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ARTICLE

Alterations of Microvascular Density in Bone Metastases of Adenocarcinomas*

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Bone may provide an extremely fertile microenvironment for angiogenesis. Experimental investigations indicate angiogenesis as a major regulator of bone metastasis development. Vascularization and angiogenic potential is known for most of the primary tumor types, but no studies investigated angiogenesis in bone metastases of human cancers. We have evaluated microvessel density of bone metastases of various cancer types (all adenocarcinomas) and compared to their primary tumors in paraffin samples of 39 patients. Microvessel density was determined by using the hot spot method and the blood vessel marker, CD34. The most vascularized adenocarcinoma was found to be renal cell cancer followed by lung adenocarcinoma, while breast cancer was heterogenous in this respect. Two patterns of modulation of the angiogenic phenotype in the bone metastases emerged in this study, which seemed to be cancer type specific: decreased angiogenic potential characterizing 45% of renal cell cancers and breast cancers of high vascularity in their primary, and increased angiogenic potential characterizing 40% of lung adenocarcinomas and breast cancers of low vascularity in their primary lesion. Our data demonstrate that i., the vascularization of bone metastases is frequently altered compared to the primary tumors, ii., patterns are different in the case of various cancer types. The tumor-type specific alterations of the angiogenic phenotype of cancers, metastatic to the bone, can have a clinical significance when angiosuppressive therapies are considered. (Pathology Oncology Research Vol 10, No 3, 149–153)

Keywords: bone metastasis, microvessel density, breast cancer, lung cancer, renal cell cancer

Introduction

Vascularization of cancer metastasis influences tissue perfusion, tumor growth, oxygenization, bioavailability of drugs as well as drug sensitivity, therefore has clinical significance. Predictive pathology analyzes the primary tumor and determines features, including vascularization (microvessel density, MVD), to predict future outcome of the disease and response to therapies with the silent notion that the metastatic tumor will have geno- or phenotypic characteristics similar to the one observed in the primary tumor.[1]

Bone metastasis is a frequent complication of several common human malignancies, including carcinomas of the breast, prostate and lung.[2] After the initial arrest and growth in the new environment, the induction of angiogenesis becomes crucial for the continued secondary tumor growth. Micrometastatic cells must be able to induce angiogenesis to form macrometastases in their new target tissue.[3] Experimental observations suggest that bone metastatic tumor cells have the capacity to induce endothelial cell proliferation, migration as well as differentiation.[4,5] The ability of a tumor to induce angiogenesis is dependent not only on the angiogenic potential of the neoplastic cells, circulating promoters and inhibitors of angiogenesis, but also on the tissue environment.[6,7]

Since there is no human data on the MVD in bone metastasis of cancers, the aim of this study was to evaluate microvessel density in bone metastases of various cancer types including breast, lung and renal cancers, compared to their primary tumors.

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Materials and Methods

Patients

Bone metastasis tumor samples were retrospectively selected from the pathology departments of three institutes (Departments of Orthopedics and Traumatology, Semmelweis University and National Institute of Traumatology). Samples were open biopsies of bone metastases obtained during transfocal stabilization of impending or completed pathological fractures, or resected bone metastases.

We have selected 39 patients where both the primary tumor and the bone metastatic samples were available for analysis (78 tumor samples in total): primary breast cancers (BRC) and their metachronous bone metastases (n=18), primary renal cell cancers (RCC) and their synchronous bone metastases (n=11), and primary non-small cell lung cancers (NSCLC) and their metachronous bone metastases (n=10). Informed consent was obtained from all patients. Histological sections were reviewed. Necrotic areas were found in 5 of 11 primary and in 1 of 11 bone metastatic RCCs, in 5 of 10 primary NSCLCs, in 8 of 18 primary BRCs and in 5 of 18 bone metastatic BRCs. Necrotic areas were not detected in bone metastatic NSCLCs. The characteristics of patients included in the study are shown in *Table 1*.

Immunohistochemistry

The immunohistochemical procedure was performed on formalin fixed, paraffin embedded 4 μm sections with the avidin-biotin-peroxidase method. Staining for vascular endothelial cells was performed using a monoclonal mouse anti-human CD34, Class II antibody (DAKO, Glostrup, Denmark), at a 1:40 dilution in TRIS buffered saline (TBS). For the detection of estrogen receptors (ER) in primary and metastastic breast cancers a mouse monoclonal antibody against ER (Novocastra Laboratories, Newcastle upon Tyne, UK) has been applied at a 1:40 dilution in TBS on adjacent sections. Staining for VEGF in primary and metastatic breast cancer cases was performed on adjacent sections using a polyclonal goat anti-human VEGF antibody (RD Systems, Abingdon, UK) at a dilution of 1:80 in phosphate buffered saline (PBS).

After deparaffination and rehydration endogenous peroxidase was blocked by incubation with 1.5% hydrogen peroxide in methanol. Epitope retrieval for the detection of CD34 positive vessels and VEGF was achieved in citrate buffer (0.1 M, pH 6) using a microwave oven, as previously described[8] or by using a pressure cooker for the detection of ER.

After washing in TBS, sections were blocked with 3% bovine serum albumin (BSA) for 30 min to inhibit nonspecific immunoreactivity, followed by an overnight incubation at 4°C with anti-CD34 or anti-ER antibody. The bound antibodies were detected using the avidin-biotin complex/horse radish peroxidase (HRP) (LSAB2 kit, DAKO) for the detection of CD34 positive vessels and ER, or by using the HRP-AEC System Goat Kit (RD Systems) for the detection of VEGF according to the manufacturer's instruction, and finally visualized using 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories, Inc. Burlingame, CA). Counterstaining was performed with hematoxylin. As negative controls, we used non-immune IgG_1 immunoglobulin instead of primary antibodies or omitted primary antibody.

Assessment of microvessel density

Intratumoral microvessel density was determined according to previously described guidelines.[9] The areas containing the greatest numbers of microvessels (vascular hot spots) were identified by scanning the stained sections at low magnification using a light microscope (Olympus B061, Olympus Optical Co. Ltd, Tokyo, Japan). Once

Table 1. **Characteristics of patients**

	RCC	*NSCLC*	*BRC*
n	11	10	18
gender (F/M)	1/10	1/9	17/1
age (mean)	64	55	56
histology	clear cell carcinoma	adenocarcinoma (7) sq. cell carcinoma (3)	ductal invasive cc. lobular invasive cc. (2)
volume of the primary	130.9±161.6	33.7±49.7	19.2±22.0
volume of the metastasis (cm^3±SD)	60.4±71.5	25.9±30.6	50.0±83.7
type of metastasis	synchronous	metachronous	metachronous
irradiation	no	no	no
endocrine therapy	0/11	0/10	7/18
chemotherapy	0/11	2/10	7/18

RCC = renal cell cancer, NSCLC = non-small cell lung cancer, BRC = breast cancer

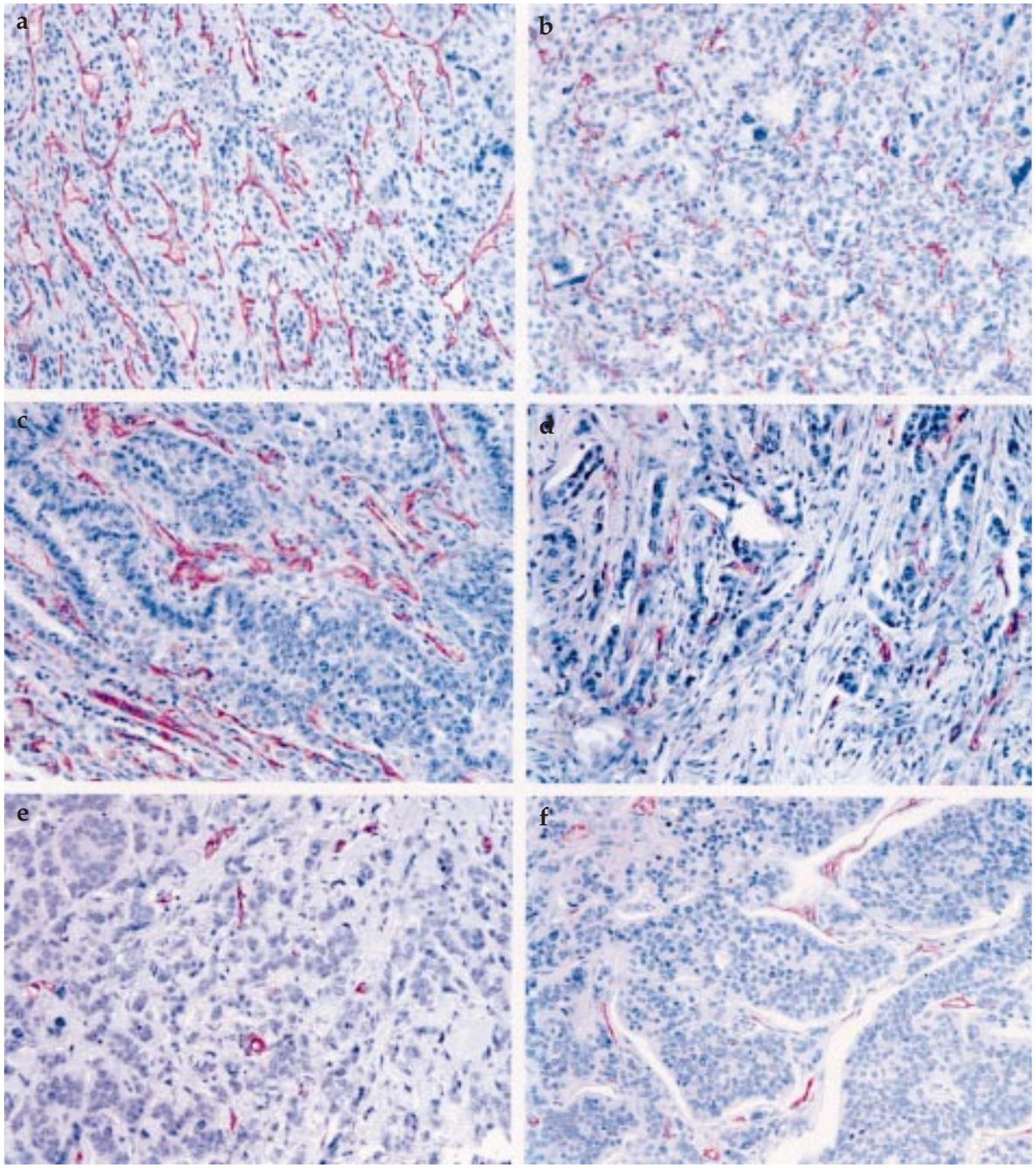

Figure 1. *Detection of microvessels in primary tumors as well as in bone metastases using CD34 marker (200x).* ***(a,b)*** *renal cell carcinoma;* ***(c,d)*** *adenocarcinoma of the lung;* ***(e,f)*** *invasive ductal carcinoma of the breast;* ***(a,c,e)*** *primary tumors;* ***(b,d,f)*** *bone metastases*

these areas were recognized, individual stained microvessels were counted at x400 magnification using a square grid graticule. This corresponded to a field size of 0.0625 mm^2 (all figures in text are quoted per mm^2). Any CD34 positive endothelial cells or endothelial cell clusters clearly separated from adjacent microvessels, tumor cells and connective tissue elements were considered as single countable microvessels; branching structures were counted as one, unless there was a break in the continuity of the vessel, in which case it was counted as two distinct ves-

sels. Three fields per tumor section were counted in the areas that appeared to contain the greatest number of microvessels on scanning at low magnification. Microvessel density was defined as the mean score from all three fields/mm^2.

Table 2. **Patterns of the alterations of microvascular densities in bone metastases**

	Total	*RCC*	*NSCLC*	*BRC*
MVD increased (>30%)	13/39	0/11	4/10	9/18
MVD decreased (>30%)	15/39	5/11	2/10	8/18
MVD unaltered (<30%)	11/39	6/11	4/10	1/18

MVD: microvessel density, RCC: renal cell carcinoma, NSCLC: non small cell lung cancer, BRC: breast cancer

Statistical analysis

Comparisons were made by Wilcoxon's rank sum test for paired variables and by Mann-Whitney U-test for unpaired variables. Spearman's rank-order correlation coefficient was used to assess the relationship between vascularity of primary tumors and their matched bone metastases. Two-tailed P was considered significant when it was lower than 0.05. All analyses were undertaken using the SPSS 10. package for Windows.

Results

CD34 immunostaining was successfully performed in all cases and in all tumor types on both the primaries as well as the bone metastases (*Figure 1*). Considering all the 39 cases as a group, we found a nonsignificant alteration of MVD in the bone metastases compared to their primary tumors. We considered the changes of MVD to be biologically relevant when difference was greater than 30% compared to the primary tumor. Based on such thresholding we have found that the frequency of unaltered, increased or decreased MVD in bone metastases of cancers was very similar *(Table 2*).

Not only primary RCCs but also their bone metastases were the most vascularized tumors compared to lung adenocarcinomas and BRC cases (*Figure 2*), but BRC was highly heterogenous in this respect, containing primary tumors with the lowest MVD among the adenocarcinomas analyzed. Accordingly, it was evident that different types of adenocarcinomas have to be analyzed separately.

We found a statistically significant decrease (27.59%) in MVD in the bone metastases of RCCs compared to their primary tumors ($p<0.05$) (*Figures 1a,b,2*). Using the thresholding principle described above, we found that MVD either decreased (in 5 of 11 cases) or was not altered (6/11 of the cases) in bone metastases, with no increased MVD values observed in any of the cases studied (*Table 2*). Furthermore, not only MVD but also the volume of bone metastases was found to be decreased with a mean of 53.85% (in 6 cases of 11) compared to primary RCCs (*Table 1*).

When we have analyzed the lung adenocarcinoma cases, we found a significantly increased MVD in their bone metastases compared to the primary tumors (49.12%, $p<0.05$) (*Figure 1 C, D, Fig. 2*). In this tumor type the decrease in MVD was rare (2/10), and increased MVD was relatively frequent (4 of 10 cases) (*Table 2*). The size of bone metastases of lung adenocarcinomas decreased with a mean of 25,83% compared to primary tumors, which seemed to be an independent feature of MVD (*Table 1*).

Breast carcinoma cases did not show significant alterations of MVD (*Fig. 2*) or size (*Table 1*) in their metastases compared to their primary tumors (*Figure 1e,f*). However, two completely different subgroups could be identified based on their MVD in the primary tumors and the alteration of MVD in the bone metastases: subgroup I was characterized by high MVD while subgroup II with low MVD. Interestingly, in subgroup I the MVD decreased, while in subgroup II MVD increased significantly ($p<0.05$) in the bone metastases compared to their primary tumors, respectively (*Table 2*).

Discussion

To our knowledge, this is the first study investigating microvessel density in bone metastases of solid cancers in human. We have analyzed microvessel density in human primary breast, lung and renal cell cancers and in their bone metastases. We found different associations of vascularity

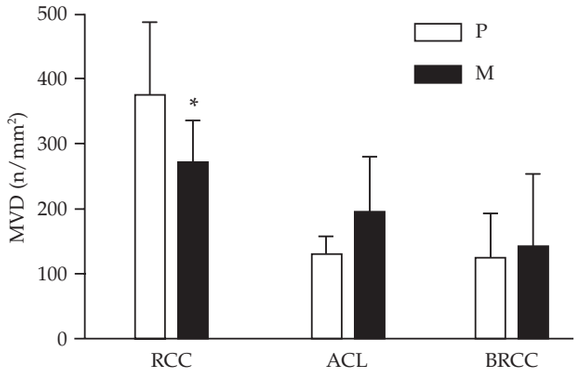

Figure 2. *Microvessel density in primary tumors and in their corresponding metastases. Data are shown as mean ± SD. * p<0.05. RCC: renal cell carcinoma, ACL: adenocarcinoma of the lung, BRC: breast cancer, MVD: microvessel density.*

between primary neoplasms of various histological origin and their matched bone metastases. In around one third of the cancer cases the initial MVD pattern was maintained in the bone metastases but in a significant proportion of cases a different MVD pattern developed. Maintained MVD pattern was a characteristics of about half of the cases of RCC and NSCLC but was unusual in BRC. Increased MVD in bone metastases characterized about one half of NSCLC cases, while decreased MVD was a characteristics of RCCs. Unlike in RCC and NSCLC, two alteration patterns of MVD were associated primary BRCs. Decreased MVD in bone metastases of BRC occurred in cases with high MVD in the primary tumor, and vice versa: metastases with increased MVD developed exclusively from primary BRCs characterized by low MVD.

It seems that the new microenvironment in the bone tissue does not affect the angiogenic phenotype of a significant proportion of RCC and NSCLC cases. On the other hand, significant modulation was induced by bone tissue in this phenotype in BRC and in a significant proportion of RCC and NSCLC cases. Two patterns of such modulation of the angiogenic phenotype emerged in this study which seemed to be cancer-type specific: decreased angiogenic potential characterizing RCC and the BRCs of high vascularity, and increased angiogenic potential characterizing lung adenocarcinomas and BRCs of low vascularity. Despite decreased MVD, not only primary RCCs but also their bone metastases were the most vascularized tumors compared to BRC and NSCLC cases. A possible cause for altered angiogenic phenotype in bone metastasis could well be the hormonal- or chemotherapy. However, this was definitively not the case for RCC and NSCLC, since the majority of the analyzed cases were chemotherapy-naive.

Our data demonstrate the role of microenvironment in regulating angiogenesis in bone metastases, however, this effect seems to be cancer-type specific.[10] With the advent of the angiosuppressive therapy entering clinical practice of metastatic RCC,[11] NSCLC, or colorectal cancer,[12] physiological modulation of the angiogenic phenotype of cancers in the bone microenvironment may have clinical significance.

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