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Advances in Brief

Transendothelial Function of Human Metastatic Melanoma Cells: Role of the Microenvironment in Cell-Fate Determination[1]

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Abstract

On the basis of the ability of aggressive melanoma cells to participate in vasculogenic mimicry, particularly their expression of endothelial-associated genes, we examined the plasticity of human metastatic cutaneous melanoma cells with respect to vascular function. Fluorescently labeled metastatic melanoma cells were challenged to an ischemic microenvironment surgically induced in the hind limbs of nude mice. The data reveal the capability of these melanoma cells to express cell-fate determination molecules, normally expressed during embryonic vasculogenesis, and to participate in the neovascularization of circulation-deficient muscle. These results demonstrate the powerful influence of the microenvironment on the transendothelial differentiation of aggressive melanoma cells, and may provide new perspectives on tumor cell plasticity that could be exploited for novel therapeutic strategies.

Introduction

A common necessity for development, wound healing, and tumor progression is a blood supply formed by vasculogenic and/or angiogenic events, involving the cooperative interactions of cells with their microenvironment (reviewed in Refs. 1–4). Without question, the constituents of the microenvironment play a key role in the regulation of these events through complex signaling cascades and cell-matrix dynamic reciprocity (2). The vascularization of tumors, in particular, has been the focus of myriad studies with the ultimate goal of targeting key factors and cells responsible for the formation of vasculature (5–7). However, recent evidence has confounded our understanding of tumor vasculature and underscored the complexity of the events involved, including reports of mosaic vessels (tumor- and endothelial-lined vasculature; Ref. 8), tumor-lined vasculature (9, 10), angiogenic *versus* nonangiogenic pathways of tumor dissemination (11), and tumor cell vasculogenic mimicry (12).

Our knowledge of the molecular determinants of cancer has benefited significantly from microarray analysis technology in recent years. The first molecular classification of human malignant cutaneous melanoma revealed the aberrant expression of multiple molecular phenotypes by the aggressive, but not the nonaggressive, melanoma cells reminiscent of an embryonic-like cell (13). However, follow-up investigations addressing the putative biological significance of some of these aberrant genes have been slow to emerge.

The present study follows a unique strategic approach using an ischemic microenvironment and challenges malignant melanoma cells to make cell-fate decisions regarding their participation in neovascularization and/or tumor formation. The results demonstrate the influence of the microenvironment on the transendothelial differentiation of aggressive melanoma cells and may provide new perspectives on tumor-cell plasticity that could be exploited for novel therapeutic strategies.

Materials and Methods

Cell Culture. The human cutaneous nonaggressive and nonmalignant C81–61 and aggressive, malignant C8161 cell lines have been characterized previously according to their phenotype, invasive and metastatic potential, and clinical significance (14). These cell lines were maintained in either RPMI 1640 for the C8161 or Ham's F-10 for the C81–61 (InVitrogen Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA) and 0.1% gentamicin sulfate. Cell cultures were determined to be free of *Mycoplasma* contamination using the GenProbe rapid detection system (Fisher, Itasca, IL). The C8161 cells that were stably transfected with EGFP[3] (pEGFP-N1; Clontech, Palo Alto, CA) were maintained as above with the addition of 400 μg/ml G-418 (Mediatech, Herndon, VA). Transient tumor cell expression of either the green (Ad5 RSV-GFP) or RFP (Ad5 RSV-RFP) was achieved by adenoviral infection (University of Iowa Gene Transfer Vector Core, Iowa City, IA).

Ischemic Mouse Model. All of the procedures were performed on nude ($HFh11^{nu}$) mice (Jackson Laboratories, Bar Harbor, ME) according to The University of Iowa Animal Care and Use Committee guidelines. Surgical procedures were followed as has been described previously (15).

Under anesthesia (91 μg of ketamine + 10 μg of xylazine/gram) the mouse was placed ventral side up, and a 1–1.5-cm incision made in the skin in the left inguinal area. The femoral artery was then ligated twice with 6–0 silk and transected in two places distal to the ligature. Any other large blood vessels distal to the ligature that were visible were also transected. The wound was closed with 4–0 silk, and the hind limbs were gently immobilized and scanned using a laser Doppler imager (Moor Instruments, Inc., Portland, OR), which measures the flux (blood $\times$ area^{-1} $\times$ time^{-1}) of blood in the limb as described previously (15). The mean flux in the operated limb immediately after surgery ranged from 6–13% compared with the unoperated control limb for the mice studied. Four to 8 h after surgery, 1×10^3 or 2×10^3 Ad-RFP-transfected C8161 and C81–61 human melanoma cells were injected i.m. into the ischemic limb.

Five and 20 days later, four mice that had received 1×10^3 and 4 mice that had received 2×10^3 Ad-RFP cells were anesthetized according to modifications of previously described methodologies (8, 15). A PE10 catheter was inserted into the abdominal aorta. After anchoring the catheter, the vessel was anterogradely perfused with 250 μg of F-BSLB$_4$ (Vector Laboratories, Burlingame, CA) or a mixture of 250 μg of F-BSLB$_4$ and 500 μg of FITC-labeled *Ulex europaeus* agglutinin (F-Ulex; Vector Laboratories; $n = 2$) in 200 μl of 0.9% NaCl. Four mice were coperfused with F-BSLB$_4$ and 3×10^5 of 4.2-μm and 2×10^5 of 6.0-μm polystyrene microspheres (Molecular Probes, Eugene,

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[3] The abbreviations used are: EGFP, enhanced green fluorescent protein; Ad, adenovirus; RFP, red fluorescent protein; Eph, Eph receptor tyrosine kinase; BSLB$_4$, *Bandeira simplicifolia* lectin B$_4$; F-BSLB$_4$, FITC-labeled BSLB$_4$.

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OR). Immediately before use, microspheres were vortex mixed, sonicated for 2 min, and then revortexed and diluted in the F-BSLB$_4$/0.9% NaCl solution. Four to 7 min later, the hind limbs were perfused with 3–5 ml of 1% paraformaldehyde in PBS. The muscle and overlying skin from the ischemic limb, and contralateral limb muscle were harvested and postfixed 4–12 h in 2% paraformaldehyde.

Immunohistochemistry. The identification and localization of cytokeratins 8, 18, and Notch 3 and Notch 4 were performed by using antibodies to each protein (clone CK5 for cytokeratin 18 from Sigma Chemical Co., St. Louis, MO; Notch-3 and -4 from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the Vectastain ABC and AEC kits (Vector Laboratories). The specificity of the Notch-3 and -4 antibodies was verified previously by Western analysis (16). Cell cultures and tissue were fixed in 3.7% formaldehyde followed by a wash in 0.1% Triton X-100 in PBS for 5 min, and frozen unfixed tissues were used as well. The cultures were then incubated with either a primary antibody or a nonspecific IgG antibody for 2 h, followed by processing using the Vectastain ABC and AEC kits according to the manufacturer's protocol. The identification of mouse endothelial cells in paraffin sections of EGFP-labeled tumor cells in reperfused tissues was performed using BSLB$_4$ with Alexa 594 (red) dye (Molecular Probes). Images were obtained using a Zeiss Axioskop 2 microscope (Carl Zeiss, Inc., Thornwood, NY), a Spot 2 camera (Diagnostic Instruments, Inc., Sterling Heights, MI), and Zeiss Axiovision 2.0.5 software.

Confocal Microscopy. Vibratome sections (200–250 μm thick) of mouse tissues were examined using a Zeiss LSM510 confocal microscope (Carl Zeiss, Inc.), using both the rhodamine and fluorescein filters for each image collected during the scanning process. The tomographic images were then converted into 32 projected images calculated from the original images throughout 53 degrees of rotation about the X-axis using the Zeiss LSM510-associated software. Individual projected images at each point of rotation were captured and an animated movie of the projected images revolving through a 53-degree arc was subsequently created.

Results and Discussion

A series of novel experiments were designed to test the putative biological significance of vasculogenic mimicry by aggressive human melanoma cells. In the present study, Ad-RFP (RFP-expressing Ad) or EGFP-labeled human cutaneous metastatic melanoma cells were injected i.m. into hind limbs of nude mice with surgically induced ischemia (resulting in blood flow <15% of normal). Five days postischemia, the presence of microspheres in limb vasculature (perfused via the aorta) demonstrated reperfusion of the limb as demonstrated by the presence of perfused beads within the vasculature (Fig. 1*A*). Mice were perfused via the aorta with F-BSLB4 and *Ulex europaeus* agglutinin (Fig. 1*B*) to visualize the luminal side of the vascular wall, and confocal microscopy revealed Ad-RFP-labeled melanoma cells adjacent to the luminal label (Fig. 1, *C* and *D*). The three-dimensional reconstruction of confocal images into both vertical and horizontal planes delineates melanoma cells within vascular walls *versus* those external to the vasculature (Fig. 1*D*). To exclude the possibility that the Ad-vector targets murine vessels, similar experiments were performed using metastatic melanoma cells stably transfected with EGFP. Five days postischemia, histological cross-sections of muscular tissue, stained with mouse endothelial cell-specific BSLB4 (followed by streptavidin-conjugated Alexa dye 594), showed human melanoma cells adjacent to, and overlapping with, mouse endothelial cells in a linear arrangement (Fig. 1*E*). When poorly aggressive EGFP-labeled melanoma cells were introduced at the site of ischemia in separate experiments, they were not found 5 days postischemia (Fig. 1*F*).

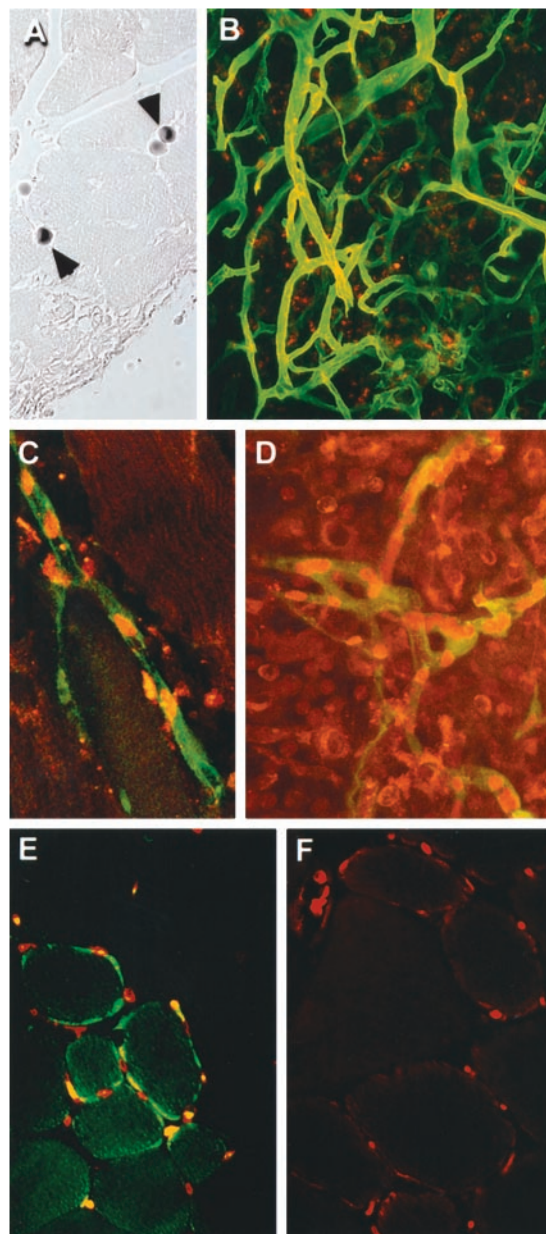

Fig. 1. Microscopic analyses of revascularization of nude mice ischemic hind limbs 5 days after surgical severing of femoral arterial branches followed by inoculation with 1×10^3 Ad-RFP- or EGFP-labeled human metastatic cutaneous melanoma cells (C8161) i.m. *A*, bright field microscopy of histological section shows perfusion of 10-mm beads (*arrowheads*) in neovasculature of ischemic muscle; *B*, confocal microscopy of mouse neovasculature perfused with F-BSLB$_4$ and *Ulex europaeus* agglutinin delineating mouse vessels (*green*) and Ad-RFP-labeled tumor cells (*red*) with (*C*) branched neovasculature-containing tumor cells, confirmed in (*D*), with three-dimensional reconstruction showing melanoma cells in the lumenal side of the vascular wall (AVImovie@http://www.anatomy.uiowa.edu/pages/directory/faculty/images/vert_plane.avi). *E*, cross-section of EGFP-labeled metastatic melanoma cells colocalized with mouse endothelial cells labeled with BSLB$_4$ and streptavidin Alexa dye 594 (*red*); *F*, poorly aggressive EGFP-labeled melanoma cells (C81–61) were not found with mouse endothelial cells (*red*). (*A*, ×20; *B–F*, ×40.)

The evidence shown here demonstrates the powerful influence of the microenvironment on the transendothelial differentiation of malignant melanoma cells needed for neovascularization and reperfusion of ischemic limbs. On the basis of the microarray analysis of the malignant cutaneous melanoma cells used in this study (13), we suggested that the molecular signature of these tumor cells resembles that of a pluripotent, embryonic-like phenotype. An example of the plasticity of the aggressive melanoma phenotype has been demonstrated by the ability of these cells to engage in vasculogenic mimicry, the *de novo* formation of vasculogenic-like networks concomitant with the expression of vascular endothelial-cadherin (*VE-cadherin*) and other endothelial-associated genes (13, 17, 18), and participation in neovascularization shown in the present study. It is evident from our investigation that the malignant, aggressive melanoma cells re-

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sponded very differently to the ischemic environment than did the nonaggressive melanoma cells. Although a comparative study of the molecular profiles of these two phenotypes would have predicted a differential response, a functional assay was needed for proof of principle. Furthermore, recent studies have challenged previously held dogma regarding tissue-restricted differentiation of postnatal stem cells with convincing evidence that demonstrates pluripotency for mesenchymal, neural, and hematopoietic stem cells (for review, see Ref. 19). In addition, there are now documented examples of environmentally induced changes in differentiated cells, called transdifferentiation, which may help to elucidate the plasticity of cellular phenotypes (for review, see Ref. 20). Indeed, our results coincide with the recent observation that cardiomyocytes induced endothelial cells to transdifferentiate into cardiac muscle in ischemic hearts (20). This study may provide clues as to the potential inductive nature of the ischemic skeletal muscle in our experiments. Other studies supporting our unique observations have shown that fibrocytes can induce an angiogenic phenotype in cultured endothelial cells and promote angiogenesis *in vivo* (21); and transformation of fibroblasts into endothelial cells during angiogenesis has also been shown (22). Most intriguing is the potential significance of the expression of CD34, a progenitor cell antigen, normally found in endothelial cells and now reported in invasive malignant melanoma (23).

During embryogenesis, the formation of primary vascular networks occurs via vasculogenesis, the *in situ* differentiation of mesodermal progenitor cells (angioblasts or hemangioblasts) to endothelial cells that form a primitive network (for review, see Refs. 1, 3). Subsequent growth and remodeling of the vasculogenic network into a refined and efficient vasculature occurs via angiogenesis, the sprouting of new vessels from a preexisting network. Our present concept is that all blood vessels are derived from the same precursor cells in the embryo. However, how the ultimate vascular fate of these precursors is determined has remained somewhat elusive. In our investigation of ischemic limb reperfusion, we chose to examine selected Notch proteins known to promote the differentiation of endothelial cells into vascular networks (24, 25). As shown in Fig. 2 by immunohistochemistry, Notch 3 (Fig. 2*A*) and Notch 4 (Fig. 2*B*) were strongly expressed in the malignant melanoma cells but not in the nonspecific antibody control (Fig. 2*C*) nor in the nonaggressive melanoma cells (Fig. 2*D*). Notch signaling molecules are integrally involved in cell-fate determination of stem cells and nonterminally differentiated cell types (24, 25). Alterations in Notch expression and signaling have also been implicated in human T-cell leukemia (26), cervical carcinoma (27), murine mammary carcinomas (28), and prostatic tumor progression (29). A recent report has provided new information regarding the signaling pathways involved in arterial-venous decisions by angioblast precursor cells, including Notch-Gridlock pathways and EphB2 and EphB4 expression (30). Previous observations from our laboratory have shown the necessity for EphA2 in melanoma vasculogenic mimicry (18), which may coincide with some of the findings in the angioblast cell-fate determination study.

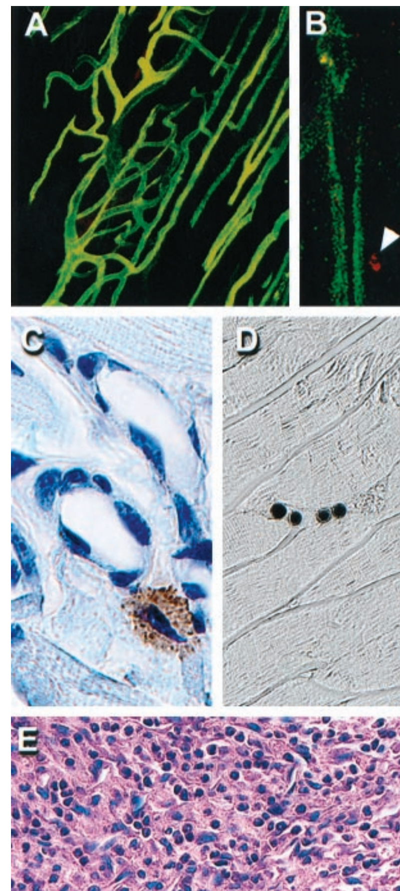

Fig. 3. Microscopic analyses of reperfused nude mice ischemic hind limbs at 20 days postischemia. *A*, confocal microscope-projected image of mouse vasculature (*green*) showing the relative absence of metastatic melanoma cells (C8161) with (*B*) demonstrating one Ad-RFP-labeled tumor cell extravascularly (*red*, *arrowhead*); *C*, cross-section of similar area showing one melanoma cell extravascularly (*brown* immunostaining for cytokeratins 8, 18), and (*D*) bright-field view showing perfused vasculature containing beads. *E*, microscopic view of H&E-stained tumor formed i.m. by C8161 metastatic melanoma cells. *A*, *B*, and *D*, ×20; *C*, ×63; *E*, ×40.

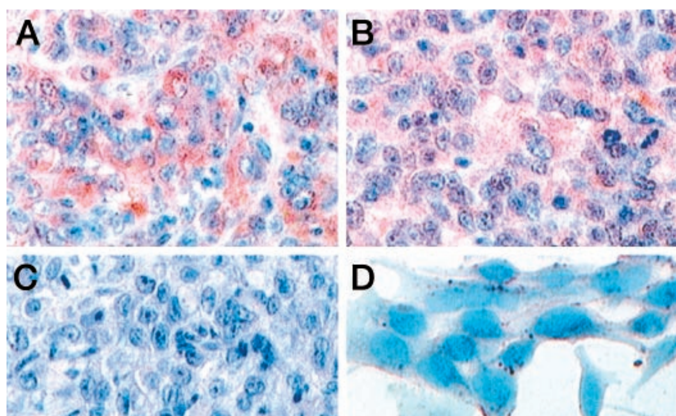

Fig. 2. Immunohistochemical localization of Notch proteins in 5-day reperfused mouse ischemic hind limbs and in cell culture. Notch 3 (*A*) and Notch 4 (*B*) expression (*red*) is intense in tissue sections containing metastatic melanoma cells (C8161) in reperfused ischemic muscle; *C*, control for Notch staining of a serial section treated with a nonspecific antibody; *D*, nonaggressive melanoma cells (C81–61) were essentially negative for Notch 3 (data not shown) and Notch 4 staining in tissue culture. *A*, *B*, and *C*, ×40; *D*, ×63.

Evaluation of the mouse reperfused limbs 20 days postischemia (Fig. 3) showed well-formed vasculature with no detectable colocalized metastatic melanoma cells (Fig. 3*A*). Rarely, a melanoma cell was found external to the hind limb vasculature (Fig. 3, *B* and *C*), which had been completely reperfused, as shown by the perfusion of beads within the vasculature (Fig. 3*D*). By this time period, melanoma tumors had developed in other areas of the musculature (Fig. 3*E*), which suggested that the influence of the microenvironmental cues under ischemic conditions ceased to control the transendothelial phenotype function of the aggressive, malignant melanoma cells. Collectively, these observations advance our understanding of the remarkable inductive nature of the microenvironment on aggressive tumor cells that express vasculogenic/angiogenic molecules, together with cell-fate-determination signaling proteins, associated with a transendothelial function. These findings present new possibilities for therapeutic strategies and novel perspectives on tumor-cell plasticity.

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