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Tumor Lymphangiogenesis and Metastasis to Lymph Nodes Induced by Cancer Cell Expression of Podoplanin

Leah N. Cueni,* Ivan Hegyi,† Jay W. Shin,* Andrea Albinger-Hegyi,‡ Silke Gruber,§ Rainer Kunstfeld,§ Holger Moch,¶ and Michael Detmar*

From the Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology (ETH) Zurich, Switzerland; the Department of Dermatology,† Inselspital Bern, University Hospital, and University of Bern, Bern, Switzerland; the Department of Oto-Rhino-Laryngology,‡ Head and Neck Surgery, and the Department of Pathology,¶ Institute of Surgical Pathology, University Hospital Zurich, Zurich, Switzerland; and the Department of Dermatology,§ Medical University of Vienna, Vienna, Austria*

The membrane glycoprotein podoplanin is expressed by several types of human cancers and might be associated with their malignant progression. Its exact biological function and molecular targets are unclear, however. Here, we assessed the relevance of tumor cell expression of podoplanin in cancer metastasis to lymph nodes, using a human MCF7 breast carcinoma xenograft model. We found that podoplanin expression promoted tumor cell motility *in vitro* and, unexpectedly, increased tumor lymphangiogenesis and metastasis to regional lymph nodes *in vivo*, without promoting primary tumor growth. Importantly, high cancer cell expression levels of podoplanin correlated with lymph node metastasis and reduced survival times in a large cohort of 252 oral squamous cell carcinoma patients. Based on comparative transcriptional profiling of tumor xenografts, we identified endothelin-1, villin-1, and tenascin-C as potential mediators of podoplanin-induced tumor lymphangiogenesis and metastasis. These unexpected findings identify a novel mechanism of tumor lymphangiogenesis and metastasis induced by cancer cell expression of podoplanin, suggesting that reagents designed to interfere with podoplanin function might be developed as therapeutics for patients with advanced cancer. ***(Am J Pathol 2010, 177:1004–1016; DOI: 10.2353/ajpath.2010.090703)***

Podoplanin is a small mucin-type transmembrane glycoprotein that, in normal human tissues, is expressed by diverse cell types, including kidney podocytes,[1] lung alveolar type I cells,[2] and lymphatic endothelium.[3] Studies in podoplanin knockout mice demonstrated that podoplanin is essential for the development of the lung and the lymphatic vascular system.[4,5] Besides these important physiological functions, podoplanin appears to have a role in cancer pathogenesis. Podoplanin expression was found to be up-regulated in mouse skin during wound healing and chemically induced skin carcinogenesis,[6] as well as in several human cancers, including squamous cell carcinomas of different organs,[7–9] mesotheliomas,[10] certain germ cell tumors,[7] and tumors of the central nervous system.[11] Several studies indicated that the expression of podoplanin in tumors might be related to their malignant progression. High levels of podoplanin expression were detected in highly metastatic clones of mouse colon adenocarcinoma and melanoma cell lines, compared with parental cells.[12,13] Also, data from studies of some human cancers suggested a possible association of podoplanin expression with invasion and metastasis of tumors.[14,15] The exact molecular function of podoplanin in cancer cells, however, remained unclear.

Podoplanin was shown to be involved in the regulation of cytoskeletal organization and cell motility. Via its short cytoplasmic domain, podoplanin interacts with the membrane cytoskeleton linkers ezrin and moesin, induces formation of filopodia-like plasma membrane extensions, and increases motility in a variety of cell types.[4,9,16–18] Also, down-regulation of the cell–cell adhesion protein E-cadherin, which is associated with epithelial-mesenchymal transition, was observed in certain cells and tu-

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Address reprint requests to Michael Detmar, M.D., Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology, ETH Zurich, Wolfgang Pauli-Strasse 10, CH-8093 Zurich, Switzerland. E-mail: michael.detmar@pharma.ethz.ch.

mors in response to podoplanin expression.[9,18,19] Together with the localization of podoplanin to the invasive front of tumors, which was observed in human squamous cell carcinomas,[7,9,17] these data indicate that podoplanin might promote the detachment of tumor cells and the invasion of adjacent tumor stroma, which are crucial steps toward metastasis. Podoplanin also induces the aggregation of platelets[20] and could thereby facilitate the arrest, extravasation and subsequent metastasis of podoplanin-expressing tumor cells circulating in the blood stream.[21]

The relevance of podoplanin to cancer spread via the lymphatic system, however, has not been clarified. In the present study, we have assessed the effect of podoplanin on lymphatic metastasis in a human breast carcinoma xenograft model. Surprisingly, we found that podoplanin expressed by tumor cells induced tumor lymphangiogenesis and increased metastasis to regional lymph nodes without promoting primary tumor growth. We also show that high cancer cell expression levels of podoplanin correlate with metastasis to lymph nodes and poor prognosis in patients with oral and oropharyngeal squamous cell carcinomas (OSCC). Importantly, by comparative transcriptional profiling of tumor xenografts, we identify novel potential molecular mediators of podoplanin-induced effects.

Materials and Methods

Generation of Podoplanin-Overexpressing MCF7 Cell Clones and Cell Culture

MCF7 human breast carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (all from Gibco, Grand Island, NY). A clone of MCF7 cells stably expressing the pTet-Off-regulator plasmid (Clontech, Mountain View, CA) was kindly provided by Dr. Sam W. Lee (Massachusetts General Hospital, Boston, MA). Cells were transfected with the pTRE2hyg response plasmid (Clontech) containing or not full-length cDNA of human podoplanin (GenBank Accession number NM_006474; Origene, Rockville, MD). Stably transfected cell clones were selected using 300 μg/ml G418 (Sigma, St. Louis, MO) and 130 μg/ml hygromycin B (Calbiochem, San Diego, CA). Primary dermal human lymphatic and blood vascular endothelial cells were isolated and cultured as described.[22]

Real-Time Reverse Transcription-PCR

RNA was isolated from subconfluent cell cultures using TRIzol reagent (Invitrogen, Carlsbad, CA) or from tumor tissue using the EZ1 RNA Universal Tissue Kit (Qiagen, Hilden, Germany) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the TaqMan Universal PCR Master Mix or the Power SYBR Green PCR Master mix (Applied Biosystems), in a 7900HT Fast Real-Time PCR system (Applied Biosystems). β-actin served as internal control for normalization of signals.

TaqMan-based real-time PCR was performed for human podoplanin (forward primer 5′-AGGCGGCGTTGCCAT-3′; reverse primer 5′-GTCTTCGCTGGTTCCTGGAG-3′; probe 5′-FAM-CCAGGTGCCGAAGATGATGTGGTG-BHQ1-3′), human vascular endothelial growth factor (VEGF)-C (forward primer 5′-CACCACCAAACATGCAGCTG-3′; reverse primer 5′-TGAAAATCCTGGCTCACAAGC-3′; probe 5′-FAM-CGGCCATGTACGAACCGCCAG-BHQ1-3′) and human β-actin (forward primer 5′-TCACCGAGCGCGGCT-3′; reverse primer 5′- TAATGTCACGCACGATTTCCC-3′; probe 5′-JOE-CAGCTTCACCACCACGGCCGAG-BHQ1-3′). Sybr Green-based real-time PCR was performed for human endothelin-1 (forward primer 5′-ACTTCTGCCAC-CTGGACATC-3′; reverse primer 5′-CTCTTGGAC-CTAGGGCTTCC-3), human villin-1 (Hs_VIL1_1_SG QuantiTect Primer Assay, Qiagen), mouse tenascin-C (Mm_Tnc_1_SG QuantiTect Primer Assay; Qiagen), human endothelin receptor B (Hs00240747_m1; Applied Biosystems), or human endothelin receptor A (Hs00609865_m1; Applied Biosystems). Cycling parameters were as follows: 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds denaturation at 95°C and 1 minute annealing and extension at 60°C. All reactions were performed in triplicates.

Protein Extraction, Immunoprecipitation, and Immunoblots

Proteins were extracted from cultured cells for 30 minutes on ice using cell lysis buffer containing 20 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 25 mmol/L NaF, 1 mmol/L PMSF, 1 mmol/L $NaVO_3$, 10% glycerol, and a protease inhibitor cocktail. To extract protein from tumors, tissues were homogenized in lysis buffer (Ray Biotech, Norcross, GA) supplemented with protease inhibitor cocktail, followed by incubation for 30 minutes on ice. Endothelin receptor B was immunoprecipitated from endothelial cell lysates (500 μg total protein) using a sheep polyclonal antibody (Abcam, Cambridge, UK). Immunoblotting was performed using antibodies against human villin-1 (0.56 μg/ml; Sigma), human podoplanin (D2-40, 1:1000; Covance, Emeryville, CA), human RhoA (1 μg/ml; BD Transduction Laboratories, Lexington, KY), human endothelin receptor B (Abcam) or β-actin (clone AC-15, 1:5000; Sigma), and corresponding HRP-coupled secondary antibodies (GE, Little Chalfont, UK). All blocking and antibody incubation steps were performed in 5% nonfat dry milk in PBS, except for the detection of RhoA, for which blocking and primary antibody incubation were done in 10% bovine serum albumin (BSA) in PBS. For detection, the ECL Plus Detection System (GE) was used.

RhoA Activity Assay

Protein was extracted from cultured cells at approximately 30% confluency, for 5 minutes on ice. Cell lysis buffer was used, containing 50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 2.5 mmol/L $MgCl_2$, 2.5 mmol/L $CaCl_2$, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxy-