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Pericytes from human non-small cell lung carcinomas: An attractive target for anti-angiogenic therapy

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Abstract

Anti-angiogenic strategies have largely focused on endothelial cells and progenitors. However, pericytes are also an important component of vasculature. Perivascular cells from normal tissues have been widely reported, yet have not been extensively studied from human tumors. We have investigated pericytes from tumors of patients with lung cancer, the leader of cancer-related deaths in both men and women. Antibodies and magnetic beads were used to isolate cells from non-small cell lung carcinomas (NSCLC). The morphology of the pericytes was distinct with multiple elongated cytoplasmic extensions. Molecular expression of angiogenic genes was quantified by RT-PCR. Flow cytometric analysis shows that NSCLC pericytes express antigens such NG2 and VEGFR1 and present the ganglioside 3G5. The value of pericytes as models of tumor vasculature was demonstrated in cell-culture-based angiogenesis assays such as tube formation and proliferation. Results show that pericytes from some NSCLC but not all were able to maintain tubes networks on Matrigel. Pericyte function can be influenced by angiogenic growth factors or anti-angiogenic agents. Pericytes displayed invasive action against NSCLC clusters in the absence of other cell types. Perivascular cells contribute to the progression of disease and are an attractive target for anti-angiogenic therapy.

Keywords: Pericytes; Non-small cell lung carcinoma; Adenocarcinoma; Squamous cell carcinoma; Angiogenesis; Vasculature; Assays; Models

Introduction

Lung cancer has recently received greater visibility in the field of cancer as the leader of cancer-related deaths for both men and women. The lack of effective treatments illustrates the need for future therapies against novel targets that have previously been overlooked. While anti-angiogenic strategies have supplemented approaches that heretofore have been directed towards cancer cells, the endothelium has been the focus of vascular destabilization. This report details the characteristics and function of perivascular cells or pericytes from malignant tissue. Pericytes are a vital structural component of vasculature and have been identified, isolated, and investigated from multiple tumors surgically resected from patients with NSCLC.

Pericytes were first identified in the microvasculature in 1873 (Rouget, 1873). Since then, pericytes have been described as Rouget cells or myofibroblasts. Pericytes are recognized in tissue sections through immunohistochemical staining for desmin or alpha-smooth muscle actin (αSMA) (Nehls and Drenckhahn, 1993). Electron micrographs show that pericytes

Abbreviations: EC, endothelial cells; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; HMVEC, human microvascular endothelial cells; HDF, human dermal fibroblast; bFGF, basic fibroblast growth factor; MSC, mesenchymal stem cell; PDGF/PDGFβ, platelet-derived growth factor; PDGF-R, platelet-derived growth factor receptor; EGF, epidermal growth factor; ANG, angiopoietin; MMP, matrix metalloproteinase; HGF, hepatocellular growth factor; IL-8, interleukin 8; IGF-1, insulin-like growth factor-1; CD54/ICAM, intercellular adhesion molecule-1; αSMA, alpha-smooth muscle actin; CD106/VCAM, vascular cell adhesion molecule; TGF-β, transforming growth factor-beta; NG2, neurite growth proteoglycan2; FC, flow cytometry; SAGE, serial analysis of gene expression.

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wrap around the abluminal surface of blood vessels and directly interact with endothelial cells (EC) through tight junctions, gap junctions, and by interdigitation (Majno and Palade, 1961; Tilton et al., 1979; Cuevas et al., 1984). Pericytes function in the regulation of blood pressure through contractile action and vascular remodeling in close association with EC (Tilton et al., 1979; Allt and Lawrenson, 2001).

The vasculature of tumors is abnormal compared with the vasculature of the corresponding normal tissue. The pericytes in solid tumor vasculature are irregular in several aspects. The association of tumor pericytes with tumor EC is poorly defined, and cytoplasmic projections of pericytes that in normal tissue interact with EC instead extend deep into tumor tissue (Morikawa et al., 2002). While pericyte recruitment and coverage in the vasculature of human tumors are often very heterogeneous, lung carcinomas show greater incorporation of pericytes to microvasculature than do glioblastomas or renal cell carcinomas (Eberhard et al., 2000). The role of pericytes in tumor vasculature has been studied in several rodent models. In the transgenic RIP1Tag2 mouse model of pancreatic cancer, pericytes were identified associated with tumor vasculature. In addition, the treatment of mice with compounds interfering with the PDGF pathway was able to destabilize the tumor vasculature, including pericytes, leading to tumor regression (Bergers et al., 2003; Pietras and Hanahan, 2005). The ability to target pericytes, in addition to EC, would likely increase the effectiveness of anti-angiogenic therapies.

Pericytes are an integral component of vasculature and have been implicated in the pathology of several diseases including diabetic microangiopathy, hypertension, inflammatory diseases, and tumor growth (Sims, 1991; Herman, 1993; Hirschi and D'Amore, 1996). The goal of the current study was to identify, isolate, and characterize pericytes directly from human lung tumors. NSCLC specimens were obtained fresh from surgery, and through a series of isolation steps for specific cell types, a population of pericytes was selected and cultured from patient samples. The pericytes were further selected using an antibody to NG2, an accessible cell surface proteoglycan. NG2 expression by pericytes has been identified during vascular development by human brain tumors and promotes EC motility and angiogenesis (Fukushi et al., 2004; Chekenya et al., 2002). Growth factors involved in angiogenesis were investigated to determine how they might affect pericyte function. The pericytes isolated from fresh NSCLC specimens were characterized for morphology, expression of molecular markers, and performance in standard angiogenesis assays. Comparisons were made between pericytes isolated from lung tumors of several pathologies: adenocarcinoma (AD), squamous cell carcinoma (SQ), or neuroendocrine (NE).

The angiogenesis field has largely focused on EC and precursors with less attention to the secondary and stabilizing component of vasculature, pericytes. Pericytes isolated from human tumors are an important tool for studying the angiogenesis process and offer a new source of therapeutic targets for strategies directed against NSCLC.

Materials and methods

Tissue dissociation

Lung tumor specimens were received on wet ice and processed within 24 h of surgery. Tumors were obtained from two female and four male patients with informed consent ranging in age from 50 to 77 presenting with NSCLC: 2 SQ, 3 AD, and 1 NE. The tumor samples weighed between 1 and 4 g and were kept in cold RPMI1640 media (Invitrogen Corp., Carlsbad, CA) with penicillin/streptomycin (Invitrogen) prior to processing. The tumor specimens were minced and tweezed apart with crossed scalpels in a Petri dish. The tissue was divided equally into eight 50 ml conical tubes, each containing 30 ml of DMEM high glucose media plus 1% FBS (Invitrogen), 20 mM HEPES (Invitrogen), 0.4 mg/ml elastase (Roche Applied Science, Minneapolis, MN), 4 mg/ml collagenase (Roche Applied Science), and penicillin/streptomycin (Invitrogen). Tumor minceates were digested for 60–90 min at 37°C in a rotational incubator with pipetting every 10–15 min to break up the formation of clumps often aggravated by the presence of tar. The resulting cell suspensions were sequentially filtered through nylon membranes (Tetko, Inc, Briarcliff Manor, NY) with mesh sizes of 500, 250, 100, and 40 μm. The single cell suspensions were divided into eight 50-ml conical tubes, centrifuged at 1200 RPM for 10 min at 4°C. The resulting pellets were suspended in 30 ml of cold PBS (Invitrogen)/0.5% BSA. All remaining procedures except red blood cell (RBC) lysis were carried out at 4°C using PBS/0.5% BSA buffer kept cold on ice. The cell populations were washed twice in PBS/0.5% BSA. The combined pellets were suspended in 6 ml of RBC lysis buffer (Biosource Int'l, Camarillo, CA) and incubated at room temperature for 10 min with occasional agitation. The cells were then divided into two 50-ml conical tubes in a total volume of 30 ml cold PBS/0.5% BSA. Cells were washed by centrifugation and suspension in 30 ml cold PBS/0.5% BSA. Finally, the combined cell pellets were suspended in 1 ml of cold PBS/0.5% BSA.

Isolation of cells

The cell isolation procedures were carried out in a cold room. Magnetic beads were washed, and unconjugated antibodies anti-CD64 (BDPharmingen, San Diego, CA) and anti-NG2 were coupled to beads (Dynal Biotech) according to the manufacturer's instructions. White blood cells were depleted from the normal lung tissue and NSCLC tissue single cell suspensions using magnetic beads coupled with antibodies directed toward CD14, CD45, and CD64 (Dynal Biotech, Brown Deer, WI). Two hundred and twenty microliters of a 1:1:1 mixture of each bead was added to the single cell suspensions in buffer (1 ml) and rotated end-over-end at 4°C for 30 min at slow speed. Beads with attached cells were removed by magnet, and the remaining cell suspension was washed according to the manufacturer's instructions. Cells were pelleted by centrifugation for 8 min at 1200 RPM. The re-suspended cells were exposed to beads conjugated with an antibodies to the epithelial marker (BerPE4) (Dynal Biotech) or to CD31 (Dynal Biotech) for epithelial and endothelial cell collection. The cells remaining in suspension were placed in a 25-cm^2 flask in media formulated to enhance pericyte growth containing 2 ng/ml each bFGF, EGF, IFG-1, 5 mg/ml insulin, 1 μg/ml hydrocortisone, and apo-transferrin (ScienCell Research Labs). After approximately 1 week, the cells reached 60–80% confluency. Pericytes were selected using magnetic beads conjugated to anti-NG2 antibody (Chemicon International, Temecula, CA). Pericytes were maintained in complete pericyte media with supplements (ScienCell Research Laboratories, San Diego, CA). The pericytes collected by brief exposure to 0.25 mM trypsin/50 mM EDTA (Invitrogen) and maintained for up to ten passages.

Immunohistochemistry

NSCLC with well-differentiated adenocarcinoma pathology or lung tissue was flash frozen in OCT compound and sectioned. Slides were dried at room temperature for 10 min, washed twice in TBS, once in TBST (TBS/0.05% Tween-20), and fixed in zinc/formalin buffer for 10 min. Slides were rinsed twice in TBS, blocked for 10 min, rinsed twice again, and incubated with antibodies against CD31 (clone JC70A, DAKO, Carpinteria, CA), αSMA (clone 1A4, DAKO), desmin, or NG2 (Chemicon, Temecula, CA.), for 1 h in a humidified chamber. Slides were