Tryptase-positive mast cells correlate with angiogenesis in early breast cancer patients

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Abstract. Literature data indicate that mast cells (MCs) are involved in tumor angiogenesis due to the release of several pro-angiogenetic factors among which tryptase, a serine protease stored in MCs granules, is one of the most active. However, no data are available concerning the role of MCs in angiogenesis in primary human breast cancer. In this study, we have evaluated the correlations between the number of MCs positive to tryptase (MCDPT), the area occupied by MCs positive to tryptase (MCAPT) and microvascular density (MVD) and endothelial area (EA) in a series of 88 primary T1-3, N0-2 M0 female breast cancer, by means of immunohistochemistry and image analysis methods. Data demonstrated a significant (r = from 0.78 to 0.89; p-value from 0.001 to 0.002 by Pearson's analysis respectively) correlation between MCDPT, MCAPT, MVD, EA to each other. No correlation concerning MCDPT, MCAPT, MVD, EA and the main clinicopathological features was found. Our results suggest that tryptase-positive MCs play a role in breast cancer angiogenesis. In this context several tryptase inhibitors such as gabexate mesilate and nafamostat mesilate might be evaluated in clinical trials as a new anti-angiogenetic approach.

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Introduction

Angiogenesis plays a crucial role in tumor growth *in situ* and at distance and is a well established anti-tumor target (1-4). Mast cells (MCs) intervene in tumor angiogenesis (5-7), throughout several classical pro-angiogenic factors such vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), interleukin-6 (IL-6), and non classical pro-angiogenic factor, such as tryptase and chymase, stored in their secretory granules (8-11).

Data from experimental tumor models suggest that MCs accumulate near to tumor cells before the onset of angiogenesis and that they are required for macroscopic expansion and metastatic spread of primary tumor cells (12,13). Evidence for a specific role for MCs has been reported in animal and human cancers, such as mast cell tumors, head and neck, gastric, lung and cutaneous malignancies, where MCs density is highly correlated with the extent of tumor angiogenesis (14-18).

In benign breast lesions and in primary breast cancer, tryptase-positive MCs are more numerous as compared to chymase-positive MCs (19). Recently, it has been demonstrated that tryptase-positive MCs are involved in angiogenesis in sentinel lymph nodes with micrometastases from patients with breast cancer (20). However no data have been published regarding the correlations between MCs positive to tryptase and angiogenesis in primary breast cancer tissue.

In the present study, we have evaluated the correlations between the number of MCs positive to tryptase (MCDPT), the area occupied by MCs positive to tryptase (MCAPT), microvascular density (MVD) and endothelial area (EA) in a series of primary T1-3, N0-2 M0 female breast cancer, by means of immunohistochemistry and image analysis methods.

Table I. MCDPT, MCAPT, MVD and EA expression as a function of clinicopathological characteristics in a series of 88 breast cancer patients.

Variable	No. of patients	No. of tumors with high MCDPT[a] (%)	No. of tumors with high MCAPT[b] (%)	No. of tumors with high MVD[c] (%)	No. of tumors with high EA[d] (%)
Age (years)					
Range 26-85	88				
Median 58	88				
<58 years	41	22 (54)	23 (56)	21 (51)	20 (49)
≥58 years	47	24 (51)	25 (53)	23 (49)	22 (47)
Menopausal status					
Premenopausal	34	17 (50)	18 (53)	18 (53)	19 (56)
Postmenopausal	54	29 (54)	30 (56)	28 (52)	28 (52)
Histological type					
Ductal	65	33 (51)	33 (51)	31 (48)	30 (46)
Lobular	23	12 (52)	11 (48)	11 (48)	11 (48)
Tumor size					
pT_1	39	22 (56)	23 (59)	21 (54)	19 (49)
pT_2	31	16 (51)	17 (55)	15 (48)	15 (48)
pT_3	18	9 (50)	9 (50)	8 (44)	8 (44)
Nodal status					
pN_0	37	19 (51)	17 (46)	18 (49)	20 (54)
pN_{1-2}	51	26 (51)	28 (55)	27 (53)	25 (49)
Cytohistological grade					
G_1	31	17 (55)	18 (58)	16 (52)	15 (48)
G_2	37	19 (51)	21 (57)	20 (54)	19 (51)
G_3	20	10 (50)	11 (55)	9 (45)	9 (45)
Estrogen receptor status					
Negative	27	16 (59)	16 (59)	15 (56)	14 (52)
Positive	61	31(51)	33 (54)	33 (54)	35 (57)
Progesterone receptor status					
Negative	32	15 (47)	16 (50)	14 (44)	14 (44)
Positive	56	31 (55)	30 (54)	29 (52)	27 (48)
c-erbB-2					
Negative	53	29 (55)	30 (57)	28 (53)	29 (55)
Positive	35	17 (49)	18 (51)	16 (46)	16 (46)

[a]Median cut-off value, 7 cells per 400 field. [b]Median cut-off value, 136.67 μ^2 per 400 field. [c]Median cut-off value, 31 vessels per 400 field. [d]Median cut-off value, 146.54 μ^2 per 400 field.

Patients and methods

Patients. The clinicopathological features of the patients are summarized in the Table I. Biopsy specimens were collected from 88 female breast cancer patients who had undergone breast cancer surgery, classified accordingly the presence of a primary, invasive breast tumor (stage T1-T3), the presence or not of metastases in axillary lymph nodes (stage N0-N2), the absence of distant metastases (M0), the presence of unilateral breast cancer and the absence of previous or concomitant primary cancer. Patients were staged according to the International Union Against Cancer Tumor Node Metastasis (UICC-TNM) classification. Patients had not received neo-adjuvant therapies, they were subjected to a modified radical mastectomy (37 patients in which the tumor had a diameter >3 cm) or to a quadrantectomy followed by 5-6 weeks of radiation therapy (51 patients) and axillary lymph nodes were surgically removed when sentinel lymph node were positive. On the basis of clinicopathological features patients were then evaluated to receive adjuvant hormonal therapy or chemo-therapy or both.

Immunohistochemistry. The histological diagnosis was made on haematoxylin-eosin-stained slides and histopathological grading was performed according to the criteria described by Bloom and Richardson, as well, moderately and poorly differentiated state (21).

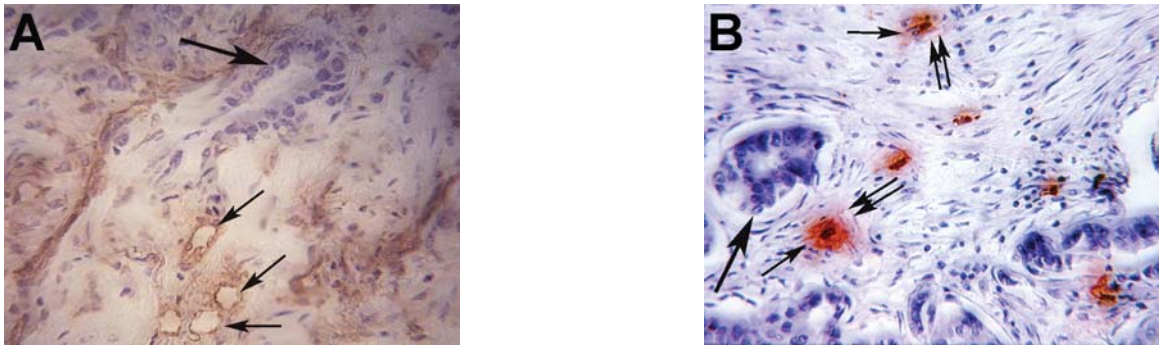

Figure 1. (A) A highly vascularized breast cancer sample stained with an anti-CD-34 antibody. Big arrow indicates ductal breast cancer cells, whereas small arrows indicate a single brown stained microvessel. (B) A breast cancer sample stained with an anti-tryptase antibody. Big arrow indicates ductal breast cancer cells, single small arrows indicate tryptase-positive mast cells and double small arrows indicate adjacent microvessels. Magnification: A and B, x400.

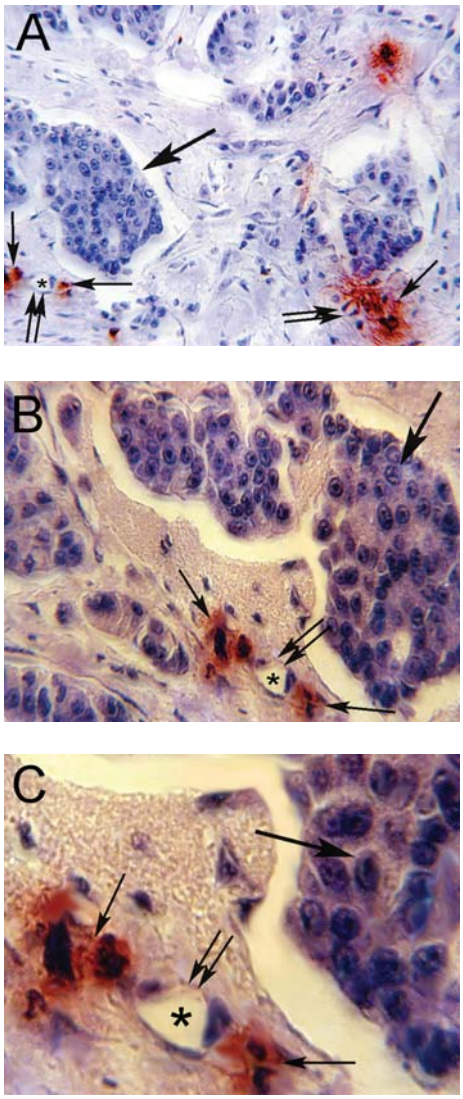

Figure 2. Breast cancer sample stained with an anti-tryptase antibody. (A) A big arrow indicates breast cancer cells, single small arrows indicate tryptase-positive mast cells and double small arrows indicate adjacent microvessel with an asterisk in its lumen. (B) A particular of the same section at an higher magnification, where big arrow indicates breast cancer cells, single small arrows indicate tryptase-positive mast cells and double small arrows indicate the adjacent microvessel with an asterisk in its lumen. (C) A particular of the same section at an higher magnification, where a big arrow indicates breast cancer cells, single small arrows indicates red tryptase-positive mast cells and double small arrows indicate the adjacent microvessels with an asterisk in its lumen. Original magnification: A, x250; B, x600; C, x1000.

For the evaluation of MCDPT, MCAPT, MVD and EA, a three layer biotin-avidin-peroxidase system was utilized, as previously described (22). Briefly, six-micrometer thick serial sections of formalin-fixed and paraffin-embedded biopsy tumor samples were deparaffinized. Then, for antigen retrieval, sections were microwaved at 500 W for 10 min, after which endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution. Next, slides were incubated with human-specific monoclonal antibodies anti-CD34 (QB-END 10; Bio-Optica Milan, Italy) diluted 1:50 for 1 h at room temperature and anti-tryptase (clone AA1; Dako, Glostrup, Denmark) diluted 1:100 for 1 h at room temperature. The bound antibody was visualised using biotinylated secondary antibody, avidin-biotin peroxidase complex and and 3-amino-9-ethylcarbazole or 3,3 diaminobenzidine. Nuclear counterstaining was performed with Gill's haematoxylin no. 2 (Polysciences, Warrington, PA, USA). Primary antibody was omitted in negative controls.

Morphometrical assay. An image analysis system (Quantimet 500 Leica, Wetzlar, Germany) was utilized. The five most vascularized areas ('hot spot') were selected at low magnification and both individual vessels (Fig. 1A) and MCs (Fig. 1B) were counted at x400 magnification (15). Single brown stained endothelial cells, endothelial cell clusters and microvessels, clearly separated from adjacent microvessels, tumor cells and other connective tissue elements were counted (22). Areas of necrosis were not considered for counting. In serial sections each single tryptase-positive MC was counted. Single brown stained endothelial cells and red MCs positive to tryptase were also evaluated in terms of immunostained area at x400 magnification (22).

Statistical analysis. MCDPT, MCAPT, MVD, EA mean values ± 1 standard deviations (SD) were evaluated by two independent observers (G.R. and F.A.Z.) for each tumor sample and in all series of sections. Correlations between MCDPT, MCAPT, MVD and EA to each other were calculated using Pearson's (r) analysis. The correlations between the above indexes and the clinicopathological features listed in Table I were analyzed by Chi-square test. All statistical analysis were performed with the SPSS statistical software package (SPSS, Inc., Chicago, IL).

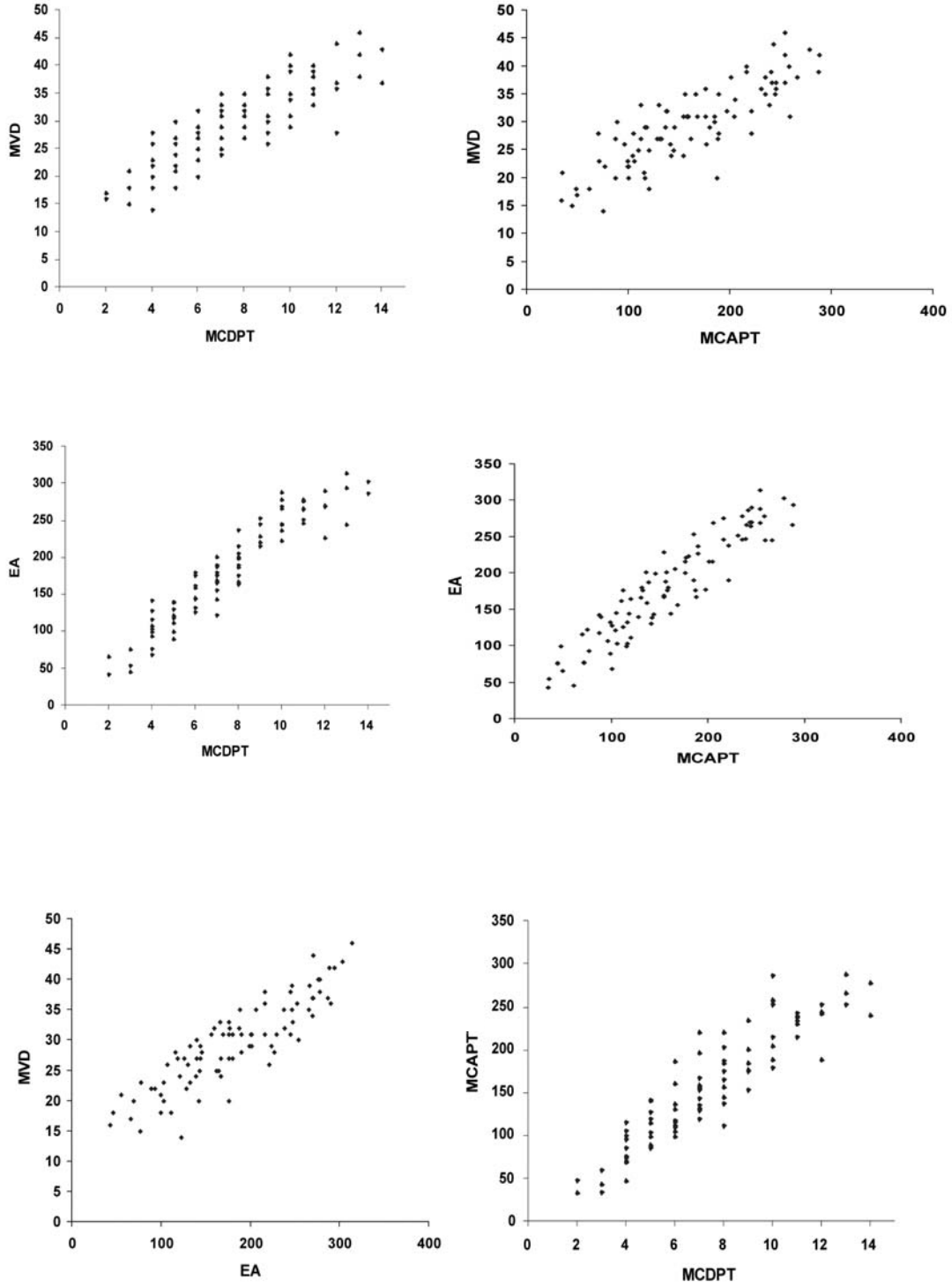

Figure 3. Correlation analysis between MCDPT and MVD (r=0.83, p=0.001); MCAPT and MVD (r=0.80, p=0.001); MCDPT and EA (r=0.86, p=0.001); MCAPT and EA (r=0.81; p=0.001); MVD and EA (r=0.88; p=0.001); MCDPT and MCAPT (r=0.75, p=0.002).

Table II. MCDPT, MCAPT, MVD and EA means ± 1 standard deviations in a series of 88 breast cancer patients.

MCDPT x400 magnification (0.19 mm^2 area)	MCAPT x400 magnification (0.19 mm^2 area)	MVD x400 magnification (0.19 mm^2 area)	EA x400 magnification (0.19 mm^2 area)
8±6[a]	173.42 μ^2 ± 131.16[a]	32 ± 17[a]	189.33 μ^2 ± 115.64[a]

[a]Mean ± 1 standard deviation.

Results

Immunohistochemical staining by using the antibodies anti-CD34 and anti-tryptase allow to demonstrate that in highly vascularized breast cancer tissue tryptase-positive MCs are well recognizable and generally they are located in perivascular position (Figs. 1 and 2).

Mean values ± 1 SD of all the evaluated parameters are listed in Table II. There was a significant correlation between MCDPT and MVD (r=0.83, p=0.001), between MCAPT and MVD (r=0.80, p=0.001), between MCDPT and EA (r=0.86, p=0.001), between MCAPT and EA (r=0.81; p=0.001), between MVD and EA (r=0.88; p=0.001) and between MCDPT and MCAPT (r=0.75, p=0.002) (Fig. 3). No correlation concerning MCDPT, MCAPT, MVD, EA and the main clinicopathological features was found.

Discussion

In this study, for the first time, we found in a series of 88 primary T1-3, N0-2 M0 female breast cancer, that the number of MCs positive to tryptase (MCDPT), the area occupied by MCs positive to tryptase (MCAPT), microvascular density (MVD) and endothelial area (EA) correlate to each other.

Tryptase is one of the most powerful angiogenic mediators released by human MCs and it may be angiogenic via several mechanisms (23-26). Blair *et al* have demonstrated that direct addition of tryptase to microvascular endothelial cells cultured on Matrigel caused a pronounced increase of capillary growth, which was suppressed by specific tryptase inhibitors, and directly induced endothelial cell proliferation in a dose-dependent fashion (10). Tryptase is involved in tissue remodelling and it may also act indirectly on tissue neovascularization by releasing latent angiogenetic factors bound to the extracellular matrix (27). Tryptase is an agonist of the proteinase-activated receptor-2 (PAR-2) in vascular endothelial cells and breast cancer cells (28-33). Activation of PAR-2 induce cell proliferation and release of IL-6 and granulocyte-macrophage colony stimulating factor (GM-CSF), which, in turn, act as angiogenic molecules (34).

It has been previously demonstrated that in several types of malignancies such as multiple myeloma, oral squamous cancer, melanoma, gastrointestinal cancer and lung cancer there is a correlation between tryptase-positive MCs and microvessel counts and both increase in function of malignancy (7,14-18,35-38). Accordingly, in this study we have demonstrated that also in breast cancer tissue there is a strong association between the number of MCs positive to tryptase and MVD. When we evaluated MCs positive to tryptase and angiogenesis in terms of morphometrical parameters we showed a strong association between the area occupied by MCs positive to tryptase and the area occupied by immunostained endothelial cells in terms of morphometrical angiogenesis. Interestingly tryptase positivity is observed near or around microvessels with a small evident lumen. Taken together these data suggest that MCs positive to tryptase may play a role in primary breast cancer angiogenesis, but they are not correlated with the main clinicopathological features (39). In this context several tryptase inhibitors such as gabexate mesilate and nafamostat mesilate (40,41) might be tested in clinical trials as a new anti-angiogenetic approach.

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