Primary Human Osteosarcoma Cell Responses to Alterations in Substrate Rigidity

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INTRODUCTION:

Relevant research over the last decade has shown that, in addition to chemical cues, substrate rigidity can also control various functions of a healthy cell, such as migration, proliferation, apoptosis and differentiation. Besides physiological conditions, microenvironment mechanics are also important in disease states, such as cancer. 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. Despite the evident implication of mechanics in carcinogenesis, the up-to-date reports on cancer cell mechanosensing are limited and often contradictory. 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. It is, thus, of particular value to determine the responses of primary cells extracted directly from an excised human tumor.

We hypothesized that primary cancer cells derived from a human osteosarcoma would exhibit altered phenotypic and functional responses to substrates of diverse, physiologically-relevant rigidities. By constructing gelatinous substrates that closely mimic the rigidity of various bodily tissues, such as brain (1kPa), connective tissue (7 kPa) and collagenous bone (55 kPa), we demonstrate that primary osteosarcoma cells show phenotypic and functional responses preferential for stiffer substrates, especially those with mechanical properties closer to their native microenvironment.

METHODS:

Cells. Following the informed consent of the patient, primary human cells were isolated from a surgically excised, non-metastastatic, femoral parosteal osteosarcoma of a female patient. Osteosarcoma samples were mechanically dispersed and isolated adherent cells were expanded in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 5% penicillin (GM) and streptomycin in a humidified environment at 37°C and 5% CO_2. Cells from the second passage and GM supplemented with 25 mM HEPES were used for all the experiments.

Substrate Construction and Characterization. Glass coverslips were activated with 3-aminoplopyltrimethoxysilane and glutaraldehyde. Gels comprised of acrylamide (5-8%) and bis-acrylamide (0.03-0.5%) were polymerized onto the activated coverslips. For traction force and gel rigidity experiments, fluorescent 0.2 μm latex beads were added. Gels were mounted onto custom-made chambers, chemically activated and coated with 0.15 mg/ml Collagen-I. Collagen-coated glass was used as a control substrate. A stainless steel microball (640 μm diameter) was dropped onto the fluosphere-bearing gels. The vertical displacement of flusopheres immediately underneath the microball was recoded before and after removing the microball using a calibrated inverted Leica DMIL microscope. The Young's moduli (E) of the elicited gels were 0.9, 6.9, and 55 kPa (herein referred to as 1, 7, and 55 kPa, respectively).

Cell Morphology and Function Measurements. Osteosarcoma cell area and circularity were measured (n=24-52) using ImageJ. Apoptosis and necrosis of adherent osteosarcoma cells were revealed using Annexin V and propidium iodide staining, respectively. Apoptosis, necrosis and total cell death (apoptosis plus necrosis) were quantified and expressed as the percentage of Annexin V^+ and PI^+ cells relative to the total number of cells analyzed (n=200-370). Finally, to determine cell traction forces onto the flexible substrates, cells were first 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 cell trypsinization. Cell traction forces were computed based on fluosphere displacements using the unconstrained Fourier transform traction cytometry algorithm.

Statistics. One-way analysis of variance with a Newman-Keuls post-hoc test were performed for comparisons among multiple groups using Sigma Stat 3.5 software. The level of significance was set at $p \leq 0.05$.

RESULTS:

Primary osteosarcoma cell morphology was rigidity dependent. 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 ($p<0.05$). Additionally, as demonstrated by the calculated cell circularity, only the 1 kPa substrate prohibited cells from spreading thus assumed a significantly rounder morphology (0.6 ± 0.03; mean ± SEM), whereas the stiffer substrates allowed for elongated cell morphologies ($p<0.001$). Cell apoptosis and total cell death were 4- and 3-fold decreased for cells on the 55 kPa and glass substrates (2%) compared to 1 and 7 kPa substrates (8%), respectively ($p<0.04$). Necrotic cell counts were minimal in all conditions, thus did not contribute significantly to the total cell death. Finally, 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.

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. Human mesenchymal stem cell lineage differentiation was recently shown to be size dependent. Substrate rigidity has elicited diverse cell shape responses, as smooth muscle cells' size increased in response to increased substrate rigidity, whereas osteoblasts showed no such response. 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. 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 observed for osteosarcoma cells on the 1 kPa rigidity substrate. Cell apoptosis and death 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. Cells are in constant physical interaction with their microenvironment. We showed that the traction forces of primary osteosarcoma cells correlate with increases in substrate rigidity.

These encouraging results need to be further enriched with similar experiments on primary isolates from osteosarcomas as well as other tumors. 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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