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Increased plasticity of the stiffness of melanoma cells correlates with their acquisition of metastatic properties

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Abstract

The stiffness of tumor cells varies during cancer progression. In particular, metastatic carcinoma cells analyzed by Atomic Force Microscopy (AFM) appear softer than non-invasive and normal cells. Here we examined by AFM how the stiffness of melanoma cells varies during progression from non-invasive Radial Growth Phase (RGP) to invasive Vertical Growth Phase (VGP) and to metastatic tumors. We show that transformation of melanocytes to RGP and to VGP cells is characterized by decreased cell stiffness. However, further progression to metastatic melanoma is accompanied by increased cell stiffness and the acquisition of higher plasticity by tumor cells, which is manifested by their ability to greatly augment or reduce their stiffness in response to diverse adhesion conditions. We conclude that increased plasticity, rather than decreased stiffness as suggested for other tumor types, is a marker of melanoma malignancy. These findings advise caution about the potential use of AFM for melanoma diagnosis.

From the Clinical Editor: This study investigates the changes to cellular stiffness in metastatic melanoma cells examined via atomic force microscopy. The results demonstrate that increased plasticity is a marker of melanoma malignancy, as opposed to decreased stiffness.

Key words: Melanoma; Atomic force microscope; Cell stiffness; Malignant progression

Solid tumors are characterized by increased stiffness compared to normal tissues, which results mainly from increased stroma rigidity due to fibrosis and extracellular matrix (ECM) remodeling.[1,2] Changes in stroma composition and rigidity are sensed by cancer cells and translated into modifications of their cytoarchitecture and consequently of their own stiffness. By modulating their elastic properties tumor cells can better sustain increased stromal pressure within a developing tumor, and enhance their deformability and invasion potential.[3] Several studies have shown that the degree of stiffness of tumor cells in vitro correlates with their motility properties, so that softer cells are more motile than stiffer cells, and this has been linked to their ability to invade and metastasize in vivo.[4-8] In this respect, metastatic carcinoma cells isolated from pleural effusions of patients were shown to be significantly softer than normal mesothelial cells present in the same fluids.[5,9] In an effort to relate cell stiffness data obtained in vitro on isolated cells to the observation that tumors are in general stiffer than the surrounding normal tissues, several groups have performed elasticity measurements on whole tumor tissues and have demonstrated that stiffness is not uniform within a tumor but it varies in different regions depending on the presence of stromal components.[2,10] In particular, Plodinec and colleagues demonstrated that areas in the core of breast carcinomas composed mainly of cancer cells and containing minimal ECM appeared softer than peripheral tumor regions enriched in stromal tissue.[2] In addition, the tumor cores were less stiff than healthy mammary tissues, suggesting that carcinoma cells are in fact softer than their normal counterparts. This conclusion remains

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speculative, however, since a direct comparison should take into account the structural differences between the normal mammary gland and a tumor, in particular the fact that normal mammary cells are organized in ducts and alveoli surrounded by ECM and stromal cells and do not form the packed aggregates analyzed in tumor cores. In any case, it is clear that tissue stiffness changes during tumorigenesis, and that the analysis of the stiffness of tumors and isolated tumor cells may have both diagnostic and prognostic value.

Since the majority of the works published so far analyzed cells originating from different carcinomas, we wondered whether similar changes in tumor cell stiffness occur also during malignant progression of human melanoma, a tumor derived from melanocytes, cells of neuroectodermal origin. Human melanoma progression is characterized by successive stages of cell transformation from radial growth phase (RGP) tumors that grow locally in the epidermis and are not invasive, to vertical growth phase (VGP) tumors that degrade the ECM and invade the dermis, to metastatic tumors (met) that can enter the lymphatic and blood circulation and metastasize to other organs.[11] Cell lines isolated from RGP, VGP or metastatic tumors are available and at low passages in culture they maintain the malignant features of the tumor they were derived from, i.e. VGP and metastatic but not RGP cells are tumorigenic in vivo when injected subcutaneously in immunocompromised mice, and only metastatic cells can form metastases in vivo.[12]

In this study we employed Atomic Force Microscopy (AFM) to analyze the stiffness of normal human epidermal melanocytes (NHEM), RGP, VGP and metastatic melanoma cells in relation to adhesion to ECM proteins, cell morphology and cell motility. In particular, we analyzed a VGP and a metastatic cell line derived from a primary melanoma and a metastasis from the same patient to better characterize how cell stiffness may evolve during malignant progression in cells originating from a single tumor. We show that the initial stages of melanocyte transformation are characterized by decreased tumor cell stiffness, whereas the acquisition of metastatic competency is associated with increased average stiffness and increased heterogeneity of stiffness values in the whole cell population. We hypothesize that progression to the metastatic stage requires that melanoma cells become able to modify their stiffness over a broad range of values in response to external cues in order to promptly adapt and survive in different environments encountered during metastatic dissemination.

Methods

Cell culture

NHEM (Normal Human Epidermal Melanocytes, NHEM) were purchased from PromoCell. The human melanoma cell lines WM115 (VGP)[12] and WM239A (metastatic)[12] kindly provided by Dr. M. Herlyn (Wistar Institute, Philadelphia PA, USA), and the human RGP melanoma cell line SBCl2[13] kindly provided by Dr. B. Giovanella (Stehlin Foundation for Cancer Research, Houston, Texas, USA) were cultured in Tu 2% medium (WM series) or RPMI supplemented with 10% FBS (SBCl2) and antibiotics as previously described.[14]

Migration assay

A Costar Transwell system (8 μm pore size) was used. 30,000 cells were plated in the upper chamber in serum- and insulin-free medium. Tu 2% medium without insulin was added in the lower chamber as chemoattractant. After 24 h migration was stopped, cells that did not migrate were removed from the upper side of the filter with a cotton swab and cells that had migrated and were attached to the lower side of the filter were stained with 0.5 % Crystal Violet and counted in four fields per well. In experiments with human Transforming Growth Factor β1 (TGFβ1, Roche) cells were incubated in serum- and insulin-free medium in the presence of 5 ng/ml TGFβ1 dissolved in PBS containing 5 μg/ml BSA for 24 h before plating in Transwells and starting migration. During migration TGFβ1 (5 ng/ml) was present in both the upper and lower chambers. For statistical analyses, ANOVA/Student–Newman–Keuls (for multiple comparisons of migration abilities of the four cell types), or one-sample *t* test (migration in the presence of TGFβ1) was performed.

Immunocytochemistry

Cells were plated in 8-well glass chamberslides coated with 5 μg/ml fibronectin (Sigma) in the presence of normal growth medium, or in chamberslides coated with 100 μg/ml poly-L-lysine (Sigma) and blocked with 1% (w/v) BSA (Sigma) in medium lacking serum and insulin (melanoma cells) or growth supplement (NHEM). After incubation for the indicated times cells were fixed with 2% paraformaldehyde for 10 min and permeabilized with PBS-0.2% TritonX100. After blocking (2.5% BSA, 2.5% normal donkey serum, 0.2% TritonX100 in PBS) for 1 h cells were incubated overnight at 4 °C with Rabbit anti-Paxillin 1:100 (Cell Signalling), followed by incubation with Phalloidin-AlexaFluor488 1:400 (Life Technologies) together with the secondary antibody anti-rabbit-AlexaFluor568 1:300 (Life Technologies). Slides were mounted with ProLong Gold Antifade Reagent containing DAPI (Life Technologies) and analyzed using Zeiss AxioVision software.

Soft agar assay

Soft agar assays were performed in 6-well plates with Noble Agar (Difco Laboratories) according to standard procedures (0.6% agar for bottom layer, 0.3% agar for cell suspension). Cells (5000/well) were plated in triplicate. After 3 weeks pictures were taken using a Leica stereomicroscope, and the number of colonies with more than 30 square pixels was counted using ImageJ software. For statistical analysis, multiple comparison ANOVA/Student–Newman–Keuls test was performed.

Animals and in vivo metastasis experiments

WM115 and WM239 cells (2×10^6) were injected in the tail vein of 10-week-old NOD/SCID mice. Five mice per group were analyzed. Mice were sacrificed 6 or 9 weeks after injection and lungs were fixed in 4% formalin (Sigma) and embedded in paraffin. The presence of metastasis was analyzed after H&E staining of lung sections according to standard protocols. All