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Chaotic neovascularization induced by aggressive fibrosarcoma cells overexpressing S-adenosylmethionine decarboxylase

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ABSTRACT

S-adenosylmethionine decarboxylase is a key enzyme in the biosynthesis of polyamines essential for cell proliferation. Overexpression of S-adenosylmethionine decarboxylase in rodent fibroblasts led to aggressive transformants (Amdc-s cells) that had unforeseen high invasive capacity in nude mice, invading rapidly from the subcutaneous injection site into the peritoneal cavity and its organs. In vitro, these cells were much more invasive than Ras-oncogene-transformed fibroblasts, or human HT-1080 fibrosarcoma and MDA-MB-231 breast cancer cells. In immunohistological characterization, Amdc-s-induced tumors showed chaotic neovascularization, with abundant pleomorphic vessel-like structures that had noncontiguous or totally missing laminin (basement membrane) and CD31 (endothelial cell) immunoreactivity. Gene expression and protein analyses of Amdc-s cells showed them to overexpress several pro-angiogenic molecules, including vascular endothelial growth factor (VEGF-A), and to exhibit profound down-regulation of the anti-angiogenic thrombospondin-1 (TSP-1). By reintroduction of TSP-1 into Amdc-s cells, the high invasiveness was efficiently inhibited in vitro. Interestingly, Amdc-s cells showed up-regulation of hepatocyte growth factor (HGF) and also expressed the MET receptor, creating thus an autocrine loop able to regulate VEGF-A and TSP-1 levels. Further, we found Amdc-s cells to express increased amounts of matrix metalloproteinase-2 (MMP-2) and the large isoform of tenascin-C (TN-C), which may also contribute to the angiogenic switch and invasiveness. Consequently, Amdc-s cells offer an excellent model to sort out the key molecules of aggressive tumor growth, and thereby help in designing rational, novel anti-vascular and other cancer therapies.

1. Introduction

Among the most important themes in tumor progression are neovascularization, the growth of new blood vessels, and invasion, the ability of tumor cells to spread into the underlying tissues. A number of molecules have been documented either stimulating or inhibiting these processes. Increasing evidence supports the hypothesis that the net balance of these various molecules creates the microenvironment in which threshold changes can then induce the angiogenic switch, and enable migration of the cells through tissue boundaries, thus enhancing the aggressive growth. It would be indispensable to identify the genes whose expression is responsible for the acquisition of the angiogenic, invasive, and further metastatic potential in cancer cells, as it may not only help to understand the underlying molecular mechanisms, but also provide new therapeutic opportunities.

Without the development of an adequate vascular network tumor cells can not grow beyond a critical size (1–2 mm^3), but become necrotic and/or apoptotic (Hanahan and Folkman, 1996; Carmeliet and Jain, 2000). Tumor cells are thought to induce new blood vessel formation mainly through aberrant deployment of normal angiogenesis. In normal tissues the inhibitory or anti-angiogenic factors, such as thrombospondin-1 (TSP-1), predominate or maintain a finely tuned balance, but in tumors the balance is changed in favor of angiogenesis, as a result of down-regulation of the angiogenic inhibitors (Bouck et al., 1996; Nyberg et al., 2005; Folkman, 2006) or increased expression of the pro-angiogenic factors, such as vascular endothelial growth factor (VEGF-A) and basic fibroblast growth factor (bFGF) (Papetti and Herman, 2002; Ferrara and Kerbel, 2005; Hillen and Griffioen, 2007). The angiogenic growth factors then activate endothelial cells, leading to the activation of proteases, such as matrix metalloproteinases (MMPs), which degrade the vessel wall. This allows

Abbreviations: AdoMetDC, S-adenosylmethionine decarboxylase; BCE, bovine capillary endothelial cells; bFGF, basic fibroblast growth factor; CM, conditioned medium; ECM, extracellular matrix; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HGF, hepatocyte growth factor; HRP, horseradish peroxidase; MMP, matrix metalloproteinase; ODC, ornithine decarboxylase; TN-C, tenascin-C; TSP, thrombospondin; VEGF-A, vascular endothelial growth factor.

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the endothelial cells to migrate into the surrounding matrix, proliferate, and eventually differentiate into a new, lumen-containing vessel, according to the classical vessel sprouting model (Bergers and Benjamin, 2003).

We have previously reported that mouse fibroblasts engineered to overexpress human ornithine decarboxylase (ODC), the rate limiting enzyme in the biosynthesis of polyamines (Pegg, 1988; Heby and Persson, 1990), become malignantly transformed (Auvinen et al., 1992) and induce large, highly vascularized tumors, fibrosarcomas, in nude mice (Auvinen et al., 1997). Further, we have found that a corresponding overexpression of human S-adenosylmethionine decarboxylase (AdoMetDC), the second key enzyme of polyamine biosynthesis, results in a far-more aggressive transformed phenotype, as seen in the exceptionally high invasive activity of the transformants in nude mice (Paasinen-Sohns et al., 2000). Thus, the AdoMetDC-induced tumor model, particularly when compared to the ODC-induced one, opens up a fascinating possibility to identify the key molecular players in the aggressive tumor growth.

In this study, we first analyzed by histological and immunohistochemical methods the tumors induced by the AdoMetDC-transformed fibroblasts (Amdc-s cells), concentrating on their invasion pattern and vascularization, and then performed molecular analyses of the cells by DNA microarray profiling and protein expression studies.

2. Materials and methods

2.1. Cell lines and tumorigenicity assays

Amdc-s cells are NIH3T3 cells transfected with human AdoMetDC cDNA and 4N cells represent their normal counterparts (Paasinen-Sohns et al., 2000). Odc-n cells (Auvinen et al., 1997) are derived from the tumors induced by human ODC overexpressing NIH3T3 cells (Odc) (Auvinen et al., 1992) in nude mice, Ras E4 cells are NIH3T3 cells transfected with c-Ha-*ras*^{Val12} oncogene (pGEJ6.6) and N1 cells represent their normal counterparts (Holtta et al., 1998; Sistonen et al., 1989). The polyamine patterns and some other characteristics of the ODC-n (Auvinen et al., 1995; Auvinen et al., 1997; Ravanko et al., 2000; Kielosto et al., 2004) and Amdc-s (Paasinen-Sohns et al., 2000; Ravanko et al., 2000; Ravanko et al., 2004; Nummela et al., 2006; Kielosto et al., 2009) cells have been published previously. It is also noteworthy here that, the transformed phenotype of Amdc-s cells can be reversed by incubation with a specific inhibitor of AdoMetDC, CGP 48664 (Svensson et al., 1997), as shown by analyses of the cellular morphology (Supplementary Fig. 1A) and invasive potential in Matrigel (Supplementary Fig. 1B). Tumorigenicity assays of Amdc-s cells were performed as described earlier (Paasinen-Sohns et al., 2000). Shortly, tumors were established in nude mice by subcutaneous injection of 10^7 Amdc-s or control cells into both flanks.

2.2. Histological and immunohistochemical analyses

To highlight the tissue morphology, 5 μm sections were cut from the paraffin blocks and stained with hematoxylin and eosin (HE) according to standard procedures. For immunohistochemical analyses, paraffin sections were dewaxed in xylene, rehydrated through a graded series of ethanol and the antigens were retrieved by microwaving in 10 mM sodium citrate buffer, pH 6.0 at 780 W for 5 min and 450 W for 10 min (the endothelial cell marker CD31) or digested with 0.5% trypsin (Invitrogen, Carlsbad, CA) for 30 min at 37 °C (laminin and TN-C). To block endogenous peroxidase, the slides were treated with 0.3% H_2O_2 in methanol for 30 min. After blocking with 1–3% normal serum in PBS for 2 h at room temperature (RT), the slides were incubated with 10 μg/ml of rat CD31 antibody (BD Pharmingen, San Diego, CA), 1:1000 dilution of laminin antibody (Sigma–Aldrich, St. Louis, MO), or 1:50 dilution of TN-C antibody (Chemicon International, Temecula, CA) for 2.5 h at RT or o/n at 4 °C (TN-C). Control slides were treated with normal sera (DAKO, Glostrup, Denmark; 1:500 dilution). The immunoreactivity was detected using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) or TSA Indirect Tyramide Signal Amplification Kit (PerkinElmer, Waltham, MA; TN-C), with 3-amino-9-ethylcarbazole (AEC) as a chromogen (Sigma–Aldrich). The sections were counterstained with Mayer's hematoxylin and mounted with Aquamount (BDH, Poole, UK). Images were obtained using the Nikon-Eclipse E800 M microscope (Tokyo, Japan), equipped with Nikon D1 camera and the Nikon Capture software.

2.3. Matrigel invasion assay

The ability of the cells to invade in thick three-dimensional (3D)-Matrigel matrix was assayed as previously described (Kielosto et al., 2004; Ravanko et al., 2004; Nummela et al., 2006). In brief, 24-well tissue culture plates were coated with 300 μl Growth Factor Reduced Matrigel (BD Biosciences, Palo Alto, CA). After polymerization, 10^4 cells were seeded on top of the gel and covered with a second layer (250 μl) of Matrigel. Finally, DMEM with 5% serum was added on top of the Matrigel, and the medium was changed every third day. The patterns of cell growth were monitored daily by microscopy and photographed (after seven days) using the Olympus CK2 inverted microscope (Tokyo, Japan), equipped with phase-contrast optics and a digital camera.

2.4. Preparation of conditioned medium and endothelial cell migration assay

To prepare conditioned medium (CM), the cells were grown up to 80% confluency, rinsed twice with DMEM and incubated with serum-free DMEM for 18–24 h. The CM was collected and concentrated to 1/10 volume with Ultrafree-15 Biomax concentration device (MWCO 10 kDa; Millipore, Bedford, MA). The capacity of CM to induce proliferation and migration of bovine capillary endothelial (BCE) cells in 3D-collagen gel was analyzed as described previously (Auvinen et al., 1997). In the experiments, 12 μg of protein per sample in an equal volume was added into the wells at 24 h intervals (for four days). Photomicrographs of the migrating cells were taken after six days of culture using the Olympus CK2 microscope.

2.5. Protein analyses of cell lysates and conditioned media

Detergent soluble proteins (Paasinen-Sohns et al., 2000; Paasinen-Sohns and Holtta, 1997) and whole cell extracts (Kielosto et al., 2004) were prepared and analyzed by Western blotting as detailed previously. CM proteins were separated by gel electrophoresis, and transferred to nitrocellulose membrane as above, and stained with Ponceau S to verify equal loading (before Western blotting). As Ponceau stain is not very sensitive, CM samples were also run on a duplicate gel and stained with Coomassie Brilliant Blue (Novex, San Diego, CA) or the Bio-Rad Silver Stain or Bio-Rad Silver Stain Plus kits (Bio-Rad Laboratories, Hercules, CA). Polyclonal antibodies to VEGF (147; Santa Cruz Biotechnology, Santa Cruz, CA), MMP-2 (AB808; Chemicon International, Temecula, CA), and TIMP-2 (AB801; Chemicon International), and monoclonal antibodies to TSP-1 (Ab-4; NeoMarkers, Suffolk, UK; for specificity of the antibody see Supplementary Fig. 2), TN-C (Mtn15; a kind gift from Dr. P. Ekblom, University of Lund, Sweden) (Talts et al., 1998), and MT1-MMP (113-5B7; Calbiochem, San Diego, CA) were used for detection