Distribution of the $M$, 52,000 Estrogen-regulated Protein in Benign Breast Diseases and Other Tissues by Immunohistochemistry

Marcel Garcia, Guillermo Salazar-Retana, André Pages, Gilbert Richer, J. Domergue, Anne Marie Pages, Ghislaine Cavalle, Jean Michel Martin, Jean-Louis Lamarque, Bernard Pau, Henri Pujol, and Henri Rochefort


ABSTRACT

A secreted glycoprotein with a molecular weight of 52,000 is induced by estrogen in breast cancer cells and has been purified to prepare monoclonal antibodies. The protein has been detected in some breast cancers but not in normal breast and uterus. In order to study its potential value as a marker, we have tested by immunohistochemistry frozen sections of several normal and malignant tissues and of benign mastopathies. Among different tissues tested, the $M$, 52,000 protein was detected only in liver, sweat glands, and some sebaceous glands, and in malignant melanomas and some breast tumors. Other estrogen-responsive tissues (ovary, placenta, endometrium, etc.) gave negative results.

Immunodensitometric assay of the $M$, 52,000 protein in biological fluid revealed an elevated concentration in cyst fluid (0.5 to 7.4 ng/ml), pleural effusions of certain metastatic breast cancer, and sweat.

By immunohistochemistry, the $M$, 52,000 antigen was also detected in 42% of 129 benign mastopathies. Gynaecomastia, fibrous disease, fibroadenoma, and adenosis were mainly negative, whereas ductal hyperplasia and cysts were positive. The $M$, 52,000 protein was found mostly in proliferative ducts and in cysts but not in lobular hyperplasia and non-proliferative lesions without cyst. More $M$, 52,000 protein was found in postmenopausal patients than in premenopausal patients.

We conclude that the $M$, 52,000 protein is a marker associated with mammary cysts and proliferative ducts. On the basis of the increased risk of breast cancer in proliferative mastopathies, we suggest that the $M$, 52,000 protein is useful for predicting high-risk mastopathies acting as a marker associated with the proliferation of ductal tissue.

INTRODUCTION

An increasing number of monoclonal antibodies to mammary cancer antigens or purified proteins are available (1-7). These antibodies are used as markers of mammary tissue, as cancer associated markers, or as markers of hormone dependence in breast cancer; however, few markers if any (8) are available for detecting the early steps of cancerogenesis and for discriminating high-risk mastopathies (9, 10) from benign mastopathies, which have been defined as non-disease entities (11). Recently, Dupont and Page (12) have indicated that proliferative lesions are more exposed to breast cancer risk and some can be considered to be precancerous lesions, whereas the majority of these mastopathies (70%) are nonproliferative and not exposed to breast cancer risk. Since the current pathological grading of benign lesions is time consuming and may be subjective, biochemical markers would be more useful in helping pathologists to define these high-risk mastopathies.

We have developed several monoclonal antibodies (13) to an estrogen-regulated protein secreted by metastatic breast cancer cells in culture and defined by its molecular weight of 52,000 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14-16). Using immunoperoxidase staining of frozen sections of tissue biopsies in a preliminary study, we have detected this protein (or an immunologically related protein) in 75% of the breast cancers analyzed but not in endometrium or normal resting mammary glands (17).

In order to specify the clinical potential of this protein as a marker, we have studied its distribution in several normal and tumoral tissues and in 129 benign mastopathies of different histological types.

MATERIALS AND METHODS

Breast Biopsies. Breast biopsies were performed for diagnostic purposes in patients undergoing surgery for lumps. Most of the tissues were sent to pathologists for routine analysis and the remaining portions were immediately frozen in liquid nitrogen. Serial frozen sections were made from a tissue block, and one of 3 adjacent sections containing epithelial structures was analyzed for histological grading by a pathologist while the others were processed independently for immunodetection of the $M$, 52,000 protein and reviewed by two different observers.

Thirteen samples were subsequently excluded because they were found to be invasive or in situ mammary cancers or they lacked breast epithelial cells; 129 samples remained for analysis.

Immunohistochemical Staining of the $M$, 52,000 Protein. Eight-mm thick frozen sections, quickly fixed by cold acetone, were stained by the Sternberger (18) peroxidase-antiperoxidase method as previously described (17), using the DTE3 or M1G8 antibody (5 pg/ml) in some cases. Each staining series was associated with a positive control performed with a positive breast cancer tissue and a negative control performed with an irrelevant monoclonal antibody (IgG1; MOPC21, Bionetics) of the same subclass. In 30 cases duplicate experiments on serial sections from the same tissue block gave similar results. The staining was quantified according to the estimated percentage of positive epithelial cells in the total of 2,000 to 50,000 cells examined and was graded from 0 (no positive cells) to + (1 to 5% positive epithelial cells) and to ++ (>5% positive epithelial cells). In positive benign mastopathies, the staining intensity was not taken into consideration since it was high in the majority of cases.

Each section was graded for staining independently by two observers. In case of a discrepancy, a consensus was reached by the two observers or additional sections were analyzed.

Pathological Examination. This was performed after fixation and staining by hematoxylin and eosin. Each biopsy was classified according to the methods of Azzopardi (9) and Haagensen et al. (10) on the basis of cystic or noncystic lesions, and according to the criteria of Page et al. (19) on the basis of proliferative or nonproliferative lesions. The clinical history of each patient, hormonal status, age, and treatment were recorded.

Immunodensitometric Assay of the $M$, 52,000 Protein in Fluids. This was performed by a two-site solid phase assay as previously described (13). An adaptation of this assay to biological fluids was performed after validation of the assay by recovery experiments. Data were expressed as $M$, 52,000 protein ng per ml, according to a standard curve assayed in parallel using the secreted $M$, 52,000 protein purified from human breast cancer (MCF7) and quantified by silver-stained sodium...
Distribution of the $M_{52,000}$ Protein in Benign Breast Disease

The binding of the monoclonal antibody D7E3 was detected in frozen tissue sections by an indirect immunoperoxidase technique described in “Materials and Methods.” Tissues were classified as positive when at least 1% of one cell type population was specifically stained.

<table>
<thead>
<tr>
<th>Negative</th>
<th>Positive</th>
</tr>
</thead>
</table>
| Breast (8)
Endometrium (9)
Ovary (4)
Kidney (3)
Liver (1)
Thymus (4)
Placenta (3)
Brain (1)
Prostate (1)
Parotid (2)
Appendix (1)
Amygdala (1)
Spleen (2)
Skeletal muscle (3)
Lymph nodes (3)
Epidermis (10)
Dermis (10)
Hair follicle (10)
Sebaceous glands (3) |

- Numbers in parentheses, number of different patients.

Statistics. Statistical significance of the difference between two groups was determined by the $\chi^2$ test.

RESULTS

Tissue Distribution of the $M_{52,000}$ Protein. Preliminary studies indicated that the $M_{52,000}$ protein was not an estrogen-regulated marker like the progesterone receptor or the $M_{24,000}$ protein (4), since it was absent from endometrium (17). We completed the tissue distribution analysis of the $M_{52,000}$ protein by studying different normal and tumoral tissues (Table 1). The protein, which was initially detected in some proliferative mammary cancer cells, was not detected in other endocrine-related tissues (endometrium, placenta, ovary, prostate) and other tissues. The only other positive tissues were liver and skin. In liver, the staining was homogeneous throughout the tissue with a relatively high nonspecific background. In skin, the only positivity was seen in some sebaceous and in sweat glands. This last staining is in agreement with the high content of the $M_{52,000}$ protein in the different histological types is summarized in Table 2. All gynecomastias were negative; fibroadenoma (Fig. 1d) and fibrous mastopathies were most often negative. When the different histological types were pooled into proliferative (high risk) and nonproliferative (low risk) lesions, there was a clear correlation between proliferation (Table 3) and risk of developing breast cancer (Fig. 2) on one hand and positivity for the $M_{52,000}$ protein on the other hand. Among the 23% proliferative lesions, 79.3% were positive for the $M_{52,000}$ protein. The negative cases were all lobular hyperplasias. Thirty-two % of the nonproliferative lesions were positive for the $M_{52,000}$ protein (Table 3). This positivity was due mostly to the presence of cysts. The value of the $M_{52,000}$ protein staining in predicting high-risk mastopathies is therefore relatively high but may vary according to the histological type of the lesion. The $M_{52,000}$ negative lesions have a 91.5% chance of being nonproliferative. The only proliferative lesions that were not detected by this immunostaining were simple lobular hyperplasia (sclerosing adenosis) or lobular hyperplasia with atypia. In case of $M_{52,000}$ protein-positive lesions, the predictive value is not good; however, by excluding nonproliferative cysts, a positive $M_{52,000}$ protein staining indicates a 60.5% chance of proliferative disease and a 39.5% chance of nonproliferative lesion. We conclude that the $M_{52,000}$ protein is associated with the proliferation of ductal mammary cells and in the presence of cyst(s) 2 pathological criteria that are known to increase breast cancer risk.

Clinical Parameters and the $M_{52,000}$ Protein in Benign Mastopathies. The clinical follow-up of the patients has been too short to allow a general conclusion about the actual prog-

Table 1. Immunoperoxidase staining of $M_{52,000}$ protein in human tissues

<table>
<thead>
<tr>
<th>Normal tissue</th>
<th>Cancer</th>
<th>Benign tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Breast (8)</td>
<td>Liver (9)</td>
<td>Breast (8)</td>
</tr>
<tr>
<td>Endometrium (9)</td>
<td>Sweat glands (4)</td>
<td>Melanoma (3)</td>
</tr>
<tr>
<td>Ovary (4)</td>
<td>Kidney (3)</td>
<td>Melanoma (5)</td>
</tr>
<tr>
<td>Lung (1)</td>
<td>Thymus (4)</td>
<td>Lung (4)</td>
</tr>
<tr>
<td>Placenta (3)</td>
<td>Brain (1)</td>
<td>Brain (1)</td>
</tr>
<tr>
<td>Prostate (1)</td>
<td>Parotid (2)</td>
<td>Lymphoma (1)</td>
</tr>
<tr>
<td>Appendix (1)</td>
<td>Amygdala (1)</td>
<td>Bladder (1)</td>
</tr>
<tr>
<td>Spleen (2)</td>
<td>Striated muscle (3)</td>
<td></td>
</tr>
<tr>
<td>Lymph nodes (3)</td>
<td>Epidermis (10)</td>
<td></td>
</tr>
<tr>
<td>Dermis (10)</td>
<td>Hair follicle (10)</td>
<td></td>
</tr>
<tr>
<td>Sebaceous glands (3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^2$ M. Garcia et al., submitted for publication.
M, 52,000 ESTROGEN-REGULATED PROTEIN IN BENIGN BREAST DISEASE

Fig. 1. Immunoperoxidase staining with a monoclonal antibody to M, 52,000 protein on cryosections from benign mastopathies. All sections were immunostained with the monoclonal antibody D7E3 and a peroxidase-antiperoxidase technique (see "Materials and Methods"). Controls for nonspecific binding in serial sections (not shown) were negative. The sections were not counterstained. a, ductal hyperplasia. The epithelial cell layer had to be at least 3 cells thick to be accepted for inclusion in this group (19). Strong staining was restricted to the cytoplasm of epithelial cells and contrasted with the unstained pale ovoid nuclei. No reaction can be seen in the stroma. × 330. b, cystic lesion with papillary apocrine change. The cytoplasms of epithelial cells lining the cysts or forming papillary projections in the lumen were strongly stained. × 82. c, moderate adenosis. Lobular cells were not stained with the antibody, in contrast with a portion of microcyst seen on the right. × 165. d, fibroadenoma. Nonproliferative ductal cells and stromal cells were negative. × 320.

nostic value of M, 52,000 protein detection in mammary biopsies. Since the protein is regulated by estrogen in breast cancer cell lines, we anticipated variations in the M, 52,000 protein content as a function of the hormonal status of the patients at the time of biopsy. No significant difference was found between the follicular (18 cases) and luteal (17 cases) phases of the menstrual cycle.

By contrast, the percentage of tumors positive for the M, 52,000 protein was higher in postmenopausal (73%, 15 cases; P < 0.02) than in premenopausal patients (39.5% positive, 76 cases) and in patients over 45 yr of age (56%) compared to younger patients (37% positive, 73 cases; P < 0.01). The increase in the M, 52,000 protein with age may be due to a lower level of progesterone and an unopposed estrogen effect (20) or may be associated with the higher risk of benign breast disease observed after the age of 45. In the 18 cases of 70 that had a family history of breast cancer (in the mother, grandmother, sister, or aunt), there was no increase in the percentage of the M, 52,000 protein, suggesting that the protein is a parameter independent of family history. A more complete study and a prolonged follow-up of the patients are required before a definitive conclusion can be reached concerning the clinical significance of this marker.

Assay of the M, 52,000 Protein in Mammary Cyst Fluid and Other Biological Fluids. In 38 patients, tissue biopsies were not taken but fluid was aspirated by needle puncture from gross mammary cysts. The M, 52,000 protein content was measured by immunoradiometric assay and found to vary from 0.5 to 7.4 μg/ml depending on the patient, with a mean value of 3 μg/ml. This value is much higher than the basal level detected in plasma but similar to the M, 52,000 protein concentration in sweat. These results were consistent with positive immunoperoxidase staining of mammary cysts and indicated that the M, 52,000 protein is always highly concentrated in cyst fluid. The significance of the variable level is not yet known. There was no significant correlation with the menstrual cycle phase at collec-
Table 2  Immunodetection of M, 52,000 protein and breast cancer risk in benign breast disease

| Disease                  | No. of cases | 0 | +* | ++ | Risk factor
|--------------------------|--------------|---|----|----|-------------
| Gynecomastia             | 4            | 4 | 4  | 4  | x1.0        |
| Fibroadenoma             | 16           | 11| 4  | 1  | x1.0        |
| Adenosis                 | 23           | 22| 5  | 1  | x1.0        |
| Sclerosing adenosis      | 6            | 5 | 1  | 1  | x1.0        |
| Fibrous mastopathy       | 39           | 30| 8  | 1  | x1.0        |
| Total group 1*           | 88           | 72(82)| 13(15)| 3(3)|           |
| Cystic disease           |              |   |    |    |             |
| <3 mm                    | 13           | 2 | 3  | 8  | x1.4 to 4.0 |
| >3 mm                    | 5            | 2 | 4  | 3  | x1.4 to 4.0 |
| Ductal hyperplasia       | 21           | 2 | 7  | 14 | x1.8 to 3.0 |
| Papilloma solitary       | 1            | 1 | 1  | 1  | x2.7        |
| Atypical lobular hyperplasia | 1     | 1 | 1  | 1  | x4.0        |
| Total group 2            | 41           | 3 (7)| 12 (30)| 26 (63)|          |
| Total groups 1 and 2     | 129          | 75(58)| 25 (19)| 29 (22.5)|         |

a  +, 1 to 5% positive epithelial cells; ++, >5% positive epithelial cells.

b  Relative risk factor according to the methods of Azzopardi (9) and Haagensen et al. (10).

c  Staining in ductal structure.

d  Staining percentages of groups 1 and 2 are significantly different at P < 0.001.

e  Numbers in parentheses, percentage.

Table 3  Immunostaining of the M, 52,000 protein in proliferative and nonproliferative benign lesions of the breast

| Type of lesion          | Total no. of cases (125) | M, 52,000 protein | % of positive cases (%32.2) | Risk factor
|-------------------------|--------------------------|------------------|----------------------------|-------------
| Proliferative*          | 29                       | 6               | 8                          | 15          |
|                         |                          |                 | 79.3                       | 1.7         |
|                         |                          |                 |                            | 3.2         |
| Nonproliferative*       | 96                       | 65              | 17                         | 14          |
|                         |                          |                 | 32.3                       | 0.9         |
|                         |                          |                 |                            | 1.2         |
| With cysts              | 78                       | 63              | 12                         | 3           |
|                         |                          |                 | 19.2                       |             |
| With cysts              | 18                       | 2               | 5                          | 11          |
|                         |                          |                 | 88.9                       | 1.2         |

a  +, 1 to 5% positive epithelial cells; ++, >5% positive epithelial cells.

b  Relative risk factors were obtained from Dupont and Page (12) with or without a family history of breast cancer.

c  Proliferative lesions contained ductal hyperplasias without (9 patients) or with cysts (12 patients), papilloma (1 patient), sclerosis adenosis (6 patients), and atypical lobular hyperplasia (1 patient).

d  Nonproliferative lesions are the other lesions in Table 2 excluding the four gynecomastias. The difference between the M, 52,000 protein staining in proliferative and nonproliferative groups is significant at P < 0.001.

Figure 2. Relationship between M, 52,000 protein staining and the relative risk of breast cancer in 129 benign mastopathies. Benign lesions were classified into two groups according to their association with normal (88 cases) or increased risk (41 cases) of breast cancer, based on their histological type (see Table 2). □, nonimmunoreactive lesions; immunoreactive lesions: 1, 1 to 5% positive epithelial cells; 2, >5% positive cells. K, thousands.

Table 4  Immunoassay of the M, 52,000 protein in biological fluids

<table>
<thead>
<tr>
<th>Fluid type</th>
<th>No. of cases</th>
<th>M, 52,000 protein (ng/ml)</th>
<th>±SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cyst</td>
<td>38</td>
<td>2946 ± 1780</td>
<td>500–7400</td>
<td></td>
</tr>
<tr>
<td>Pleural effusion from breast cancer</td>
<td>9</td>
<td>1107 ± 1246</td>
<td>155–3940</td>
<td></td>
</tr>
<tr>
<td>Sweat</td>
<td>3</td>
<td>1966 ± 464</td>
<td>1500–2600</td>
<td></td>
</tr>
<tr>
<td>Ovary cyst</td>
<td>3</td>
<td>118 ± 80</td>
<td>7–188</td>
<td></td>
</tr>
<tr>
<td>Ascitic fluid (ovary cancer)</td>
<td>1</td>
<td>210</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal plasma</td>
<td>13</td>
<td>93 ± 22</td>
<td>69–144</td>
<td></td>
</tr>
</tbody>
</table>

Unpublished results.

Discussion

The study of the tissue distribution of the M, 52,000 protein provides the first information concerning its predictive value for high-risk mastopathies and its potential value as a tissue-specific marker for detecting the metastasis of mammary cancer and malignant melanomas. Another potential application for the detection of bone marrow and lymph nodes metastasis and/or the grading of mammary and melanoma cancers will be addressed in another collaborative study. In this study of 129 cases of benign breast disease, the M, 52,000 protein was found to be highly correlated with ductal hyperplasia which has previously been demonstrated to be exposed to breast cancer (12, 19), and with cysts (10). The predictive value of M, 52,000 protein detection in tissue biopsy appears to be excellent when the tissue is negative (91.5% chance of having nonproliferative lesions) but poorer when the tissue is positive (43% chance of proliferative lesions). This is due to the fact that all cysts, whether proliferative or not and some nonproliferative ducts, are positive for the M, 52,000 protein and that the nonproliferative group is 3.3-fold higher than the proliferative group. The positivity of cysts is in full agreement with the high concentrations of the M, 52,000 protein in cyst fluid and further studies are required to determine whether the assay of the M, 52,000
protein in cyst fluid is useful for discriminating between high- and low-risk cysts. We propose that the immunohistochemical detection of the M, 52,000 protein along with the pathological examination of tissue sections and the presence of a family history of breast cancer may be an additional high-risk factor to take into account when defining the treatment of these benign breast diseases. By improving the prediction of high-risk mastopathies, it might also help to improve the prevention of breast cancer. More extensive studies with a clinical follow-up of these patients will demonstrate whether or not the M, 52,000 protein is a precancerous marker of ductal lesions in mammary tissue.

The M, 52,000 protein is secreted in metastatic breast cancer cells under estrogen stimulation, and the hormonal regulation of this protein in the normal mammary gland (21) and in benign mastopathies is possible. Benign mastopathies contain receptors for estrogen and progesterone (22, 23); moreover, the proliferative lesions appear to contain higher receptor concentration (24, 25) and it is likely that these tissues are hormone responsive. Monoclonal antibodies to the estrogen receptor and to the M, 52,000 protein are two useful probes for detecting these proteins in biopsied benign mammary lumps. The fact that the M, 52,000 protein is found mostly in proliferative ductal lesions is also in agreement with the recent finding that the purified M, 52,000 protein can stimulate the growth of breast cancer cells in vitro (15, 26). It also suggests that an increased production of the protein may be associated with early stages of mammary carcinogenesis. The present immunohistochemical study also confirms the cellular heterogeneity of benign mastopathies, as already shown with other markers.

Negative staining may not always mean that the tissue contains no antigenically related M, 52,000 protein. Using a more sensitive two-site immunometric assay of cell extracts, we have found that normal resting mammary cells contain small amounts of the M, 52,000 protein.5 Comparative studies of cancer cell lines using immunoperoxidase staining and immunoradiometric assay indicate that the threshold between histochemically positive and negative cells is approximately 30 ng/μg DNA.6 This sensitivity is low enough to discriminate between a high and low cellular content of the protein. False negative staining may also be found when positive proliferative cells are absent in the tissue sections examined but present in another region of the mastopathy.

This is the first study showing that the M, 52,000 protein is a potential tissue marker for distinguishing high- (proliferative) from low-risk (nonproliferative) benign breast disease. More extensive studies are obviously needed in order to confirm and extend this assumption, including a clinical follow-up of these patients. Other growth-associated proteins may also be useful in studying high-risk proliferative mastopathies (27–29). The advantages of the M, 52,000 protein is its relative tissue specificity and its mitogenic activity (26), which may also facilitate its use in other diagnostic and therapeutic applications.

REFERENCES

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