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Gelatinase Levels in Male and Female Breast Cancer

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Breast cancer is a common disease in females but very rare in males, in whom it shows a more metastatic behavior, and a worse prognosis. Matrix metalloprotease-2 (MMP-2) and MMP-9 are proteolytic enzymes balanced by tissue inhibitor of MMP-2 (TIMP-2), commonly involved in cancer metastasis. This is the first study on gelatinolytic activity in male breast cancer patients, compared to that in female patients. In cancer tissues, both gelatinases were more expressed than in normal samples, being and more concentrated in male than in female patients. TIMP-2 levels were slightly increased in normal compared to those in cancer tissues and more concentrated in males than in females. Immunostaining showed that in male cancer tissues MMP-2 and MMP-9 staining was more intense and diffuse than in female cancer tissues, while no differences were observed regarding TIMP-2. In conclusion, the increased expression of gelatinases in male breast cancer patients together with anatomical features might explain the high tendency toward metastasis and the worse prognosis.

Key Words: **male breast cancer; MMP-2; MMP-9; TIMP-2; gelatinases.**

Male breast carcinoma is a rare type of malignancy and accounts for only 1% of all mammary carcinomas (1). In spite of this, it is of great interest because of its biological differences from female breast carcinoma, including higher percentages of hormone positivity and the anatomic aspects (2), whereas the histopathological features are similar. Male patients have lower overall survival than females, although the smaller tumor size and higher hormone dependency would lead one to expect the reverse (3, 4). Nevertheless, in males breast cancer is more aggressive, showing a higher ability to spread to other tissues, due to anatomic reasons (2, 4). Breast cancer cells need to cross the basement membrane (BM) and invade the surrounding tissues; this occurs in female as well as in male patients but in the latter, cancer cells reach the subareolar and axillary lymph nodes and blood circulation more easily (2). In this regard, proteolysis of the BM is a crucial step for allowing cancer cells to penetrate the extracellular matrix (ECM) tissue boundaries and spread through the surrounding tissues.

Matrix metalloproteases (MMPs) are a family of enzymes with proteolytic activity secreted in a latent form and activated at the cell surface by a membrane-type-1-MMP (MT1-MMP) (5, 6). MMP-2 and MMP-9, two members of this family, are inhibited by the tissue inhibitor of MMP-2 (TIMP-2) (7). These two enzymes, known as gelatinases, participate in a number of different functions such as tissue remodeling, embryogenesis, and development (8). The implication of gelatinases in cancer metastasis has been suggested by several reports, showing their distribution along the BM where proteolytic remodeling occurs (9, 10). The expression and the role of gelatinases in the breast during development and cancer have been largely documented, and the possibility that these enzymes may be regulated by sex hormones has been postulated: this would have important clinical implications (11). However, all these reports have been focused on breast cancer in females and no reports on male breast cancer are so far available.

The goal of this study is to investigate and quantify the expression of MMP-2, MMP-9, TIMP-2 in male breast cancer tissue, where the hormone differences offer the chance to study the same type of cancer in a different environment and condition from the more common female form.

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TABLE 1

Clinical Characteristics of Breast Carcinoma Patients

	Male	Female
Number of patients	18	18
Age, years mean value (range)	51 (41–79)	53.5 (36–81)
Pts. with estrogen receptor positivity (%)	12/18 (66.6%)	11/18 (61.1%)
Histological type		
Infiltrating ductal carcinoma	16	16
Infiltrating lobular carcinoma		1
Multifocal infiltrating carcinoma	2	1
Frequency of lymph node involvement	35%	83%
Tumor size (cm. mean value ± SD)	3.4 ± 2.4	3.3 ± 1.2

MATERIALS AND METHODS

Patients. We studied tissues from 18 male and 18 female patients with breast cancer, and from 4 male and 7 female control subjects that underwent surgery at the Surgery Department of the Oncological Institute for gynecomastia or breast reduction, respectively. All samples were studied under the same experimental conditions, being snap frozen in liquid nitrogen immediately after resection, while part of the tissue was included in 3.7% formaldehyde and processed for routine histology. Histological diagnosis, lymph node involvement, and hormone receptor status were investigated in each patient; the results are summarized in Table 1.

Gelatin zymography. Frozen tissues were ground by a tissue micro-dismembrator (B-Braun, Melsungen Ag, Germany) resuspended in TRIS Hepes, pH 7.5 buffer, homogenated in ice, centrifuged at 2000 rpm at 4°C for 5 min, and the supernatant was collected and used for the protein concentration measurement and for the gelatin zymography evaluation.

Protein concentration was measured by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL). Gelatinases quantification was performed by zymography using HT1080 conditioned medium as standard control. This conditioned medium is a well known standard for detecting MMP-2 and MMP-9 activity (12). Serial dilutions of the HT1080 preparation as well as the MMP-2 and MMP-9 commercial preparations at known concentrations were analyzed by gelatin zymography, gels were then acquired and quantified with an image-analysis-software system (Image Master 1D Prime, Pharmacia Biotech, UK). As a further control, we determined the gelatinase concentrations of serial dilutions of HT1080 conditioned medium using the ELISA kit. In each experiment, a standard curve using HT1080 at known concentrations was included, and serial dilutions of the sample were performed with a linear relationship between sample dilution and gelatinolytic activity.

Gelatin zymography was performed as previously described (13). Briefly, samples were normalized for protein concentration, and 18 μg of each were loaded per lane. Gels were incubated overnight, stained with Coomassie blue, and destained with a methanol acetic acid solution until gelatinolytic areas appeared evident as unstained bands in a blue stained gel. Gels were then acquired and processed as previously described.

Immunohistochemistry. We performed indirect alkaline phosphatase immunohistochemistry as previously described (12). Briefly, frozen tissues were included in Optimal Cutting Temperature 4583 (OCT) embedding compound (Miles Laboratories, Inc, Naperville, IL) and 5-μm-thick sections were serially cut with a microtome (Microtom, HM 505E, Carl Zeiss Oberkochen, Germany), and collected on appropriate glass slides (Sigma Chemical Co.). Sections were fixed in a cold chloroform/acetone mixture for 10 min, air-dried, incubated with different monoclonal antibodies directed against MMP-9 and TIMP-2 purchased from Calbiochem (San Diego, CA) and with a polyclonal antibody against MMP-2 purchased from Chemicon (Temecula, CA) diluted in RPMI medium with 10% added fetal calf serum (FCS). Then after gentle washing, sections were incubated with a proper secondary antibody (Dako, Denmark) for 30 min in a humidified chamber. Sections were washed and incubated with alkaline phosphatase anti-alkaline phosphatase complexes. Staining was developed with red fuchsin chromogen, and then sections were abundantly washed for 20 min. Finally, they were mounted with glycerol and examined with a Nikon Eclipse photomicroscope (Nikon, Corp.). Immunohistochemistry was quantified calculating the area of staining using an image-analysis software assisted system (Lucia, Nikon, Corp), in 10 randomly chosen microscopic fields for each tissue specimen; the mean and standard deviation (SD) are reported herein.

Detection of MMP-2, MMP-9, by ELISA. We quantified the concentrations of both gelatinases in the samples already processed for zymography. Pro-MMP-2, and pro-MMP-9, were determined by ELISA kits (Amersham Pharmacia biotech, UK) based on a double sandwich system whereby the antigen is captured by a primary antibody coated on the well and after extensive washes a secondary antibody conjugated with horseradish peroxidase is added to immobilize the immunocomplex. In both cases positivity is revealed by tetramethylbenzidine and optical density was read at 450 nm in a microtiter plate spectrophotometer. In addition, MMP quantification was confirmed by gelatin zymography, yielding overlapping results.

Statistical analysis. Student's test was used to determine the 99% confidence intervals (CI) for the MMP-2, MMP-9 and TIMP-2 levels in tissue samples. The correlation between the ELISA and the gelatin zymography quantification of pro-MMP-2 and pro-MMP-9 was studied with the Pearson correlation coefficient.

RESULTS

MMP-2, MMP-9 and TIMP-2 Concentrations in Breast Cancer Tissues

The gelatin zymography assay was optimized to quantify MMP-2 and MMP-9 in both latent and active form in breast tissue sample preparations. To ensure the reliability of the measurements, pro-MMP-2 and pro-MMP-9 concentrations were determined in parallel using the ELISA test and gelatin zymography. For each sample, ELISA results represent the average of two distinct measurements, and gelatin zymography results the average of four different experiments. In male patients, pro-MMP-2 levels measured by ELISA and gelatin zymography were 33.7 ± 15.9 and 38.5 ± 22.5 (ng/mg protein), respectively; while pro-MMP-9 levels were 1.9 ± 1.6 and 1.6 ± 1.1 (ng/mg protein), respectively. In female patients pro-MMP-2 levels measured by ELISA and gelatin zymography were 8.5 ± 8.4 and 8.6 ± 9.6 (ng/mg protein), respectively; while pro-MMP-9 levels were 0.99 ± 0.87 and 1.0 ± 0.83 (ng/mg protein) respectively. Furthermore, there was a very high correlation between ELISA and gelatin zymography results for both pro-MMP-2 and pro-MMP-9 in each sample (r = 0.98 and 0.93, respectively), as shown in Fig. 1. As shown in Fig. 2, the latent

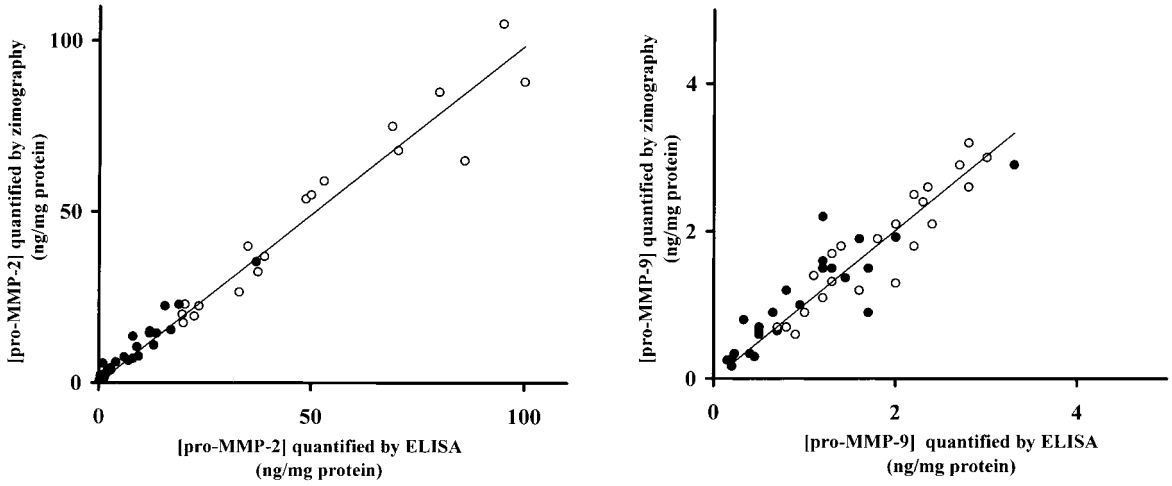

FIG. 1. Correlation between pro-MMP-2 and pro-MMP-9 quantification by ELISA and gelatin zymography. ELISA and gelatin zymography measurements of pro-MMP-2 levels show a strong correlation ($r = 0.98$), as do measurements of pro-MMP-9 ($r = 0.93$). These results were obtained in males (empty circle) and females (filled circle).

and active forms of both gelatinases were more evident in male than in female cancer tissues, while in normal subjects of both sex the gelatinolytic activity was very low. The total forms of MMP-2 and MMP-9 were quantified as above and the results are shown in Fig. 3. MMP-2 concentrations were significantly higher in male than in female breast cancer tissue (81.5 ± 36.0 vs 30.5 ± 27 ng/mg protein, $P < 0.02$), but similar in normal male and female breast tissue (4.6 ± 1.2 vs 6.7 ± 1.5 ng/mg protein). Furthermore, MMP-9 was more concentrated in male compared to female cancer tissue (2.5 ± 1.3 vs 1.0 ± 1 ng/mg protein, $P < 0.001$); similar in male and female breast tissue (0.1 ± 0.01 vs 0.1 ± 0.04 ng/mg protein). These data show that our method for quantifying latent and active gelatinases is reliable and easy, but cheaper than ELISAs.

TIMP-2 levels determined by ELISA were more concentrated in male than female cancer tissues (17.8 ± 9.2 vs 6.5 ± 4.4 ng/mg protein, $P < 0.001$); while in normal tissues they were 18.8 ± 4.6 vs 7.9 ± 6.6 ng/mg protein $P = 0.05$, in male and female subjects, respectively.

In short, the latent and the active forms of MMP-2, and MMP-9 were strongly increased in cancer compared to normal tissues in both sexes, while TIMP-2 levels were similar in cancer and normal breast tissues. Furthermore, both MMP-2 and MMP-9 concentrations were higher in males than females.

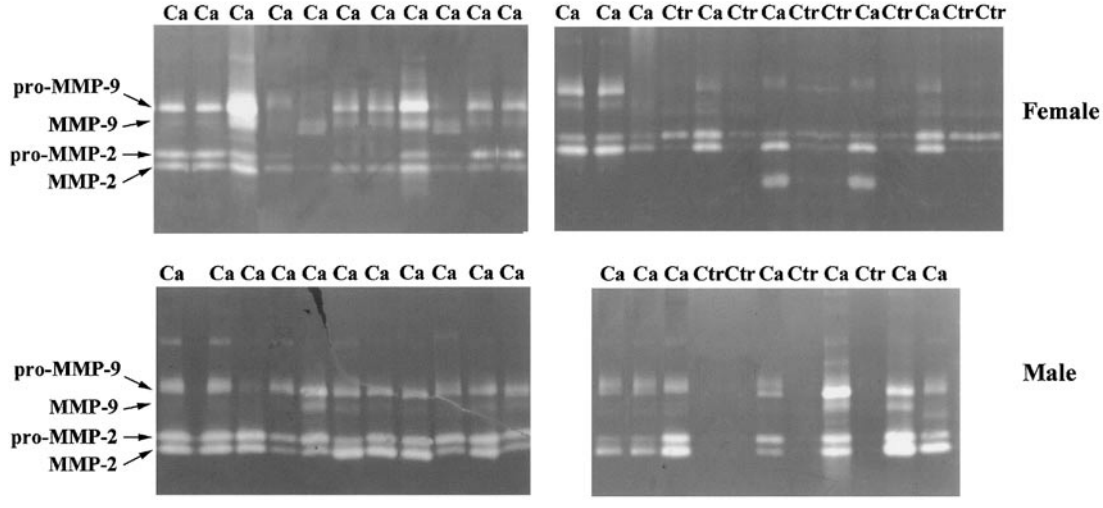

FIG. 2. Gelatin zymography of male and female tissues. MMP-2 and MMP-9 activity was more evident in cancer than in normal tissue preparations, and in male than in female cancer tissues.

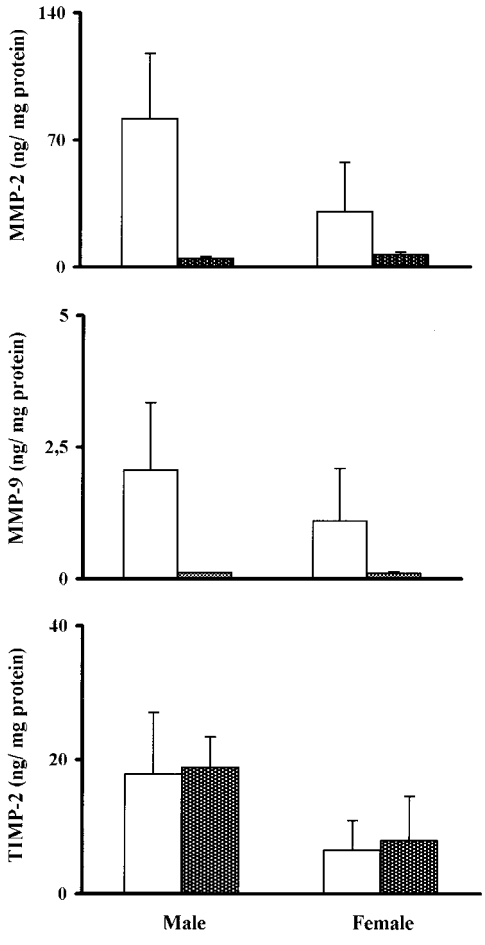

FIG. 3. MMP-2, MMP-9, and TIMP-2 levels in male and female breast carcinoma patients. MMP-2 and MMP-9 concentrations were higher in cancer (empty bar) than in normal (filled bar) tissues. Moreover, both gelatinases showed higher levels in male than in female patients. TIMP-2 was slightly more concentrated in normal subjects compared to cancer patients of both sexes.

MMP-2, MMP-9, and TIMP-2 Expression in Breast Cancer Tissue

Normal and cancerous sections of male and female breast tissue were processed at the same time under the same experimental conditions. MMP-2 and MMP-9 were strongly expressed in male breast cancer tissues but weakly expressed in the normal tissues (see Fig. 4). Both were mainly present in the extracellular space, distributed along the advancing edge of the cancer, at the cell BM contact, or present in the stroma surrounding cancer cells. In the normal male tissues, both enzymes were weakly expressed in the undeveloped gland. In female tissues, MMP-2 and MMP-9 appeared distributed with a similar pattern to that observed in the male patients: the staining was much stronger than in the normal tissues, although it was less intense than in the male cancer tissues. MMP-2 and MMP-9 staining were quantified, and as shown in Fig. 5, in male both gelatinases were more expressed than in female breast cancer tissues, this confirming above described results.

Instead, the intensity of TIMP-2 staining appeared similar between normal and cancer tissues, in both male and female patients. It was localized mainly in the stroma surrounding the glandular structure in the normal tissues, while it showed a diffuse pattern in the breast cancer tissues (Fig. 4).

In short, MMP-2 and MMP-9 were more expressed in cancerous than normal tissues, whereas TIMP-2 was similarly expressed in normal and cancer tissue. The increased expression of gelatinases suggest the presence of an impaired balance of proteolytic activity in breast cancer tissues, in particular in male patients.

DISCUSSION

Breast cancer is one of the most common malignancies in females but is very rare in males (1). Although breast cancer in males is generally smaller, more commonly hormone positive, and histologically similar to that in females, the prognosis and long term survival rates are worse (2–4). This seems to be related to more invasive and metastatic behavior of the cancer in male than female patients. MMPs have been reported to be involved in metastasis formation in several types of cancer, and several studies have reported their role in female breast tumor (14–16). However, so far no data are available on MMPs expression in males.

This is the first report in which the proteolytic activity of male breast cancer tissue has been investigated and compared to that of female patients, by means of a reliable gelatin zymography quantification method we developed to measure the latent and active forms of MMP-2 and MMP-9.This yielded overlapping results with those obtained by the commercially available ELISA. Our results suggest that there is a stronger proteolytic activity in male compared to female breast cancer patients; we base this conclusion on the following results: (1) pro-MMP-2 and pro-MMP-9 concentrations measured by ELISA and gelatin zymography were higher in male than in female patients; (2) active MMP-2 and MMP-9 tissue concentrations were more elevated in male than female; (3) MMP-2 and MMP-9 staining was more intense and diffuse in male than female breast cancer tissue.

MMP-2 and MMP-9 proteolytic activity has been suggested to play a role in remodeling of the breast during pregnancy and lactation as well as during cancer diffusion (11, 17). Proteolysis of BM components such as Laminin-5, strongly expressed in the breast, has been demonstrated to stimulate epithelial cell migration (18, 19). For instance, we have shown that

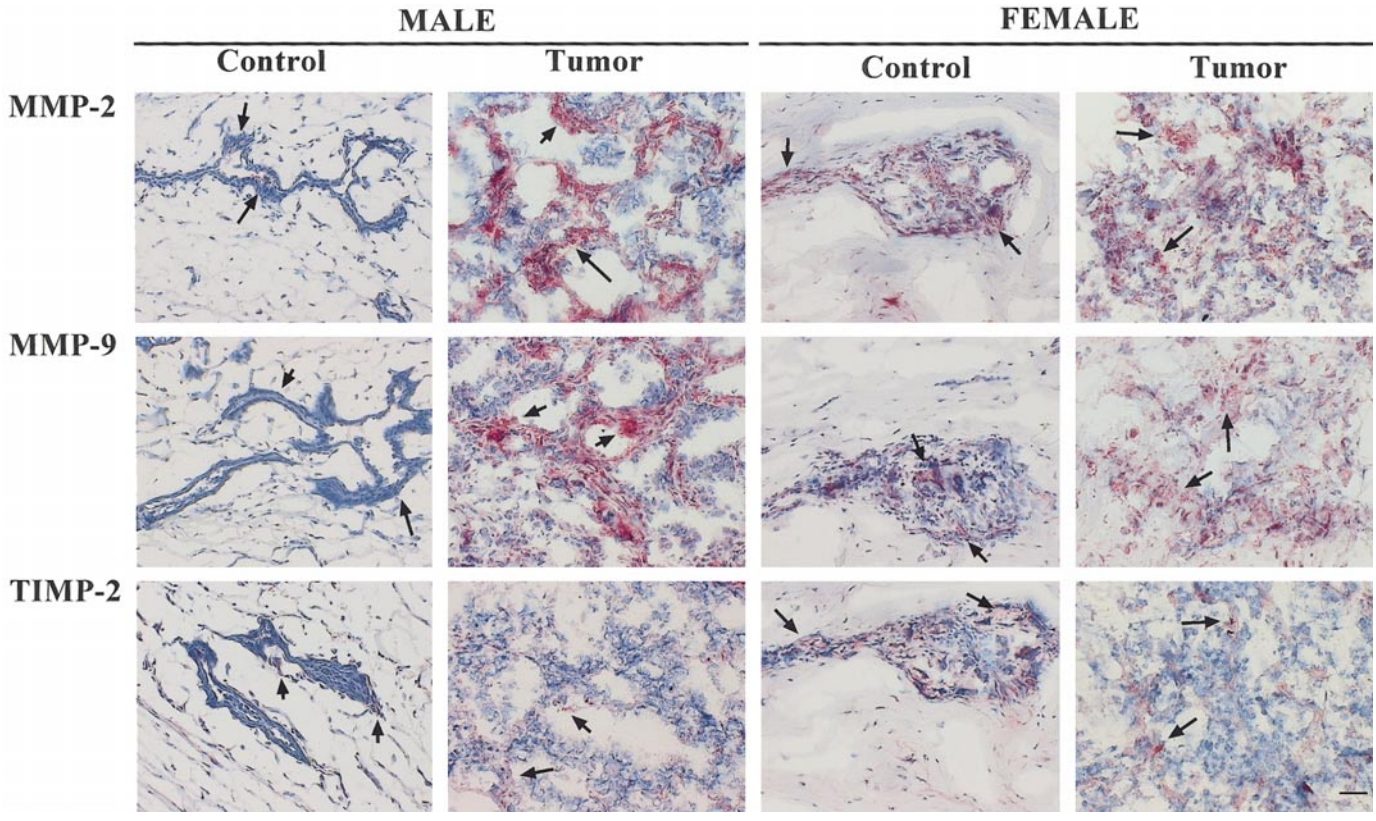

FIG. 4. MMP-2, MMP-9, TIMP-2 expression in breast cancer tissue. In male and female cancer tissues, MMP-2 and MMP-9 were concentrated along the BM (black arrows), near the advancing edge, mainly localized in the extracellular space. In males, there was more intense staining than in female patients. In control tissues obtained from healthy subjects, the expression of MMP-2 and MMP-9 was very weak. TIMP-2 has a similar distribution to that of MMP-2 and MMP-9 but a much weaker staining, not substantially different from that in control tissues. Scale bar, 5 μm.

MMP-2 is localized at the tip of the end buds, where the epithelial cells cross the BM to penetrate the fat pad so that new branching morphogenesis occurs (11). MMP-2 expression is likely under sex steroid regulation, and this is consistent with the fact that estrogen receptor-positive female breast cancer has a better prognosis because it is susceptible to anti-estrogen treatment (20). In addition, such therapy has been reported to be able to inhibit proteolytic activity and breast cancer epithelial cell motility *in vitro* (21, 22). Nevertheless, all these data contrast with the finding that male breast cancer, although featuring a higher percentage of hormone positive cases, has worse prognosis (2–4). The interaction between epithelial breast cells and stromal tissue, is crucial for gland development, but in males this interaction is limited because the gland is undeveloped (23). This substantial histological difference could be responsible for uncontrolled hormone regulation of gelatinase activity or alternatively, mechanisms resulting in evasion of hormone control could be present in male breast cancer. In either case the higher facility of cancer cell diffusion in male could be caused by anatomic reasons (2), and further facilitated by the proteolytic activity (14–16). In particular, the increased gelatinase levels in absence of a significant increase in TIMP-2 might shift the balance in favor of proteolysis, and this could be crucial to the development of metastases, as observed in other malignancies (24, 25). Several studies have been reported on the use of synthetic or natural MMPs inhibitors in cancer (26), some of these, such as Marimastat, have been used in animal models but also in small series of patients with breast and colon carcinoma, with promising results (7, 27).

In conclusion, this is the first study devoted to the study of proteolytic activity in male breast cancer, and on the basis of our results we suggest that increased MMPs activity could be responsible for the highly invasive tumor phenotype, and for the occurrence of metastasis, which strongly affect prognosis and survival. The role of sex hormones, as well as of MMPs, in the regulation of the metastatic behavior of breast cancer needs to be further investigated in view of its implications in male but also in female patients with breast cancer.

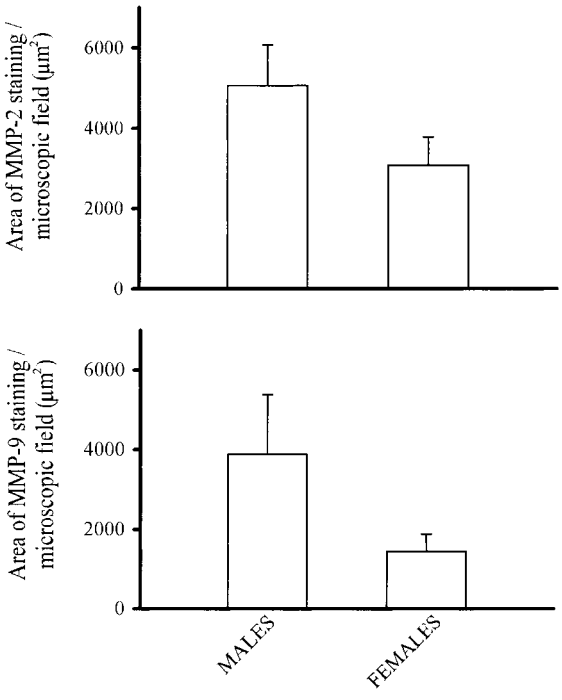

FIG. 5. Quantification of MMP-2 and MMP-9 immunostaining in male and female breast cancer tissues. Both gelatinases were more strongly expressed in male than in female breast cancer tissues.

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