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Evaluation of stromal metalloproteinases and vascular endothelial growth factors in a spontaneous metastasis model

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Abstract

This study aims to investigate MMP2 and MT1-MMP protein as well as VEGF-C and VEGF-D mRNA expression in tumor cells and distant organs considered to be targets for metastasis in a tumor spontaneous metastasis model previously described. Cultured tumor cells, able to express pro-MMP2, MMP2, pro-MMP9, and MT1-MMP, develop tumor growth and metastasis, mainly in the liver and spleen, when they are injected in the mammary pad gland of Wistar rats. Immunohistochemical studies of tumor masses showed small groups of tumor cells staining for MT1-MMP but not for MMP2. In the liver, tumor metastatic foci and a stromal positive staining for both MMP2 and MT1-MMP were shown. The spleen and lymph nodes, with only scattered metastatic cells, did not show MMPs immunostaining. Using RT-PCR, a significantly higher VEGF-C and VEGF-D gene expression was shown in the liver of tumor-bearing rats respect to normal rats, whereas spleen and lymph nodes did not show significant differences in mRNA VEGF-C/D levels. Taken together, our results suggest that the stroma microenvironment of target organs for metastasis has the ability to produce MMPs and VEGFs that facilitate the anchorage of tumor cells and promote tumor cell growth and angiogenesis.

Keywords: Metalloproteinases; Metastasis; Stroma; Tumor; Tumor microenvironment; VEGF-C; VEGF-D

Introduction

Metastasis involves a series of events concerning tumor cells, including release from the primary tumor, invasion of the surrounding tissue parenchyma, and movement into the vascular or lymphatic system. Those cells that survive within the circulation migrate into parenchymal tissue at distant sites and establish metastatic foci (Takeda et al., 2002). Invasion of the surrounding stroma requires degradation of extracellular matrix (ECM) components (collagen IV, laminin, and fibronectin), which is mediated by matrix metalloproteinases (MMPs) (Stetler-Stevenson et al., 1993). Furthermore, ECM degraded components are involved in cell–matrix interactions and play a key role in the cancer cell migration and metastasis (Nabeshima et al., 2002). In addition, ECM contains growth factors and other bioactive molecules, which are activated and released only after proteolysis of ECM components by MMPs (Brinckerhoff and Matrisian, 2002).

Matrix metalloproteinases are a family of zinc-binding endopeptidases, which are classified in soluble and membrane type MMPs (MT-MMPs) (Egeblad and Werb, 2002; Sounni et al., 2003). The activity of MMPs within tissues is regulated mainly by activation of zymogens (pro-MMPs) and inhibition by their common tissue inhibitors of metalloproteinases (TIMPs) (Seiki and Yana, 2003). Among these MMPs, MMP2 (gelatinase A) and MT1-MMP have been involved in the degradation of the ECM associated with invasion and metastasis of tumor cells (Kurschat et al., 1999). MMP2 can be expressed by tumor cells, but recent in vitro studies have demonstrated that it is also expressed in surrounding tissue parenchyma, and this is considered to be a key factor in facilitating the cancer cell invasion (Saad et al., 2002). Although MT-MMPs comprise four different species, i.e., MT1, 2-, 3-, and 5-MMPs, it has been shown that MT1-MMP plays the main role in pro-MMP2 activation in several human carcinomas (Seiki, 1999).

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Accumulating evidence indicates that the progressive growth and spread of tumors is dependent on angiogenesis. It has been demonstrated that not only tumor cells, but also inflammatory cells which infiltrate the tumor and ECM, can be a source of angiogenic factors (Bergers and Benjamin, 2003). Among these factors, vascular endothelial growth factor (VEGF) can directly stimulate vascular endothelial cell motility and proliferation (Ferrara and Henzel, 1989; Leung et al., 1989). Two cloned members of the VEGF family, VEGF-C and VEGF-D, have been shown to be important regulators of lymph vessel growth and lymphatic metastasis (Skobe et al., 2001; Stacker et al., 2001; Mandriota et al., 2001). It is known that VEGF-C and VEGF-D promote angiogenesis by binding tyrosine kinase receptors, VEGFR-2 and VEGFR-3, which are expressed on vascular and lymphatic endothelia, respectively (Kaipainen et al., 1995; Plate, 2001). Furthermore, it has been reported that VEGFR-3 expression is up-regulated on the endothelium of tumor blood vessels (Valtola et al., 1999). Thus, VEGF-C and VEGF-D are angiogenic factors strongly involved in tumor growth and metastasis.

Taking these antecedents together, it could be hypothesized that a coordinated interaction between tumor and stroma, involving the participation of MMPs and VEGFs, is a requisite for tumor invasion and metastasis. To test this hypothesis, we used an animal model of tumor metastasis previously described (Donadio et al., 2002) in order to analyze the protein expression of MMP2 and MT1-MMP as well as VEGF-C and VEGF-D mRNA expression in tumor cells and distant organs which are considered to be a target for metastasis such as spleen, liver, and lymph nodes.

Material and methods

Rats

Female, 75- to 90-day-old Wistar rats purchased from the Facultad de Veterinaria, Universidad de La Plata, Buenos Aires, Argentina, were used. Rats were maintained according to the NIH standards established in the "*Guidelines for the Care and Use of Experimental Animals*". All animal studies were reviewed and approved by the Animal Care and Use Committee of the Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina.

Cells and cell culture conditions

A rat sarcoma cell line established in our laboratory (TuE-t cell line) was maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Natocor, Córdoba, Argentina) and 40 μg/ml gentamicin in a humidified 5% CO_2 atmosphere at 37°C.

Preparation of conditioned media from TuE-t cultures

Conditioned media were prepared by incubating subconfluent TuE-t cells in 96-well plates (5×10^4 cells/well) for 18–20 h in 0.5% FBS-RPMI medium. After cell incubation, the culture media were collected, clarified by centrifugation, and stored frozen at −20°C.

Gelatin zymography

Gelatinolytic activity in the conditioned media from TuE-t cultures was analyzed by electrophoresis under non-reducing conditions on 7.5% SDS-PAGE copolymerized with 1.5% gelatin as substrate (gelatin SDS-PAGE). After electrophoresis, the gel was washed in 2.5% Triton X-100 for 10 min to remove SDS. After rinsing twice in substrate buffer [50 mM Tris–HCl (pH 7.4), 200 mM NaCl, 9 mM $CaCl_2$], gels were incubated at 37°C for 18–20 h in the same buffer under gentle agitation. Gels were stained for 45 min in 45% methanol–5% glacial acetic acid containing 0.125% (w/v) Coomasie Brilliant Blue R250 and destained in 25% ethanol–10% glacial acetic acid. White zones on the gels indicate the gelatinolytic activity of proteinases.

Tumor experiments in vivo

Female Wistar rats were injected in the mammary pad gland with $1-2 \times 10^6$ TuE-t cells in phosphate-buffered saline (PBS), pH 7.4. Animals were monitored regularly for tumor development as previously described (Donadio et al., 2002), killed, and autopsied 2 to 3 weeks after tumor cell implantation. During each autopsy, primary tumor, lymph nodes, spleen, and liver samples were carefully excised for histopathological, immunohistochemical, and RNA studies.

Histopathological studies

Tumor, lymph nodes, spleen, and liver samples were fixed in 4% buffered formalin and then embedded in paraffin. Hematoxylin and eosin-stained sections of lymph nodes, spleen, and liver samples of each tumor-bearing animal were examined for metastasis.

MMP2 and MT1-MMP immunostaining

Tumor, lymph nodes, spleen, and liver biopsies were immunostained with a monoclonal antibody against rat MMP2 (4 μg/ml, clone CA-4001, NeoMarkers, Freemont, CA), and a polyclonal antibody against rat MT1-MMP (4 μg/ml, clone V-16, Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with enhanced horseradish peroxidase-conjugated streptavidin (L-SAB+Kit, DAKO, Denmark), the color was developed with the substrate chromogen DAB (3,3′-diaminebenzidine, Sigma Chemical Co., St. Louis, MO). Slides were examined using an Axiolab Carl-Zeiss microscope. The CA-4001 monoclonal antibody recognizes both latent and active forms of MMP2. Sections were scored independently by two observers (A.C.D. and M.M.R.). In addition, TuE-t cells grown on microscope slides for 24 h in 10% FBS-RPMI medium were fixed in 4% buffered formalin. Then the slides were washed with PBS, immunostained as indicated above, and used to analyze constitutive MT1-MMP and MMP2 expression.

Semiquantitative RT-PCR of VEGF-C and VEGF-D gene expression

Total RNA was isolated from tumor, liver, spleen, and lymph node tissues as well as from TuE-t cells cultured in 10% FBS-RPMI by the acid guanidinium thiocyanate–phenol–chloroform extraction method described by Chomczynski and Sacchi (1987). Single-stranded cDNA was then synthesized from 1 μg of the total RNA using 200 units of M-MLV reverse transcriptase (RT) (Promega, Madison, USA) with VEGF-C, VEGF-D, and actin antisense primers in 20 μl reaction volume. Four microliters of each cDNA was subjected to PCR reaction using rat VEGF-C and VEGF-D specific primers: VEGF-C, antisense (GAACGTCTAATAATTGAATGAACTTGTCT) and VEGF-C, sense (TGTAAAACTTGCTGCTGCACATT) [amplify nucleotides 393–758 of rat VEGF-C, GenBank accession numberAY032729]; VEGF-D, antisense (CTCCAGGACATGGTGCTTTACA) and VEGF-D, sense (TCCAAACAGCTCTTTGAGATATCAG) [amplify nucleotides 543–897 of rat VEGF-D, GenBank accession numberAY032728]. The actin cDNA was also amplified using the specific primers: antisense (TAGTTTCATGGATGCCACAGGATTC) and sense (ATTGAACACGGCATTGTAACCAACT) [amplify nucleotides 1541–2714 of rat actin, GenBank accession numberV01217]. For VEGF-C and VEGF-D, the following PCR cycle parameters were used: denaturation at 95°C for 2 min, followed by 32 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and polymerization at 68°C for 2 min. A final extension step was performed at 68°C for 10 min. For actin, the cycle parameters were 94°C, 30 s; 56°C, 1 min; and 68°C, 1 min (28 cycles). Each