SUBSTRATE RIGIDITY DICTATES PHENOTYPE, SURVIVAL, AND MECHANICS OF PRIMARY HUMAN OSTEOSARCOMA CELLS

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Abstract: Chemical cues and physical forces tightly control the milieu where a cell is born, survives, and finally dies. Of the physical forces, tissue rigidity is known to direct cell fate. Nonetheless, malignant tumors drastically alter the local physiological tissue rigidity. Our aim was to determine the functional changes of cells derived from an osteosarcoma, a very stiff malignant formation, to substrates of varying rigidity. Cells extracted from a human femoral osteosarcoma were exposed to collagen I-coated polyacrylamide substrates mimicking the rigidity of brain (1 kPa), connective tissue (7 kPa), and collagenous bone (55 kPa). Glass was used as a control substrate. Osteosarcoma cells occupied the smallest area and were rounder when on the compliant 1 kPa substrate compared to the stiffer substrates. Additionally, cell apoptosis, as assessed by Annexin V staining, and total death rate were significantly increased on the more compliant substrates of 1 and 7 kPa. Finally, as computed via a Fourier transform algorithm, cells exerted greater traction forces on the rigid 55 kPa substratum and less on the more compliant 7 and 1 kPa substrates. Taken together, these data suggest that osteosarcoma cells survive and function more efficiently on substrates mechanically closer to their native microenvironment.

Introduction

Cell morphology, differentiation, and survival are constantly controlled by inasmuch the chemical cues as the mechanical input from the surrounding microenvironment. Over the last decade several reports have shown that substrate rigidity can control various functions of a healthy cell, such as migration, proliferation, apoptosis and differentiation [1-3]. On one of the original reports, the migration speed of mouse 3T3 fibroblasts was shown to increase proportionally to the rigidity of the polyacrylamide substrate [4]. In addition, rigid surfaces enhanced the proliferative and reduced the anti-apoptotic potential of 3T3 fibroblasts compared to the more compliant substrate conditions [2]. Interestingly, Engler et al. showed that substrate rigidity also dictates the differentiation path that a stem cell will follow. [5].

Besides physiological conditions, microenvironment mechanics are also important in disease states, such as cancer [3, 5, 6]. Evidence suggests that tumors are more rigid relative to the surrounding tissue, whereas individual cancer cells are more compliant compared to healthy cells of the same lineage [6, 7]. Despite the evident implication of mechanics in carcinogenesis, the up-to-date reports on cancer cell mechanosensing are limited and often contradictory [2, 6, 8]. Specifically, contrary to normal cells, H-*ras*-transformed NIH 3T3 cells apoptosis was unaltered on varying rigidity substrates [2]. Similarly, cells from a mouse sarcoma cell line, despite the increase in migratory speed on more rigid substrates compared to softer ones, remained minimally responsive or totally non-responsive to other migratory parameters, such as directionality and polarization [9].

All of the currently available studies on *in vitro* cell mechanosensing employ the use of normal and malignant cell lines. Nonetheless, it is known that there are inherent differences in cells derived from a cell line and primary cells of the same origin [10]. It is, thus, of particular value to determine the responses of primary cells extracted directly from an excised human tumor. By doing so, we demonstrate that primary human cells derived from a human osteosarcoma exhibit altered phenotypic and functional responses to substrates of varying rigidity.

Methods

Cells and Reagents. Primary human cells were isolated from a surgically excised, non-metastasized, femoral parosteal osteosarcoma of a female patient. Specifically, samples from the osteosarcoma were mechanically dispersed and the osseous fragments were placed on polystyrene culture dishes in growth medium (GM) comprised of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5% penicillin and streptomycin. The fragments were left undisturbed for ten days in a humidified environment at 37^{o}C and 5% CO_2. Individual cells that adhered onto the plate were washed gently with warm GM and subcultured. These cells exhibited primarily phenotypes of os-

teoblastic and fibroblastic origin (Figure 1A). To rid of contaminating fibroblasts, cells were plated on a plastic dish for 45 minutes, whereupon all non-adherent cells were removed and replated on a new tissue culture plate. The presence of fibroblasts was thus minimized in subsequent passages. Cells from the second passage as well as GM supplemented with 25 mM HEPES were used for all the experiments.

Polyacrylamide and bis-acrylamide were purchased from Merck (Darmstadt, Germany), all other chemicals and reagents were purchased from SIGMA (St. Louis, MO, USA), fluorescent carboxylate-modified latex beads and Annexin V reagent were from Molecular Probes (Eugene, OR, USA), sulfo-SANPAH was purchased from Pierce Biotechnology (Rockford, IL, USA), TEMED was purchased from Fisher Scientific, Fairlawn, IL, USA).

Substrate Construction. Polyacrylamide (PA) gels were constructed and collagen coated as previously described [11, 12]. Briefly, coverslips were treated with 3-aminoplopyltrimethoxysilane, dried, and soaked in 5% glutaraldehyde. Acrylamide (30%), bis-acrylamide (3%), HEPES (1M), and distilled water were mixed in concentrations ranging between 5-8% acrylamide and 0.03-0.5% bis-acrylamide. Gels were crosslinked using ammonium persulfate (1:250) and TEMED (1:2000). Fluorescent carboxylate-modified 0.2 μm latex beads (1:100) were added to determine traction force and gel rigidity. A 25 μl of the mixture were allowed to polymerize between the activated coverslip and a 22 mm diameter No 1 coverslip, then washed and mounted onto the custom-made plexiglas chambers bearing a 20 mm diameter annulus through the center. PA gels needed for the completion of all the experiments were constructed concurrently and used immediately. PA gels were activated for protein cross-linking with sulfo-SANPAH under a UV lamp and coated with 0.15 mg/ml Collagen I at 37^{o}C for two hours. Prior to experiments, the gels were equilibrated with GM in a humidified environment at 37^{o}C, 5% CO_2, for 45 minutes. Additionally, collagen-coated glass was used as a control substrate.

Substrate Characterization. A stainless steel microball (640 μm diameter; 7.2 g/cm3) was dropped onto the fluosphere-bearing gel surface equilibrated in GM. The vertical displacement of flusopheres immediately underneath the microball was recoded before and after removing the microball using the calibrated fine adjustment knob of an inverted Leica DMIL microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). The Young's modulus (E) was then calculated for each substrate as described [12].

Cell Morphology Measurements. Osteosarcoma cell area and circularity were measured (n=24-52) using ImageJ (NIH, Bethesda, MD, USA).

Cell Apoptosis and Death. Apoptotic and necrotic cells were identified using Annexin V and propidium iodide (PI), respectively. Specifically, following overnight adhesion onto the different substrates, live cells were incubated with an Annexin V and PI mixture for 15 minutes according to the manufacturer's instructions. Apoptosis, necrosis and total cell death were quantified and expressed as the percentage of Annexin V^+ and PI^+ cells relative to the total number of cells analyzed (n=200-370). Total cell death was expressed as the percentage of both apoptotic and dead cells relative to total cell number.

Traction Force Measurements. Forces applied by cells onto the flexible substrates embedded with fluospheres were derived as previously described [13]. Briefly, cells were allowed to adhere on gels with embedded flusopheres. Concurrent phase contrast and epifluorescent images of the cells and the fluospheres lying directly underneath them, respectively, were captured prior to and immediately following removal of the cell from the gel surface via trypsinization. Cell traction forces (Pascal; Pa) were computed based on fluosphere displacements using the unconstrained Fourier transform traction cytometry algorithm.

Image Preparation and Statistical Analyses. Digital images were acquired using an inverted Leica DMIL microscope equipped with a Leica DFC 480 CCD digital camera and subsequently processed using Adobe Photoshop version 7.0. Adjustments of brightness and contrast were performed equally throughout an entire image. Comparisons among multiple groups were performed using one-way analysis of variance with a Newman-Keuls post-hoc test. The level of significance was set at $p \leq 0.05$. Statistical analyses were performed and graphs were produced using GraphPad Prism version 4 (GraphPad Software).

Results

Based on cell morphology, many osteoblastic and fibroblastic cells could be identified immediately following isolation from the tumor sample (Figure 1A). Nonetheless, the phenotype that predominated on the subsequent passages following preplating was that of osteogenic origin (Figure 1B).

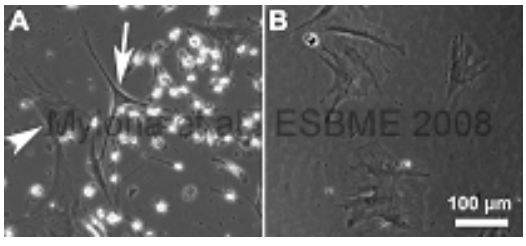

Figure 1. Primary human osteosarcoma cell morphology. (A) From the cell pool initially isolated, cells with osteoblastic (arrowhead) and fibroblastic (arrow) morphology can be identified. (B) Osteoblastic morphology predominated following cell population expansion and subculturing.

The Young's moduli of the elicited gelatinous substrates were 0.9, 6.9, and 55 kPa, herein termed as 1, 7 and 55 kPa, which closely mimic the rigidity of brain, connective tissue and collagenous bone, respectively.

Primary osteosarcoma cell morphology was rigidity dependent. Specifically, cells on the 1 kPa substrate were the smallest (1607 μm^2). Cell area significantly increased by 3.5- and 6.2-fold onto the 7 and 55 kPa substrates, respectively. Interestingly, only the very rigid glass substrate supported cells of area greater than 50000 μm^2. Additionally, as demonstrated by the calculated cell circularity, only the 1 kPa substrate prohibited cells from spreading thus assumed a significantly rounder morphology, whereas the stiffer substrates allowed for elongated cell morphologies.

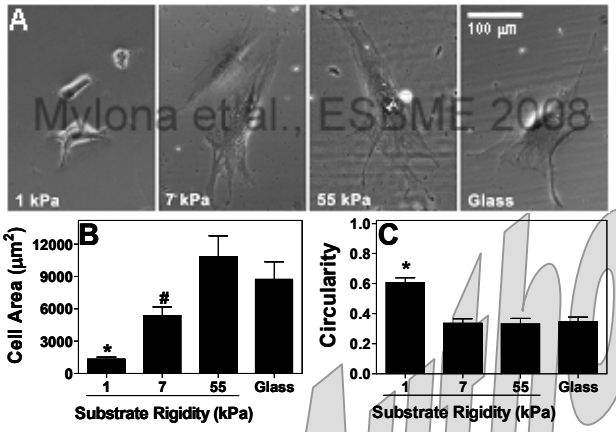

Figure 2. Substrate rigidity dictates cell morphology. (A) Osteosarcoma cell area decreases as substrate compliance increases. (B) Cell area was the largest on the rigid substrates. (C) Cells exposed to compliant surfaces were more round compared to all other substrate stiffness conditions. * Significantly different compared to all other conditions; # significantly different compared to 55 kPa ($p \leq 0.05$).

Cell apoptosis and necrosis were assessed via Annexin V and propidium iodide incorporation, respectively (Figure 3A). Cell apoptosis and total cell death were 4- and 3-fold decreased for cells on the 55 kPa and glass substrates compared to 1 and 7 kPa substrates, respectively (Figure 1B). The presence of necrotic cells was minimal in all conditions, thus did not contribute significantly to the total cell death counts (data not shown).

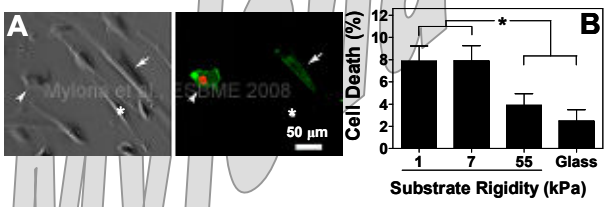

Figure 3. Osteosarcoma cell death is enhanced on compliant substrates. (A) Phase contrast and epifluorescent images of viable (asterisk), apoptotic (arrow) and necrotic (arrowhead) cells. (B) Cell death was significantly reduced on the more rigid substrates ($p \leq 0.05$).

Traction exerted by the primary osteosarcoma cells onto the surface of the three distinct gelatinous substrates was also derived via a Fourier transform traction cytometry algorithm. The magnitude and localization of traction forces differed among rigidities. Traction forces on the 55 kPa gel were localized at the leading edge and uropod of polarized cells and reached magnitudes of 2000 Pa. On the contrary, cells on the compliant 1 kPa gel were unable to exert tractions greater than approximately 500 Pa.

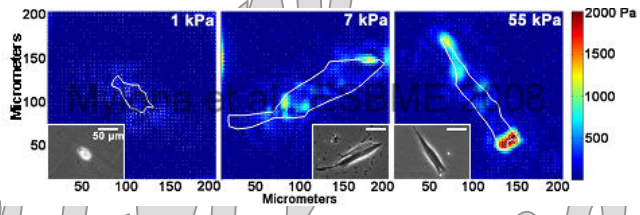

Figure 4. Rigid substrates support the exertion of greater traction forces by the cells. The depicted traction fields were computed from fluosphere displacement fields. The white outline denotes the location of the cell relative to the applied traction forces. Greater forces were exerted at the edges of the long axis of the cell body. Insets: Phase contrast images of the assessed cells.

Discussion

Traditionally, biological research has focused on the chemical effectors that control a cell's fate. The study of the mode and effects of the mechanical signals exchanged between a cell and its microenvironment is relatively recent. We utilized substrates that were chemically identical but differed in their physical parameter of rigidity. We show that primary cells derived from a human osteosarcoma responded to alterations in the mechanical properties of their substrate both morphologically and functionally.

Shape and size are both determining factors and excellent indicators of the cell's homeostatic state [14, 15]. McBeath and colleagues demonstrated that human mesenchymal stem cells' lineage commitment was dependent on the shape they were artificially forced to assume. Under the same culture conditions, cells forced to occupy a small area differentiated into adipocytes, whereas cells allowed a greater area went down the osteoblastic pathway [15]. The existing evidence on the effects of substrate rigidity on cell size is contradictory. Smooth muscle cells' size increased in response to increased substrate rigidity, whereas osteoblasts showed no such response [1, 16]. In our hands, primary osteosarcoma cells were larger at higher rigidities. It is noteworthy that peak area was observed at 55 kPa, which is the rigidity evidenced to support induction of osteogenic gene transcription [5].

Osteosarcoma cells were rounder on the very compliant surface but elongated on all other substrate rigidities. Round morphology of adherent cells is invariably associated with increased apoptosis, as for osteosarcoma cells on the 1 kPa rigidity substrate. Apoptosis and

death of cells on the rigid substrates of 55 kPa and glass was minimized. Nonetheless, osteosarcoma cells died on the 7 kPa at the same rate as the 1 kPa substrates. Apparently the 7 kPa rigidity does not provide the mechanical support for survival, albeit the better adhesion evidenced by the larger cell area and elongated shape. Interestingly, Wang at al. showed that H-*ras*-transformed fibroblasts survived equally well on all the different rigidity substrates, concluding that unresponsiveness also to mechanical, besides chemical, signals is what makes cancer cells resilient [2]. This contrast with our findings may be attributed to differences between the diverse primary cells of a lineage and the clonal cells from a cell line.

Cells are in constant physical interaction with their microenvironment. They receive mechanical signals from their surroundings and tune their own mechanical responses accordingly [6, 17]. Recently developed traction cytometry algorithms enabled the visualization and quantification of the cell traction forces [13]. It was thus shown that increasing the substrate rigidity leads to greater traction forces being applied by the cell. In addition, the traction responses of malignant mammary epithelial cells to a soft substrate were greater than those of their normal counterparts [6]. We show that the traction forces of primary osteosarcoma cells correlate with increases in substrate rigidity. The physiological significance of this response is currently under investigation.

Cancer perturbs normal homeostasis, which is otherwise tightly controlled by fine-tuned chemical and physical mediators. Elucidating the mechanical cues working in carcinogenesis and metastasis will potentially provide with preventative means to their onset and progression.

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