peroxidase in 7,12-Dimethylbenz(a)anthracene-induced Rat Mammary Tumors

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SUMMARY

An estrogen-induced, intensely staining peroxidase 3,3'-diaminobenzidine-positive reaction product is found to be characteristic of hormone-dependent, 7,12-dimethylbenz(a)anthracene-induced mammary tumors of the rat. This product is demonstrated in thick sections of such tumors from intact or estrogen-treated castrate rats but is not seen in tumors that are in regression due to castration or estrogen deprivation. It is, furthermore, absent from tumors whose growth is unaffected by castration. The subcellular localization of this enzyme activity is restricted mainly to the nuclear envelope and cisternae of the granular endoplasmic reticulum in addition to secretory granules. This provides the first evidence for a criterion that would allow differentiation of hormone-dependent and hormone-independent mammary cancer on histological sections and, as such, may have considerable potential as an aid in the classification of human breast cancer.

INTRODUCTION

The DMBA4-induced mammary cancer in the rat has become a valuable model for the study of hormone dependence of breast cancer as applied to the disease in man. Using this model system, the first evidence was obtained for the presence of estrogen receptors in breast cancer (9, 12, 17), thus providing the basis for recent successes in the prediction of clinical responses of human breast cancer to endocrine intervention (4, 5, 10, 13, 15).

In an effort to find another possible method for distinguishing hormone-dependent from hormone-independent breast cancer, we have been studying the peroxidase activity of DMBA-induced mammary tumors of the rat. Since estrogen induces a specific peroxidase in the rat uterus (2, 3, 14, 16, 18), it was conceivable that the hormone-dependent mammary tumor, which is likewise stimulated to grow on administration of estrogen, might also contain an estrogen-inducible peroxidase. Furthermore, since this enzyme can be readily visualized on suitably prepared fixed sections (2, 3), peroxidase reactivity could be readily applied to routine screening of breast cancer sections by pathology laboratories.

This report describes our observations of an estrogen-induced rat mammary tumor peroxidase and its subcellular localization.

MATERIALS AND METHODS

Tumors were induced in 50-day-old female Sprague-Dawley rats (Sprague-Dawley, Madison, Wis.) by a single intragastric feeding (8) of 22 mg of DMBA in 2 ml sesame oil. Tumor dimensions were measured with a caliper and volumes were calculated based on an ellipsoid tumor shape. Estradiol administered to bilaterally ovariectomized animals was given at a dose of 2.5 µg/day, 6 days/week.

Biopsy samples of tumors were surgically removed from ether-anesthetized animals and fixed in a mixture of cold 4% glutaraldehyde-1% formaldehyde in 0.1 M cacodylate buffer, pH 7.2 (11). After 1 hr in this fixative, the tissue was washed overnight in the same buffer and cut into 100-µm slices using an Oxford vibratome (Oxford Vibratome Co., San Mateo, Calif.), the slices were incubated in Graham and Karnovsky’s (6) 3,3'-diaminobenzidine-H2O2 medium for the demonstration of peroxidase. After a buffer rinse, the tissue was postfixed in 2% OsO4 in distilled water, dehydrated in ethanol, and embedded in Epon. Thin sections were examined and stained lightly with lead citrate (21) in Hitachi HU-11C or Siemens Elmiskop 101 electron microscopes.

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4 The abbreviation is: DMBA, 7,12-dimethylbenz(a)anthracene.

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RESULTS

In the growing tumor (Figs. 1 and 2), each acinus consists of a single layer of cuboidal epithelial cells that surrounds the regularly shaped lumen containing amorphous secretory product and filamentous material (Fig. 1, inset). The cells are well developed, containing considerable cytoplasm with relatively numerous mitochondria. The rough endoplasmic reticulum is flat, while the abundant agranular endoplasmic reticulum appears as small vesicles. Free ribosomes are numerous and evenly distributed throughout the cytoplasm. The well-developed Golgi complexes, composed of small vesicles and parallel flat saccules, are located at the apical region of the cell. Lipid droplets are seen throughout the cytoplasm.

In the tumor regressing after ovariectomy (Figs. 3 and 4), the dilated, irregularly shaped lumens are surrounded by 1 or 2 layers of flattened epithelial cells. Numerous mast cells are seen in the interalveolar spaces (Fig. 3, inset). The flattened acinar cells contain prominent nuclei comprising the bulk of the cell, due to reduction in the amount of cytoplasm. The Golgi complexes are poorly developed and located at the supranuclear region of the cell. The granular endoplasmic reticulum is poorly developed.

When stained for peroxidase activity, 100-μm-thick sections of the growing tumor of the intact animal show an intensely positive reaction (Fig. 5). On the other hand, the regressing tumor in the castrate animal shows no evidence for peroxidase-staining cells (Fig. 6). Semithin sections (1 μm) of the growing tumor show that not all epithelial cells are equally positive for peroxidase activity (Fig. 7, inset), as adjacent cells may be highly reactive or largely unreactive. Intracellular peroxidase activity is most prominent in the nuclear envelope, the dilated cisternae of the granular endoplasmic reticulum, and the secretory granules located in the apical cytoplasm of the cell (Figs. 7 and 8). Golgi saccules and vesicles show only slight activity (Fig. 7).

In marked contrast, none of the epithelial cells of the regressing tumor exhibits peroxidase reaction product (Fig. 9, inset). No enzyme activity is observed in the acinar lumen, in the poorly developed cisternae of the agranular endoplasmic reticulum, or in other organelles (Fig. 9). Mitochondrial cristae, however, stain positively for cytochrome c oxidase activity.

In the clearly hormone-dependent tumor growing during estradiol administration in the castrate rat (Chart 1), a biopsy sample (Chart 1, Biopsy 1) showed intense peroxidase activity similar to the activity seen in the tumor of the intact animal (Fig. 5). After termination of estrogen administration, the tumor again regressed and no peroxidase reaction was evident in the subsequent biopsy sample (Chart 1, Biopsy 2).

A number of tumor biopsies were obtained from animals that were subjected at the same time to ovariectomy to test the in vivo dependence of the tumors on ovarian hormones. Chart 2A shows the growth record of one such tumor demonstrating hormone-independent growth; thick sections of this tumor were completely negative for peroxidase activity. The tumor for which the growth record is shown in Chart 2A was also studied for peroxidase activity. This tumor, which originally showed a hormone-dependent component as evidenced by its partial regression following ovariectomy, subsequently showed hormone-independent growth in the castrate rat. In this hormone-independent state the tumor showed no evidence of peroxidase reaction product when assayed before estradiol administration (Chart 2, Biopsy 1), or during short-term (Chart 2, Biopsy 2) or long-term estrogen administration (Chart 2, Biopsy 3).

With a limited number of tumors assayed in this manner, there was a good correlation between hormone-dependent growth in vivo and positive peroxidase reaction, as well as with hormone-independent growth and a lack of peroxidase stain.

DISCUSSION

The results presented here show that the growing, hormone-dependent, carcinogen-induced mammary tumor of the rat gives an intensely positive reaction for peroxidase activity and that this activity is indeed intracellular, being localized in the nuclear envelope and cisternae of the granular endoplasmic reticulum as well as compartmentalized in secretory granules. The tumor regressing after castration, frequently infiltrated by mast cells, not only shows the characteristic ultrastructural changes reported by others (22) but also loses the peroxidase reactivity seen in such a tumor before castration. That the peroxidase reactivity is not simply a characteristic of a growing tissue or tumor, as has been suggested (19), is indicated by the observations of no detectable peroxidase activity in growing mammary tumors whose growth is independent of ovarian hormones. Furthermore, in hormone-dependent tumors, peroxidase activity was present when the tumor was stimulated to grow in the castrate by estrogen administration, and this activity disappeared in the same tumor after estrogen administration.
was withheld. It is, therefore, reasonable to conclude that the mammary tumor peroxidase activity is probably an estrogen-induced characteristic of a hormone-dependent rat mammary tumor. Within a single tumor specimen, not all acinar cells show equal peroxidase reactivity. Whether this implies that not all epithelial cells within an apparently hormone-dependent tumor are equally dependent on estrogen or whether some cells may be in a quiescent state cannot be answered at the present time. However, similar differences in sensitivity were observed in the peroxidase response of the uterine epithelium to estrogen (3). There is some controversy (20, 23) as to whether the hormone-dependent carcinogen-induced rat mammary tumor is dependent upon prolactin, estrogen, or both hormones in concert for its growth regulation. In view of the apparent estrogen induction of uterine peroxidase, it seems probable, but by no means proven, that estrogen is directly involved in the induction of the peroxidase reaction product in the hormone-dependent tumors reported here.

Previous studies (22) were unable to identify any characteristic differences between hormone-dependent and hormone-independent rat mammary tumors on examination at the light or electron microscopic level. It now appears that examination of histological sections stained for peroxidase reactivity may provide such a differentiating criterion for the growing tumor in the intact or estrogen-treated animal. Although others have reported histological identification of mammary tumor enzymes such as alkaline phosphatase and β-glucuronidase (1), these were not correlated with hormone dependence of the tumors. In an elegant study of biochemical characteristics of carcinogen-induced rat mammary tumors, Hilf et al. (7) identified a number of enzymes, notably glucose 6-phosphate dehydrogenase, isocitrate dehydrogenase, and glucosylphosphate isomerase, whose activity decreased in the DMBA-induced tumors in the absence of estrogen. However, with these enzymes there was significant activity in the regressing tumor of the castrate, and the maximum decrease seen on castration was about 2-fold. Using the peroxidase assay reported here, the regressing tumor in the castrate appears to be completely devoid of activity, at least by the histochemical methods used.

Whether the presence of peroxidase reaction product is a sine qua non marker for hormone dependence of a tumor in the intact or estrogen-treated rat, and, furthermore, whether this enzyme activity could provide a simple marker for hormone-dependent human breast cancer, will require additional studies. We have been able to identify areas showing a positive peroxidase reaction in thick sections of some human breast cancer specimens. Since this activity is limited to epithelial or glandular cells and human breast cancer specimens are frequently heterogeneous, it will probably be necessary to examine numerous sections of such tissue to decide whether peroxidase activity is present in the tumor. The simplicity of the peroxidase determination and the fact that it could readily be done along with routine pathological examination of the tumor suggests that further, more extensive studies of human breast cancer peroxidase activity are warranted. Certainly, the correlation of patient response to endocrine therapy with the presence of estrogen receptors in the tumor (4) has provided a much-needed criterion on which to base an endocrine approach to breast cancer therapy. It is conceivable that breast cancer peroxidase activity could provide an additional criterion, one more readily applicable to routine screening procedures already used by most hospitals.

It is important to use a low concentration (500 μg/ml) of freshly prepared 3,3'-diaminobenzidine and a short incubation time to avoid nonspecific stain development in thick sections. This can best be verified by examining the subcellular localization of peroxidase reaction product at the electron microscopic level.

REFERENCES

Rat Mammary Tumor Peroxidase


Figs. 1 and 2. Growing DMBA-induced mammary carcinoma from an intact, mature female rat. The acinar lumen (L) is surrounded by a single layer of cuboidal epithelial cells (inset). Well-developed Golgi complexes (GC), numerous lipid droplets, and cisternae of the granular endoplasmic reticulum (RER) are present in the cytoplasm of the epithelial cells. Filamentous material is present in the acinar lumen (Fig. 2). Uranyl acetate and lead citrate. Fig. 1, x 14,000; inset, x 540; Fig. 2, x 14,000.

Figs. 3 and 4. Regressing mammary tumor in the castrate. The dilated acinar lumen (L, inset) contains filamentous material and exfoliated cells and cell processes (Figs. 3 and 4). The Golgi complex (GC) and granular endoplasmic reticulum (RER) are poorly developed. Junctional complexes (Fig. 4, arrows) are present at the apices of the flattened acinar cells. Numerous mast cells (MC, inset) occupy the interalveolar spaces. Uranyl acetate and lead citrate. Fig. 3, x 7,000; inset, x 520; Fig. 4, x 14,000.

Figs. 5 and 6. Thick vibratome sections, 100 µm. After incubation in the 3,3'-diaminobenzidine-H2O2 incubation medium, the growing mammary tumor tissue shows strong peroxidase activity (Fig. 5), while a biopsy taken from the tumor from the castrate is negative for peroxidase activity (Fig. 6). x 100.

Figs. 7 and 8. Intense peroxidase activity is present in some (arrows) of the acinar epithelial cells (inset, 1 µm-thick section). At the electron microscope level, the peroxidase reaction product appears in the nuclear envelope, granular endoplasmic reticulum (RER; see Fig. 8, inset), and in secretory vesicles. The Golgi complex (GC) is weakly reactive. Lead citrate. Fig. 7, x 7,200; inset, x 500; Fig. 8, x 7,000; inset, x 13,000.

Fig. 9. The regressing tumor of the castrate is shown here. All epithelial cells are devoid of peroxidase activity (see inset). Cytochrome c oxidase activity is present on the mitochondrial cristae. L, acinar lumen. Lead citrate. x 7920; inset, x 500.
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