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A Novel *In Vitro* Model of Lymphatic Metastasis from Colorectal Cancer

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Introduction. **For many types of cancers, successful metastasis is critically dependent on tumor cell survival under flow conditions in the lymphatic system as well as attachment to the lymphatic endothelium at distal sites. In the lymphatic system, tumor cells are exposed to dynamic forces of laminar shear stress; however, there are currently no models to study the effects of these dynamic fluid forces on colorectal cancer metastasis. This study aims to establish the rudiments of an *in vitro* flow system that mimics the conditions to which tumor colorectal cancer cells (CRCCs) are exposed during lymphatic spread.**

Methods. **Human CRCCs (RKO and HCT-8) were cultured on collagen-1 coated glass slides in 10% fetal bovine serum, and grown until 50% confluence under static conditions. Subconfluent cells were then treated with laminar shear stress (1.2 dynes/cm^2) using a Flexcell Streamer (Flexcell International Corp., Hillsborough, NC) parallel plate chamber for up to 48 h, in the continued presence of serum. Control conditions consisted of cells maintained under static conditions (0 dynes/cm^2). Cells were examined with digital microscopy. Cell number was determined directly by cell count. Poly (ADP-ribose) polymerase-1, caspase-3, matrix metalloproteinase (MMP)-2, MMP-9, and vascular endothelial growth factor C levels were measured by Western blot.**

Results. **CRCCs survived under conditions of lymphatic flow (1.2 dynes/cm^2), and were confluent by 48 h. Although a small number of cells (10% to 15%) initially detached upon exposure to shear stress, the majority of cells remained attached, and mitotic cells were observed. Cells demonstrated increased attachment and spreading under lymphatic flow compared with cells kept under control conditions. Cell number increased in cells treated with both lymphatic flow and static conditions by similar amounts until confluence was achieved. Cleaved products of poly (ADP-ribose) polymerase-1 and caspase-3 were not observed. MMP-2, MMP-9, and vascular endothelial growth factor C were expressed to similar degrees at all time points in cells exposed to lymphatic flow.**

Conclusions. **Using a novel *in vitro* model of lymphatic flow, we describe colorectal tumor cell proliferation and expression of peptides critical to lymphatic spread under flow conditions. The ability to model lymphatic spread *in vitro* will allow additional studies to determine mechanisms of tumor cell survival in the lymphatic system.**

Key Words: **colorectal cancer; lymphatic; metastatic; VEGF C; lymphangiogenesis; *in vitro*; shear stress; laminar flow.**

INTRODUCTION

Lymphatic metastasis is one of the most important determinants of aggressive cancer phenotype and predicts poor outcome in patients with colorectal cancer [1]. Nodal metastasis is the result of a complex cascade of processes that include invasion of the primary tumor into the lymphatic system, transit of tumor cells through the lymphatic system, homing and attachment of tumor cells to receptive endothelium at distal sites, and implantation and proliferation of viable tumor cells into the subendothelial regional lymph nodes or distant organs, while simultaneously evading the host immune defense [2]. Progress has been made in the elucidation of the molecular processes that underlie metastatic disease, augmenting the potential to selectively target tumor cells [3–6], and potentially leading to new treatment paradigms for metastatic colorectal cancer [7]. In addition to the molec-

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ular and cellular processes at play, it has become clear that in order for successful metastasis to occur, certain biophysical factors such as the flow of lymphatic fluid must be present [8].

Less is known about the lymphatic system in comparison to the rest of the vascular system. Lymphatic biology research has recently been bolstered by the application of unique molecular markers specific for lymphatic endothelial cells, aiding in the characterization of the local tumor microenvironment [2]. A distinguishing feature of lymphatic vessels is the lower magnitude of shear stress to which lymphatic endothelium is exposed, in comparison with the higher flow of both arteries and veins. Exposure of certain cell types to varying degrees of shear stress is known to cause a variety of alterations in cell behavior [9–11]; both endothelial cells and vascular smooth muscle cells align to applied shear stress. However, the effects of shear stress on cancer cells at the low amplitude characteristic of lymphatic flow are unknown.

Whereas conventional thought held that the ability of tumor cells to enter the lymphatic system was predicated on the invasiveness of the tumor itself, it has been demonstrated that tumors actively recruit new lymphatic ingrowth in a process known as lymphangiogenesis [12]. Therefore, not only do tumor cells increase their access to the lymphatic system by direct invasion into regional lymphatics, but they also cause the ingrowth of intratumoral and peritumoral lymphatics through lymphangiogenesis [2]. The principal mediator of lymphangiogenesis in metastatic colorectal cancer appears to be vascular endothelial growth factor C (VEGF C), a ligand for VEGF receptor 3 [13, 14]. Expression of VEGF C in primary lesions has been shown to be an independent predictor of lymph node metastasis and patient survival [15, 16].

It is not currently known whether colorectal tumor cells survive, proliferate, or express VEGF C under lymphatic flow conditions. Therefore, we examined the response of colorectal tumor cells in an *in vitro* model that mimics lymphatic flow *in vivo*.

METHODS

Cells

Colorectal cancer cells (CRCCs) of the RKO and HCT-8 lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and maintained in RPMI 1640 with 10% fetal bovine serum and mycoplasma inhibitor at 37°C in a humidified incubator with 5% carbon dioxide. Cells of passages 4–12 were used for this study; 2×10^5 cells were plated on glass slides coated with collagen-1 and incubated in static conditions until 50% confluent.

Lymphatic Shear Stress Model

Cells adherent to collagen-coated slides were exposed to laminar shear stress of 1.2 dynes/cm^2 for up to 48 h in a Streamer (Flexcell International Corp., Hillsborough, NC) parallel plate laminar flow chamber [17]. The chamber was incorporated into a closed loop MasterFlex peristaltic pump circuit (Cole Parmer, Vernon Hills, IL) containing culture medium equilibrated to the incubation environment. Culture medium was propelled in a continuous, non-pulsatile fashion. Negative controls consisted of plated cells maintained in a static bath (0 dynes/cm^2) of culture medium. Cells were examined with light microscopy using a Zeiss Axioskop 40 (Carl Zeiss, Jena, Germany), and cells per 20× field were counted.

Western Immunoblotting

After treatment, cells were detached from slides by digestion with 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA). The trypsin was neutralized, and cells were collected by centrifugation at 500 *g* for 10 min at 4°C. Cell pellets were washed twice with ice-cold PBS, and lysed using buffer consisting of 25 mM tris hydrochloride pH 7.6, 150 mM sodium chloride, 1% nonidet P-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate, supplemented with 1 mmol/L of the protease inhibitor phenylmethylsulfonyl fluoride. The lysate was centrifuged at 15,000 *g* for 10 min at 4°C and the supernatant containing the protein extract was aspirated. Equal amounts of protein (25 ug, DC Protein Assay; Bio-Rad Laboratories, Hercules, CA) were loaded into a 4% to 15% gradient sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). Blots were blocked using PBS containing 0.05% Tween-20 and 5% nonfat dry milk. Primary detection was performed with the following antibodies: α-tubulin antibody (EMD Bioscience, San Diego, CA), anti-poly (ADP-ribose) polymerase (PARP)-1 antibody, anti-caspase-3 antibody (Upstate USA, Charlottesville, VA), anti-VEGF C antibody (Invitrogen), matrix metalloproteinase (MMP)-2 antibody and MMP-9 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies (Invitrogen) and SuperSignal Pico West Chemiluminescent (Pierce, Rockford, IL) were used for autoradiographic detection. Quantitative blot densitometry was performed using NIH Image software (NIH, Bethesda, MD).

Statistical Analysis

Statistical analysis was performed with SPSS (SPSS, Chicago, IL) using Student's *t*-test. Differences were considered statistically significant when *P*-values were less than 0.05. Error bars represent standard error of the mean.

RESULTS

CRCCs Survive and Proliferate Under Lymphatic Magnitudes of Shear Stress

RKO and HCT-8 CRCCs were cultured on collagen-coated microscopy slides and exposed to lymphatic magnitudes of laminar shear stress (1.2 dynes/cm^2) or control static conditions, in 10% serum for up to 48 h. HCT-8 cells treated with shear stress demonstrated an initial decrease in number, reflecting early detachment of cells, but survived and continued to proliferate after 48 h of lymphatic flow (Fig. 1A). Morphological changes of lateral spreading and cytosol lucency were observed at early time points, but absent at later time points (Fig. 1B). After the initial decrease in cell number, cells treated with lymphatic shear stress continued to grow and proliferate. Confluence was reached in both treated and untreated cultures by 48 h (Fig. 1B).