Metastatic cancer cells tenaciously indent impenetrable, soft substrates

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Abstract. We present here the first evidence of mechanical penetration by a metastatic cancer cell. During metastasis, the invasive cancer-cell penetrates tissue and extracellular matrix, changes shape and applies force. These applied forces, in turn, depend on substrate stiffness and degradability. The initial stage of metastatic penetration comprises substrate indentation, which, however, has not yet been studied. Hence, we evaluate the evolution of indentation, focusing on differences relating to the metastatic potential (MP) of the cells and substrate stiffness. We found that metastatic cells attain a mushroom-like morphology and then, over several hours, repeatedly indent the substrate in a manner suggestive of a special role for the nucleus. Cells with higher MP have previously been shown to be softer internally and externally than those with lower MP yet, paradoxically, applied stronger forces. Cells of higher MP develop stronger forces on gels stiff enough to provide grip handles yet soft enough to indent, whereas benign cells did not indent substrates at all. These findings provide insight into the central role of physical forces in the initial stages of metastatic penetration and reveal new targets for treatment.

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1. Introduction

The process of invasion is of special importance in cancer metastasis, the main cause of death in cancer patients. During metastasis, invasive cancer cells penetrate tissue and extracellular matrix (ECM) to reach potential secondary-tumor sites [1]. While penetrating, cells change shape and apply mechanical forces depending on substrate stiffness and degradability [2–4]. Substrate stiffness affects cell adhesion, morphology, motility and forces developed in the cell [5, 6]. Thus, recent studies have been focused on forces that cells apply after adhering to two-dimensional (2D) substrates [7–10] or as they move through three-dimensional (3D) matrices [11, 12]. However, the first stage of cell penetration, which comprises 2D substrate indentation, has yet to be studied. Metastatic cells have typically been shown to penetrate a matrix by degrading it or by squeezing through pores, following biochemical interactions [13]. In the system used here, we reveal a mechanically based indentation process that occurs on a non-degradable and impenetrable substrate with sub-micron holes. The system used here can model initial stages of penetration into a substrate as it can serve, for example, as a mimic for the ECM or endothelial cells on a blood-vessel wall.

Two modes of 3D cell-motility have been defined: mesenchymal and amoeboidal [14]; cancer cells can switch between modes according to environmental conditions [14, 15]. Mesenchymal motility involves proteolysis of the ECM and is characterized by elongated cell morphology. In contrast, amoeboid cells typically remain rounded and do not require proteolysis to move [15]. In amoeboid motility, cells move aside the ECM fibers and pull the cell body forward by applying contractile forces using acto-myosin machinery within membrane protrusions [16]. Accordingly, elevated acto-myosin generated forces were correlated with increased invasiveness in metastatic cells [17]. Actin-containing membrane protrusions evidently transmit extracellular forces across the membrane, through the cytoskeleton, and to the nucleus [18]. However, in invasion, the nucleus is typically suggested to follow, perhaps passively, after initial membrane protrusions are successfully dispatched [13]. Our work reveals a special role for the nucleus, where it is transported into the substrate indentation by the surrounding cytoskeleton.

Here, we show that metastatic cells repeatedly indent an impenetrable, elastic, soft substrate for several hours. Metastatic cells attained two different morphologies that allowed
indentation. Both morphologies were mushroom-like, but likely resulted from different biomechanical interactions with the substrate. Interestingly, forces applied by cells through both morphologies were similar. Applied forces increased with metastatic potential of the cells and with gel stiffness, while depth of indentation and specifics of cell morphology depended more on cell type.

2. Methods

2.1. Sample preparation

Human, highly metastatic breast-cancer epithelial cells MDA-MB-231 (ATCC HTB-26) and low metastatic potential breast-cancer epithelial cells MDA-MB-468 (ATCC HTB-132) were grown in DMEM (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, ThermoFisher Scientific, Waltham, MA) and 1 vol% of L-glutamine, sodium pyruvate and penicillin-streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel). Benign MCF-10A cells, ATCC origin (CRL-10317), were kindly provided by Professor Israel Vlodavsky from the Faculty of Medicine, Technion-Israel Institute of Technology. The benign cells were cultured in DMEM/Ham’s F-12 medium supplemented with 5% horse serum (HyClone, ThermoFisher Scientific, Waltham, MA), 1 vol% of L-glutamine and penicillin/streptomycin, 10 µg ml⁻¹ insulin, 10 ng ml⁻¹ epidermal growth factor, 0.5 µg ml⁻¹ hydrocortisone and 100 ng ml⁻¹ cholera toxin. Cells were cultured and maintained in a humidified incubator at 37 °C, 5% CO₂ and were used at passages 10–30 from stock. From here on, the cells will be referred to with simplified names: the MDA-MB-231, MDA-MB-468 and MCF-10A will be referred to as high MP, low MP and benign.

The polyacrylamide gel that was used was prepared according to an established protocol [7, 10]. The surface of a number five cover slip, 30 mm (Menzel, Germany), was first coated with hydroxyl groups using 0.1 M NaOH (Sigma, St Louis, MO), and then activated using 3-aminopropyltrimethoxysilan (Sigma, St Louis, MO) and glutaraldehyde fixed. All the solutions for preparing the gel were kept at 4 °C, and the preparation of the gel was done on ice. The gels were composed of 30 µl of 40 vol% acryl and 5/2.6 µl of 2 vol% BIS acrylamide (both from Bio-rad, Israel), used to generate the 1250/2300 Pa gels, respectively. Gelation was induced with 1:200 vol% Ammonium per sulphate as initiator and 1:500 vol% Tetramethylethylene diamine as a catalyst (both from Sigma, St Louis, MO). The gel was obtained on the cover slip, using a plastic frame (Gene Frame, 25 µl, 10 × 10 mm, ABgene Thermo-Scientific, Waltham, MA). The gel was covered with a plastic top to keep it without oxygen that inhibits the gel polymerization. The gel was prepared with fluorescent, carboxyl-coated polystyrene particles, 200 nm in diameter (Molecular Probes, Invitrogen Life Technologies, Carlsbad, CA), and in order to bring the beads to the gel surface, the gel was placed in cooled centrifuge, 2 °C, and centrifuge for 30 min at 300 g [10, 19]. Then the gel was left to polymerize at room temperature upside down to keep the particles on the surface. After the polymerization the plastic top and frame are removed. The gel was kept in phosphate buffered saline for at least 24 h before the beginning of the experiments so all the solute polyacrylamide could diffuse out. Finally, the surface of the gel is activated with Sulfo-SANPAH (Pierce, Thermo Scientific, Waltham, MA). The activated surface is then coated with collagen (Rat tail type 1, Sigma, St Louis, MO), and kept at 4 °C for 12 h.
2.2. Microscopy, image acquisition and analysis

Imaging was done with an inverted, epifluorescence Olympus IX81 microscope, using a 60× differential interference contrast (DIC) air-immersion, long working distance objective (numerical aperture 0.7). We imaged the cells every 1 h for 6.5 h, starting 1.5 h after seeding to allow initial adherence; cells exposed to fluorescence before \( t = 1.5 \) h or at shorter intervals typically remained rounded and did not attempt indentation. At each time-point three images were taken: DIC image of the cells on the gel, fluorescence image at the gel surface focal plane, and fluorescence image at a focus below the gel, at the bottom of the cell protrusion or indent. Images were taken using an XR Mega-10AWCL camera (Stanford Photonics Inc., Palo Alto, CA), at a final magnification of 107.8 nm per pixel.

Images were analyzed using a custom-designed module in MATLAB 2010b (The MathWorks, Natick, MA). We have measured areas of the cells in images and have also independently obtained the focal depths of each image. The areas and depths were used to obtain approximate radii and forces applied by the cells, using a simple Hertz model:

\[
F = \frac{4E}{3(1-\nu^2)} R^{1/2} \Delta L^{3/2} \quad [20].
\]

Here the \( E \) is the gel’s Young’s modulus, \( \nu \) is the Poisson ratio of the gel (0.5), \( \Delta L \) is the indentation depth and the \( R \) is the radius of the cell above the gel surface. We assume the cell is an elastic, isotropic body. In addition, we assume that interaction between the cell and the gel is due solely to forces applied by the cell.

2.3. Trans-retinoic acid treatment

High MP cells were plated and incubated for 5 days with trans-retinoic acid (TRA) (Merck-Millipore, Whitehouse Station, NJ) at a final concentration of 25 \( \mu \)M [21, 22]. On the day of the experiment 150 000 cells were seeded on the stiffer, 2300 Pa polyacrylamide gel.

2.4. Rheology

We have determined the Young’s modulus of the gels using TA Instruments AR-G2 rheometer (New Castle, Delaware). Gels were prepared on the rheometer plate and modulus was measured during and after gelation with a 2 cm diameter parallel plate fixture. We have run time sweep experiments during and following gelation (oscillatory stress of 3.14 Pa and angular frequency of 3.14 rad s\(^{-1}\)) to determine the Young’s modulus using the following relation:

\[
E = 2G^* (1 + \nu)
\]

where \( G^* \) is the complex modulus and is equal to the elastic modulus \( G' \) in an elastic material and \( \nu \) is the Poisson’s ratio.

2.5. Staining and confocal imaging

For experiments, prepared gels with particles were incubated for 1.5 h with the culture media and then cells were seeded on the gel within 12-well plates. After plating, all samples were incubated for 2 h to allow initial cell attachment. Samples were fixed and stained to observe cytoskeleton structure or live stained for relative gel-nucleus location. For live staining, the nucleus was stained with Hoechst 33342 (Sigma, St Louis, MO). Cells were fixed with 3.2% paraformaldehyde and permeabilized with 0.5% triton. Blocking for non-specific binding was done with 1% gelatine (Sigma, St Louis, MO). Microtubulin staining was done with the primary antibody \( \alpha \)-tubulin (Sigma, St Louis, MO) and secondary antibody was Cy3 goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Actin staining was done with the antibody Phalloidin Fluorescein Isothiocyanate (Sigma, St Louis, MO). In parallel with the secondary
antibody, cell nuclei were labeled with 4′-6-diamidino-2-phenylindole (DAPI, Sigma, St Louis, MO). Imaging was performed with a spectral-imaging Zeiss LSM700 confocal system mounted on a motorized Axio Observer Z1 microscope, using a 63 × 1.4 NA oil objective. Images were taken in stack of 16–22 slices in x,y-scale of 0.06 µm and z-scale of 0.5 µm.

3. Results and discussion

We monitored the indentation produced by metastatic breast-cancer cells on two soft, flat, polyacrylamide gels. We have chosen Young’s moduli of the gels at 1250 ± 150 and 2300 ± 200 Pa; representing the physiological range of soft tissue [23]. As a metastatic cell indents the substrate, 3D structural changes are revealed through focal-height changes in the 200 nm diameter fluorescent particles embedded at the gel surface. That depth-of-indentation allows us to evaluate the applied normal force using the Hertz model [20]. In contrast to metastatic cells, control, benign cells (MCF-10A) remained rounded and did not indent gels at any timescale (data not shown). To correlate the indentation capabilities with the metastatic potential, we modified the highly metastatic cells with TRA; TRA has been shown to reduce in vitro invasive capacity of the cells by 60–65% [22].

Most cancer cells remained rounded on the gel surface and about 15, 30 and 25% of cells indented the gel in the low MP, high MP and TRA-treated high MP cells, respectively. We monitored a total of at least 30, 55 and 50, respectively, indenting cells in four experiments on each gel; TRA-treated cells were only evaluated on the stiffer gels. Fewer cells adhered on the softer gels and less low MP cells adhered overall. High MP cells were observed to relocate on the gels after several hours, repeating indentation attempts at a different location.

Figure 1 shows two distinct morphologies observed during gel indentation, indicating different interactions with the substrate. Between 60–75% of indenting cells displayed extensive blebbing while others exhibited skirt-like structures with protruding lamellipodia (figures 1(a) and (b)). We have determined that cells are viable on our gels, regardless of the observed

Figure 1. Highly metastatic breast-cancer cells indenting a 2300 Pa gel. (a) Bleb morphology. (b) Skirt-like morphology. (c), (d) Gel-surface focal height. Particles under the cell are out of focus. (e), (f) Indent height, showing particles in focus at bottom of indented region. Scale bar is 10 µm.
blebbing (see supplementary figure 1, available from stacks.iop.org/NJP/15/035022/mmedia); blebbing could potentially indicate apoptosis. TRA-treated high MP cells exhibited almost exclusively the blebbing morphology. In both morphologies and especially in those blebbing, a condensed ring of particles was observed under the cells (figures 1(c) and (d)), which also appears elevated from the gel surface (see figure 2(b)). Within this defined margin particles are at a lower focal plane, indicating indentation (figures 1(e) and (f)). Thus, indentation occurs when a cell pulls the surrounding gel surface inwards (figure 1(c)) and upwards (figure 2(b)) to push its center vertically into the gel; a 3D sketch of the cell structure is provided in figure 2(c).

These morphologies relate to how each cell interacts with the substrate when applying force. Blebbing has previously been observed in these cells during and following adherence [24]. Cell adhesion has been shown to induce isometric tension in the cytoskeleton [25, 26]. Such symmetric forces are likely also applied during blebbing-morphology indentation, resulting in the nearly perfect circular ring in figure 1(c). In contrast, skirt-like indenters exhibit an uneven, incomplete ring under the cell (figure 1(d)). We believe the
Figure 3. Indent morphology presented as area at the gel surface versus area at the bottom of the indent and depth of indentation, both at all times. (a) Area ratio of high MP cells on the 1250 Pa (■) and the 2300 Pa (▲). The line is a guide to the eye with slope of 1.6 obtained from average of the best fits; TRA treated high MP cells on 2300 Pa (▲); (b) area ratio of low MP cells on the 1250 Pa (■) and the 2300 Pa (▲) with slope of 1.3; (c) distribution of indentation depths of high MP cells 1250 Pa gel on 1250 Pa gel (■), 2300 Pa gel (▲) and TRA-treated high MP cells on 2300 Pa gel (▲). T-test shows there was no statistical difference between the high MP cells on the two gels, TRA-treated cells were statistically different and similar to the (p = 0.05); (d) indentation depths of low MP cells on 1250 Pa gel (■), 2300 Pa gel (▲). T-test shows that indentation depth is larger on the 2300 Pa gel than on the 1250 Pa gel (p = 0.05). In addition, the TRA-treated high MP cells on the 2300 Pa gel were indistinguishable from the low MP cells on the 1250 Pa gel (p = 0.05)

skirt-like structures may result from an unstable state in the focal adhesion complexes [2] or uneven attachment to substrate ligands [25].

Confocal images (figure 2) reveal mushroom-like shape of an indenting cell. The protrusion that is locally pushing into the gel is the mushroom stem. Within the stem we find actin and microtubules followed by the nucleus, all of which can be used to apply force. Active, dynamic force application at cell leading-edges is typically associated with acto-myosin [27]. The cytoskeleton transmits force and is mechanically connected to the nucleus, [18, 28] which

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has recently been suggested to act as a direct mechanical sensor of substrate topography [29]. We suggest that the nucleus is actively transported into the protrusion, probably propelled by actin–microtubule cooperation, and can be used to apply extra force on the gel.

The depth and areas of the indentation stem depend on gel stiffness and metastatic potential. We did not observe any distinct features differentiating the blebbing or skirt-like morphologies, thus those are shown together; those were statistically indistinguishable in most cases. Figures 3(a) and (b) show the area ratio of stem top-to-bottom (i.e. surface and indent locations as defined in figure 2(c)). That ratio is constant for each cell type, larger for the high MP cells, and independent of gel stiffness. Interestingly, treating with TRA did not affect the observed areas or their ratio, further indicating it is a cell specific feature. Concurrently, the indentation depth varied significantly with metastatic potential and with gel stiffness (figures 3(c) and (d)). Indentation depths observed here (up to 9 µm) are significantly larger than dimples exhibited by cells that spread [30] or crawl [31] on a substrate (< 0.5 µm); those smaller dimples are a consequence of 2D cell adhesion and not due to an actively applied vertical force as observed here. The high MP cells exhibited a wider distribution of indentation depths on the stiffer gels yet with the same average as on the softer gels. In contrast, in the low MP cells the average depth decreased on the softer gels. Decreasing invasive potential with TRA treatment of the high MP cells reduced distribution width as well as average depth; depths following treatment are reduced to the scale of those observed in the low MP cells on the softer gel.

Cells repeatedly indent the gels over several hours applying alternately larger and smaller forces with time (see supplementary figures 2 and 3, available from stacks.iop.org/NJP/15/035022/mmedia). We observe that gel stiffness and cell type determine the force. Morphology (skirt/blebb) did not significantly affect the forces, hence both are averaged together; in the high MP cells on the stiffer gels a significant difference was observed, yet for consistency we average those together as well. In the high MP cells forces are smaller on the softer gels as compared to the stiffer, as less force is required to achieve the same indentation depth. However, for the low MP cells, the penetration depth reduces on the softer gels as does the applied force, likely indicating they are not able to grip the gel and develop force as well. As force is not exerted continuously, we define force as a function of attempt number.

Figure 4 shows repeated indentation attempts defined as local force-maxima observed during the experiment time. Only cells that exhibited indentation in at least five time-points were counted (supplementary figures 2 and 3, available from stacks.iop.org/NJP/15/035022/mmedia); fewer low MP cells attempted indentation overall and especially on the soft gels. We observe that the number of attempts does not significantly vary between the cell types and the two gels (figure 4(a)), making it an independent parameter that can be used to effectively replace time. Figure 4(b) shows that force applied by treated and untreated cancer cells decreases with attempt number and is significantly smaller on softer gels (see also supplementary figure 4). On the softer gels, a cell likely cannot grip the gel properly and therefore builds up less overall force and also reduces number of attempts; cells have also been shown to apply stronger lateral forces with higher substrate rigidity [27]. TRA treatment reduces the force applied by a high MP cell below the force applied even by a low MP cell on the same gel. Interestingly, we observe that cells apply less force with increasing numbers of attempts indicating mechano-cellular feedback. The feedback concept is strengthened when observing cell responses after several hours; responses differ for high and low MP cells. The high MP cells on both gels typically relocated to different sites after several hours (validated at 24 h). In those new sites
Figure 4. Number of attempts and forces applies during, (a) number of cell indentation attempts (top) high MP cells attempts on, from left to right, the 1250 Pa (green) and 2300 Pa (red) gels and TRA-treated high MP cells on 2300 Pa gel (blue), (bottom) low MP cells. Only cells with at least five time-points were counted. T-test shows that there is no significant difference in number of indentation attempts between the different cells on the various gels ($p = 0.01$), making it an independent parameter. (b) Average peak forces applied as a function of attempt number by the high MP cells (◼, ◣), low MP cells (▲, ▼) attempts on the 1250 Pa (empty) and 2300 Pa (full) gels and TRA-treated high MP cells on the 2300 Pa gel (●). Error bars are standard errors. $T$-tests show that differences between forces applied at attempts 1 and 2 are statistically significant ($p \leq 0.01$); in attempt 3 there was not enough data to verify significance.

cells either indented gels again or remained rounded. In contrast, the low MP cells typically remained stationary and simply ceased indenting. Hence, in both cases, cells eventually sense and respond to the impenetrability of the substrate.

4. Conclusions

In conclusion, metastatic cells will actively and repeatedly indent a soft gel, although it is non-degradable and impenetrable. Cell indentation involves active participation of the cytoskeleton and possibly also the nucleus in a likely mechano-cellular feedback. The two morphologies observed during indentation could indicate different mechanisms for force generation, yet produce similar force magnitudes. We show that although the high MP cells are softer both internally [32] and externally [21, 33] as compared to low MP and non-cancerous cells, they are able to apply stronger forces. Reducing the invasive capacity of the high MP cells with TRA did not affect the number of indentation attempts or the protrusion-stem morphology, yet reduced the indentation depth and force applied by a cell. Hence, our work reveals that the observed mechanical indentation process is directly related to the metastatic potential of a cell as well as substrate stiffness. Cells develop force more readily on stiffer gels, thus substrate stiffness or adhesion/motility molecules may also be viable targets against metastatic penetration. Our work shows the central role of mechanics during the indentation process and could also be essential during in vivo metastatic penetration, through ECM and blood-vessel endothelium, concurrent with biochemical processes.

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