Distribution of an Estrogen-induced Protein with a Molecular Weight of 24,000 in Normal and Malignant Human Tissues and Cells

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ABSTRACT

We have shown previously that estradiol regulates the synthesis of a M, 24,000 protein, of unknown function, in the human breast cancer cell line MCF-7. We have also reported the preparation of monoclonal antibodies against M, 24,000 protein, antibodies that reacted by immunohistochemistry with MCF-7 cells and certain human breast tumor samples but not with an estrogen receptor-negative cell line. In the present paper, we report much more extensive information about cell and tissue distribution of the M, 24,000 protein. Several human tissue biopsy samples and human cell lines were studied by immunohistochemistry. The results show that M, 24,000 protein is not a fetal-associated antigen nor a milk-related protein. It was occasionally found in carcinomas other than breast tumors. It was also detected in normal estrogen target organs: uterus; oviduct; vagina; and breast. Some normal skin samples were likewise positive. The monoclonal antibodies to M, 24,000 protein also reacted specifically with maternal decidual cells in placenta, suggesting that these antibodies may be useful in the study of decidual transformation.

The M, 24,000 protein was detected in six estrogen and progesterone receptor-positive cell lines but not in receptor-negative cell lines. The fraction of M, 24,000-positive cells in two breast cancer cell lines increased significantly after estradiol treatment.

The finding of M, 24,000 protein in organs with secretory capacity, the varying degrees of immunostaining within the same positive cell population as expected for cells in alternating synthetic and secretory phases, and the increase of M, 24,000 protein immunostaining after estradiol treatment are all consistent with the possibility that M, 24,000 has a secretory function regulated in part by estrogens. Moreover, since decidual cells strongly reacted with the monoclonal antibodies to M, 24,000 protein, it is possible that the protein is under progesterone as well as estrogen control. These results point to the usefulness of these monoclonal antibodies for detecting the M, 24,000 protein and for studying M, 24,000 protein as a marker for the mechanism of hormone action in human estrogen target organs, tumors, and cell lines.

INTRODUCTION

Estrogens have been implicated in the regulation of induction of several specific proteins, both proteins with well-characterized biological functions and proteins whose functions are unknown (2, 5, 7, 8, 11, 12, 22, 23). One of these estrogen-regulated proteins is characterized by its molecular weight of 24,000 on sodium dodecyl sulfate-polyacrylamide gels and initially was found in the human breast cancer cell line MCF-7 (2, 8). Its potential importance as a marker for estrogen responsiveness and for studying the mechanism(s) of hormone action has been emphasized (3).

Recently, we have produced mouse MAb against the M, 24,000 protein and have shown them to be useful in the detection of the protein by immunohistochemistry (6). These MAb displays restricted immunostaining to the cytoplasm of MCF-7 cells grown in nude mice. They did not react with an estrogen receptor-negative breast cancer cell line, and they did react with certain human breast tumor biopsy samples.

In the present study, we have tested the MAb by the immunoperoxidase technique with many normal and neoplastic human tissues and cell lines in order to define the distribution of the M, 24,000 estrogen-regulated protein.

MATERIALS AND METHODS

MAb. For details about the production of the 3 mouse MAb used in the present study, we refer to our previous paper (6).

Cell Culture. The cell lines used in this study and their laboratory of origin are listed in Table 1. Growth conditions for MCF-7, MDA-MB-231, T 47 D, HBL-100, and ZR-75 have been described (13, 23). Human esophageal carcinoma cells (KB) were grown in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% heat-inactivated fetal calf serum, 1 mm sodium pyruvate, 1% nonessential amino acids, 2 mm L-glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml). Human bladder carcinoma cells (T-24) were grown in Eagle’s minimal essential medium supplemented with 5% whole calf serum, 1 mm sodium pyruvate, 2 mm L-glutamine, 1% nonessential amino acids, porcine pancreatic insulin (6 ng/ml; Sigma Chemical Co., St. Louis, Mo.), and geramycin (25 μg/ml). The CG 5 cell line (19) (variant of MCF-7 cell line) was grown in Dulbecco’s modified Eagle’s minimal essential medium with 0.45% glucose, 10% fetal bovine serum, 20 mm 4-(2-hydroxyethyl)-1-piperazinanesulfonic acid buffer, 2 mm glutamine, geramycin (25 μg/ml), 0.05% sodium carbonate.

The MCF-7-243, MDA-MB-231, ZR-75, and T47 D cells were treated with the estrogen rescue protocol as described previously (7, 8). Briefly, 2 days after plating, the cells were incubated for 6 days with antiestrogen nafoxidine (10−6 M), changing the medium every 48 hr. Then, one group of cells was incubated with estradiol (10−8 M) for 5 days, while another control group continued for 5 days with calf serum stripped of endogenous steroids. An additional control group was incubated for 11 days with calf serum stripped of endogenous steroids by treatment with dextran-coated charcoal.

Cells were harvested by 10-min incubation at 37° with 1 mm EDTA in Ca2+- and Mg2+-free Earle’s balanced salt solution and pelleted by low-speed centrifugation. Cell pellets were washed once with Earle’s solution. The cells used for microscopic study were fixed in Bouin’s fixative and embedded in paraffin.

1 This work was supported by NIH Grants CA 11378 and CA 09402 and by the Robert A. Welch Foundation.

2 Postdoctoral Research Fellow from the Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET), Argentina.

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Received July 12, 1982; accepted December 2, 1982.

* The abbreviation used is: MAb, monoclonal antibody.

* D. J. Adams, manuscript in preparation.
Receptor Assays. Estrogen and progesterone receptor levels were assayed in the MDA-MB-231, ZR-75, T 47 D, and MCF-7 cells (from our laboratory). The cells were washed once with phosphate buffer (5 mM, pH 7.4; 10% glycerol-1 mM monothioglycerol) at 4°C, resuspended in the same buffer, and homogenized in a Teflon-glass homogenizer. Cytoplasmic estrogen and progesterone receptors were assayed by the one-point dextran-coated charcoal assay (13). Cytosol protein concentration was measured by the method of Lowry et al. (16).

Biopsy Samples. The human normal and pathological tissues were obtained from the Pathology Laboratory of the Medical Center Hospital, San Antonio, Texas. Previous tests showed that good M, 24,000 preservation was obtained using 10% formalin (6). Therefore, in the present study, routinely prepared formalin-fixed, paraffin-embedded blocks were used. Since we have shown that M, 24,000 protein cannot be detected in necrotic areas (6), only those samples with high cytological quality were selected. Some of the samples considered as normal tissues were taken from areas distant from sites of pathological lesions when lesions were nonmalignant. Fetal tissues came from 5 spontaneously aborted fetuses, all less than 5 months fetal age.

Immunostaining. All the samples were immunostained with the avidin-biotin-peroxidase complex system purchased from Vector Laboratories, Burlingame, Calif., following the instructions supplied with the kit. Some slides were counterstained with hematoxylin.

Using MAbs to M, 24,000 protein, we found that the concentration at which they are applied has a striking influence on the immunostaining. Therefore, in the present study, we used MAbs obtained from mouse ascites, partially purified by ammonium sulfate precipitation, diluted to 5 μg protein per ml. As a negative control, a MAb of unrelated specificity was routinely run: mouse MAb (IgG) against human IgE (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). In addition, 2 other controls were run: hybridoma growth medium and supernatant from culture of the parent myeloma cells (6).

RESULTS

The 3 MAbs to M, 24,000 protein gave the same immunostaining pattern on all of the different tissues examined. This was expected, since we have biochemical evidence suggesting that the 3 MAbs may have identical specificity.

The presence of the estrogen-regulated M, 24,000 protein in various human biopsy samples is shown in Table 2. The protein was detected in most estrogen target organs, although not always in every case tested. Moreover, M, 24,000 protein was not present in estrogen-producing organs like ovaries and placenta (the positive staining in placenta was restricted to decidual cells). The M, 24,000 protein was found in cells from normal organs such as breast, uterus, placenta, fallopian tubes, vagina, and skin. In all these organs, the immunoreactive site was localized to the cytoplasm of epithelial cells.

This estrogen-regulated protein was present in one of 7 normal breasts tested, and the staining in this positive case was heterogeneous, showing a positive reaction in some lobules of the mammary tree while others in close proximity remained unstained (Figs. 1 and 2). However, the small ducts inside the stained lobules were all positive (Fig. 2).
24,000 protein was confined to the cytoplasm of the epithelial cells lining the small ducts. In lobules with well-developed alveoli, no reaction was detected. The same staining pattern was observed in one of the 3 breasts with fibrocystic disease. In lactating breasts, where all the lobules had alveoli and some had milk in the lumen, no reaction was detected. Moreover, neither breast samples with gynecomastia nor breasts with fibrocystic disease showed M, 24,000 protein. In 18 of 70 breast carcinomas, the protein was found localized to the cytoplasm of malignant cells as illustrated previously (6). We are currently testing more breast samples with fibrocystic disease and gynecomastia; the results will be presented in a future publication.

In normal uteri, a positive reaction was restricted to the cytoplasm of decidual cells (Figs. 5 and 6). The reaction was not as intense as in endometria, and it was localized to the cytoplasm of ciliated epithelial cells (Fig. 11). No reaction was detected in the stroma or the muscle of the oviduct, the stroma, and the muscle of the oviduct were not immunostained.

In placenta, M, 24,000 protein was detected only in maternal villi. The presence of M, 24,000 protein was also detected in normal uteri. It was present in the cytoplasm of epithelial cells in the endometrium (Fig. 3) and in myometrial cells (Fig. 4). However, the reaction in myometrium was not as intense as in epithelial cells. The presence of M, 24,000 protein in smooth muscle cells was restricted to the myometrium, since no other organs with smooth muscle were immunostained. Fallopian tubes displayed M, 24,000 protein in some epithelial cells. The reaction was not as intense as in endometrium, and it was localized to the cytoplasm of ciliated cells. The stroma and the muscle of the oviduct were not immunostained.

Epithelial cells of the vagina also showed M, 24,000 protein in their cytoplasm (Fig. 11), but the reaction was not very strong. Positive M, 24,000 staining was observed in 2 of 7 skin samples tested (Fig. 12). In this case, the cytoplasm of epithelial cells was stained, mainly cells in the stratum spinosum and granulosum. Moreover, some ducts of sweat glands were stained, while others in close proximity were not.

Some tissues not mentioned in Table 2 were present in the different organs tested but did not react with the MAb to M, 24,000. Among these were: bone; fat tissue; blood cells; and nervous tissue (including neurons).

The presence of M, 24,000 protein in some bladder, endometrial, skin, and prostate carcinomas (Table 2) was always restricted to the cytoplasm of tumor cells. In all these cases, the reaction was heterogeneous with positive tumor cell populations in coexistence with unstained cells.

None of the following fetal tissues demonstrated immunoreactive M, 24,000 protein: skin; striated and smooth muscle; cartilage; bone; connective tissue; adipocytes; liver; intestine; heart; kidney; lung; adrenals; and umbilicus.

The presence of M, 24,000 protein is compared with estrogen and progesterone receptor status in different human cell lines in Table 1. It is clear that M, 24,000 protein was detected only in those cell lines containing estrogen and progesterone receptors; no reaction was obtained in the cell lines lacking receptors, and no reaction was obtained on any cell line using the negative control MAb, the hybridoma growth medium, or the parent myeloma supernatant. The reaction was always strong in the cytoplasm of MCF-7 cells, independent of the passage number of the cells or the laboratory or origin. An example of the specific M, 24,000 protein immunostaining in MCF-7 is shown in Fig. 13. The reaction was homogeneous in intensity and distribution throughout all the cytoplasm of all the cells. On the other hand, T 47 D and ZR-75 cells, cell lines with much less estrogen receptor than MCF-7, displayed M, 24,000 protein only in 2 to 3% of cells and with lower intensity than in the MCF-7 cells. Fig. 14 shows the reaction for M, 24,000 protein confined to the cytoplasm of a few T 47 D cells.

Because of the small number of ZR-75 and T 47 D cells appearing positive under the standard growth conditions above, we also examined these cells after estrogen rescue from antiestrogen growth inhibition, a condition which greatly increases M, 24,000 protein synthesis in MCF-7 cells (7, 8). MDA-MB-231 and ZR-75 cells were also included in this study. As expected, MDA-MB-231 cells, which lack estrogen receptors, remained negative for M, 24,000 protein (Fig. 15). However, both of the estrogen receptor-positive lines ZR-75 and T 47 D showed significant increases in M, 24,000 protein with estrogen rescue (Fig. 16), although still at lower intensity than in MCF-7.

**DISCUSSION**

The present study demonstrates that the M, 24,000 protein is not widely distributed in either normal or neoplastic tissues. Since M, 24,000 protein is estrogen regulated (8), we might expect to find it in estrogen target organs. The question is to what extent the different organs and tissues of the body are targets for estrogens. Many valuable studies show cellular localization of estrogens in different tissues (15, 21). However, the biological effects of estrogens are so diverse that, at
present, we do not have a complete list of the cells influenced by estrogens with their degree of sensitivity to the hormone.

Even so, it is clear that, in the female reproductive tract, we find the most sensitive estrogen target organs, and it is in these organs that we find M, 24,000 protein. However, M, 24,000 protein was not present in all of the samples tested even from the female reproductive tract. For example, the protein could be shown in only 3 of the 7 normal uteri examined. This may reflect variation in estrogen sensitivity during the ovarian cycle (4) or during menopause (most of the uteri we studied were taken from women near menopause). A further study of endometrial biopsies from women in known stages of the ovarian cycle will help to clarify this point.

We also have to consider the sensitivity of the immunohistochemical technique used in the present study. Although the avidin-biotin-peroxidase complex method is one of the most sensitive immunoperoxidase techniques currently in use (14), it is possible that we are detecting only highly estrogen-responsive cells. This is supported by the facts that M, 24,000 protein could be shown in ciliated epithelial cells of the oviduct, which are very sensitive to estrogen, but not in the other cells of the oviduct (4, 21), and that the reaction was stronger in epithelial cells of the endometrium than in myometrial cells. Finally, it is also possible that M, 24,000 protein is expressed only in certain estrogen target cells and not in others. This is supported by our finding of M, 24,000 protein in the female reproductive tract but not in the pituitary, which is classically considered an estrogen target organ (15, 21). There is still no obvious explanation for the absence of M, 24,000 protein in the pituitary, although the response of this organ to estrogen is somewhat different than that of those target tissues which show M, 24,000 protein.

The M, 24,000 protein is not a tumor-specific antigen, since the MAbs reacted with normal female reproductive tissues as well as with some of the neoplastic samples tested. Moreover, M, 24,000 protein is not a fetal-associated antigen, since no fetal tissues reacted with the MAbs.

The M, 24,000 protein was present in only one of the normal breasts tested, being detected in some small ducts but not in fully developed alveoli. The significance of this unusual staining pattern, observed in the sweat glands as well as in the mammary gland, is unclear. Lactating breasts were negative for M, 24,000 protein. Since the highly differentiated cells of the alveoli synthesize mainly milk proteins during lactation, the M, 24,000 estrogen-regulated protein is probably not a milk-related protein, a result that agrees with our previous study (3). Gynecomastia samples showed no M, 24,000 protein. Since postpubertal development of gynecomastia may be due to abnormal secretion of not only estrogens but also prolactin, growth hormone, and pituitary gonadotropins (10), no clear conclusion may be drawn from the 3 cases studied.

This study shows M, 24,000 protein in the cytoplasm of maternal decidual cells. The recognition of decidual cells by the MAbs could be important, since at present, there are no histochemical methods to identify true decidual cells with certainty (18). The MAbs to M, 24,000 protein could be of value in the study of decidual transformation during implantation and placentation. The functions of the decidua have been associated with nutrition for the embryo, protection of the uterus against trophoblastic invasion, and prostaglandin synthesis (9, 18). However, the specific function of decidua is still unknown, and it will be interesting to relate M, 24,000 protein to decidual cell function. Furthermore, since in humans decidualization is influenced by estrogens but mainly by progesterone (1, 9), it seems logical to suggest that the M, 24,000 protein may be regulated by progesterone as well as by estrogen.

The tissues showing M, 24,000 protein mostly appear to have exocrine-secretory capacity. The varying reactivity found within a single cell population is also suggestive of alternating phases of synthesis and secretion, as commonly seen in cells with secretory functions. If M, 24,000 protein is indeed a secretory product, its concentration in estrogen target tissues and its increase in several cell lines after estrogen stimulation are consistent with estrogen regulation of this secretory function.

The presence of M, 24,000 protein in normal skin is not completely surprising, since skin has a common embryological origin with mammary gland and sweat glands. The M, 24,000 protein was present in skin biopsy samples taken from both males and females.

At present, it is premature to evaluate the presence of these MAbs to M, 24,000 protein in diagnostic tumor histopathology. There are nonbreast carcinomas (endometrial, prostate, skin, and bladder) which react with the MAbs. Moreover, one of the 7 normal breast samples was positive (although this biopsy sample considered “normal” could have been preneoplastic, even though it was taken from an area distant from a benign lesion). Thus, the MAbs to M, 24,000 protein are not sufficiently specific to be useful in the diagnosis of breast cancer. However, they may be useful for detecting hormone responsiveness in tumors. Approximately 25% of the breast carcinomas tested showed M, 24,000 protein, roughly the percentage which responded objectively to endocrine therapy. Cell lines lacking estrogen and progesterone receptors also lacked M, 24,000 protein, but the absence of M, 24,000 protein in the absence of the receptors would not alone explain its tumor distribution, since 49 to 55% of breast tumors are estrogen and progesterone receptor positive (20). However, our results show that even some estrogen and progesterone receptor-positive cell lines had a low fraction of cells positive for M, 24,000 protein, which might have been missed in biopsy sections. The heterogeneity of the tumor cell population within a single tumor has been emphasized before (17). Thus, a study directly evaluating the usefulness of the M, 24,000 MAbs for detecting hormone-responsive tumors needs to be done. Such a study will correlate the presence of the M, 24,000 protein in breast cancer tissues, using a quantitative M, 24,000 protein radiolmmunoassay (a technique more sensitive than histochemistry), with the presence of estrogen and progesterone receptors and ultimately with response of breast tumors to endocrine treatment.

ACKNOWLEDGMENTS

The authors thank Lenore C. Weinberg and Sandra E. Foster for their excellent technical assistance.

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Fig. 1. Human normal breast stained with the control MAb. No reaction is observed. Counterstaining was required to reveal the cells in the photomicrograph. C, connective tissue between the 2 lobules. Avidin-biotin-peroxidase with hematoxylin counterstain, × 150.

Fig. 2. Serial section next to the sample shown in Fig. 1. Positive immunostaining appears in one of the lobules but not in the other (arrows). Avidin-biotin-peroxidase complex immunostaining using a MAb to M, 24,000 protein without counterstain, × 150.

Fig. 3. Human normal uterus showing positive reaction of moderate intensity on myometrial cells. C, connective tissue. Arrow points to a blood vessel. Avidin-biotin-peroxidase complex immunostaining with hematoxylin counterstain, × 160.

Fig. 4. Human normal uterus showing positive reaction of moderate intensity on myometrial cells. Arrow points to a blood vessel. Avidin-biotin-peroxidase complex immunostaining with hematoxylin counterstain, × 140.

Fig. 5. Fallopian tube stained with the control MAb. No positive immunostaining was observed. Avidin-biotin-peroxidase complex immunostaining with hematoxylin counterstain, × 140.

Fig. 6. Same as Fig. 5, square, but stained with a MAb to M, 24,000 protein. A positive reaction on the cytoplasm of some epithelial cells is observed. Avidin-biotin-peroxidase complex immunostaining with hematoxylin counterstain, × 450.

Fig. 7. Placenta showing an area occupied by maternal decidual cells (arrows) and villi (left). H & E, × 100.

Fig. 8. Serial section next to the sample shown in Fig. 7 but immunostained using a MAb to M, 24,000 protein. Decidual cells appear selectively immunostained. Note lack of reaction on placental villi (V). Avidin-biotin-peroxidase complex immunostaining without counterstain, × 100.

Fig. 9. Decidual cells showing different immunostaining intensity on cytoplasm. Avidin-biotin-peroxidase complex immunostaining using a MAb to M, 24,000 protein with hematoxylin counterstain, × 200.

Fig. 10. Same as Fig. 9, square, showing in detail the different immunostaining intensity of cytoplasm of decidual cells. Avidin-biotin-peroxidase complex immunostaining without counterstain, × 500.

Fig. 11. Vagina showing M, 24,000 protein on epithelial cells. Note the lack of staining on vaginal stroma (S). Avidin-biotin-peroxidase complex immunostaining without counterstain, × 150.

Fig. 12. Normal skin showing M, 24,000 protein on epithelial cells. There is no reaction on the stroma (S). Avidin-biotin-peroxidase complex immunostaining without counterstain, × 150.

Fig. 13. MCF-7 cell pellet immunostained with a MAb to M, 24,000 protein. Note the strong reaction in the cytoplasm of the cells and its absence in cell nuclei. Avidin-biotin-peroxidase complex immunostaining without counterstain, × 550.

Fig. 14. T47D cell pellet immunostained with a MAb to M, 24,000 protein. Immunostaining is observed in only a few cells (compare with MCF-7 in Fig. 13). Avidin-biotin-peroxidase complex immunostaining without counterstain, × 600.

Fig. 15. MDA-MB-231 cell pellet immunostained with a MAb to M, 24,000 protein. No reaction is observed. Avidin-biotin-peroxidase complex immunostaining, × 450.

Fig. 16. T47D cell pellet after estradiol treatment, immunostained with a MAb to M, 24,000 protein. Note the increment of strongly immunostained cells as compared with Fig. 14. Avidin-biotin-peroxidase complex without counterstain, × 600.
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