Direct mapping of melanoma cell – endothelial cell interactions

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Abstract

The most life-threatening aspect of cancer is metastasis; cancer patient mortality is mainly due to metastasis. Among all metastases, presence of brain metastasis is one with the poorest prognosis; the median survival time can be counted in months. Therefore, prevention or decreasing their incidence would be highly desired both by patients and physicians. Since the central nervous system (CNS) lacks classical lymphatic circulation, metastatic cells invading the CNS must breach the blood-brain barrier. The key step in this process is the establishment of firm adhesion between the cancer cell and the cerebral endothelial layer.

Using the atomic force microscope, a high resolution force-spectrograph, our aim was to explore the connections among the cell morphology, cellular mechanics and biological function in the process of transendothelial migration of metastatic cancer cells. By immobilization of a melanoma cell to an atomic force microscope's cantilever, intercellular adhesion was directly measured at quasi-physiological conditions. Hereby we present our latest results using this melanoma -decorated probe. Binding characteristics to a confluent layer of brain endothelial cells was directly measured by means of single cell force spectroscopy. Adhesion dynamics and strength was characterized and we present data about spatial distribution of elasticity and detachment strength.

These results highlight the importance of cellular mechanics in brain metastasis formation and emphasize the enormous potential towards exploration of intercellular dynamics related processes.

Keywords: atomic force microscope, single-cell force spectroscopy, nanomechanics, cancer, direct mapping, living cell, cell adhesion, cerebral endothelial cell, melanoma

Introduction

One of the most feared complications of cancer incompatible with life are brain metastases (BM), often causing life impairing neurological symptoms. Presence of BM has a very poor prognosis; the median survival time can be counted in months, rarely few years [1]. Different cancers show different propensities to form BM. Although melanoma is only 1-2% of all cancers, it has the highest tendency to invade the central nervous system (CNS) [2]. Autopsy studies showed that up to 90% of patients with end-stage melanoma had BM [3]. Despite our growing knowledge about biology of BM formation, the precise details that trigger and guide tumor cell towards the brain are still under debate.

Formation of BM requires escape of tumor cells from primary sites, their hematogenous dissemination, attachment to the blood vessel wall, transmigration through cerebral endothelial layer into the brain parenchyma which must be followed by invasion and interaction with the microenvironment resulting in survival and proliferation [4]. This multi-stage process has a crucial step, namely the firm adhesion of blood travelling tumor cells to the cerebral endothelial cells (CEC) which form the cellular basis of the first defense line of the brain, the blood-brain barrier (BBB). By forming a precisely regulated barrier between the circulation and the CNS, the BBB restricts the free movement of solutes and cells between the two compartments.

Cancer cells and especially melanoma cells must have special attributes which somehow facilitate breaching the BBB. Tumor cell – endothelial cell interactions are key steps of BM formation, therefore a detailed understanding of this interaction could lead to the identification of mechanisms which could reduce transmigration and thus BM formation. Biochemical characteristics are strongly linked to biophysical manifestations, hence nanomechanical characterization of a tumor cell – endothelial attachment has important information on their dynamics of attachment. *In vitro* cell co-culture models dealing with the whole transmigration process have already been successfully established [5]. Characterization of the elasticity, adhesion dynamics or strength might provide additional and complementary information.

In order to investigate mechanics from single molecule [6] to single cell [7] level the atomic force microscope (AFM) is an outstanding system. Being a member of the scanning probe microscope superfamily, the AFM soon after its invention [8], became the most reliable and accurate nanoforce-tool in the research of cellular biomechanics [9]. Using AFM-based single cell force spectroscopy, interaction of living cells can be monitored in liquid environment and at human body temperature [10,11]. Moreover, high resolution topographical maps can be reconstructed based on the measured mechanical parameters [12,13].

It has been shown previously that short term adhesion of a human melanoma cell to CECs layer is highly dependent on contact time and applied load [14]. In this study, we report direct mechanical mapping of a human CEC layer using a human melanoma cell as a probe. Spatial distribution of several nanomechanical parameters are shown in order to characterize the elasticity and linkage strength between the melanoma cell and the CEC layer.

Materials and Methods

Cell culture

hCMEC/D3 human microvascular cerebral endothelial cells (shortly D3, [15]) were grown on rat tail collagen-coated dishes in EBM-2 medium (Lonza) supplemented with EGM-2 Bullet Kit (Lonza) and 2.5% FBS (Sigma).

Highly invasive A2058 human melanoma cells (obtained from the European Collection of Cell Cultures) were maintained in EMEM (Sigma) supplemented with 5% FBS (Sigma). These cells were derived from metastatic site (lymph node) of a 43 years old caucasian man. Tumor cells were labeled with CellTracker^{TM} Red CMTPX Dye (Life Technologies), ensuring that they can be clearly identified prior to immobilization at the end of the cantilever.

AFM

All experiments were carried out with an Asylum Research MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA; driving software IgorPro 6.32A, Wavemetrics), mounted on a Zeiss Axiovert 200 optical microscope.

The experiments were performed with rectangular tipless cantilevers, having a nominal spring constant of 50 pN/nm, resonant frequency of 10 kHz in air (MikroMasch, Tallinn, Estonia), which drops to 3 kHz in water. The spring constant of the cantilever was determined each time by thermal calibration [16,17].

Cancer cells were immobilized at the very end of the tipless cantilever using a Concanavalin-A mediated linkage which is described elsewhere [7,14]. During culturing cells were kept at 5% CO_2 atmosphere at 37°C. All experiments were conducted in serum free Leibovitz medium at 37°C within one hour after the cells were taken out from the incubator. According to our experience, they preserve their viability far beyond this period.

Single Cell Force Spectroscopy

During a force measurement cycle, a cancer cell immobilized at the end of a tipless cantilever was brought into contact with surface-adherent endothelial cells. The schematic representation can be found on Figure 1.

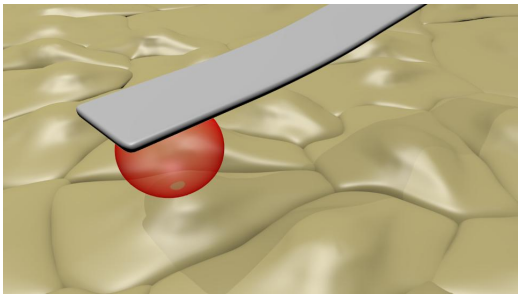

Figure 1. Schematic representation of experimental mapping setup.

Each cycle consisted of approaching the cell -decorated cantilever to the endothelial layer until the pre-set deflection was reached and pulling it back to initial position. Force curves were recorded at constant loading speed (8 µm/s) and sampling frequency (2 kHz). Total force distance was kept at 3 µm with maximum load of 2.0 nN. 32 lines by 32 points maps were recorded at each selected area of 90 µm by 90 µm, total data collection being less than 15 minutes.

Figure 2 shows a typical force-distance curve: contact point, is marked with "**o**", approaching phase is drawn in red while blue represents

retraction, as pointed by accordingly colored arrows.

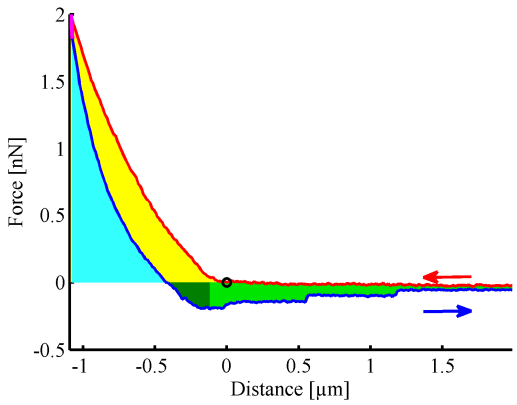

Figure 2. A representative force-distance curve. Contact point is marked with "o", yellow area marks the plastic work, cyan+yellow area marks the total elastic work, dark and light green area represents the total adhesion work.

Data analisys

A home made MATLAB routine was used to extract all the parameters. Elastic work (yellow+cyan area, Figure 2), represents the deformation of the cells by the applied force, plastic work (cyan area, Figure 2) when the cell regain its original state, their difference (yellow area, Figure 2) is the plastic deformation of the two cells.

Values of total adhesion force were considered as the difference between the maximal downward deflection of the cantilever compared to the initial non-contact level.

Total adhesion work (sum of light and dark green) at the detachment phase represents the energy needed to totally separate the two cells in contact. Darker green area can be associated mostly with cell deformation while lighter green with individual rupture events breaking up during retraction. Late ruptures, at large distances from contact point are mainly due to membrane tethers pulled out from cells.

For correlation analysis, Pearson's cross correlation coefficient was calculated for each parameter pair reported. Perfect correlation was denoted with 100% while perfect anti-correlation with -100%.

All rainbow-colored topographical maps represent low (purple) to high (red) values.

Experiments were repeated more than five times, each time similar features were observed. In this study, only a representative set is presented.

Results

Firm adhesion establishment to cerebral endothelial cells is critical for metastasizing tumor cells to reach the brain parenchyma. In this study, single tumor cell-decorated tipless cantilevers (Figure 3) were used as a probe to directly map adhesive and elastic properties of a confluent endothelial layer.

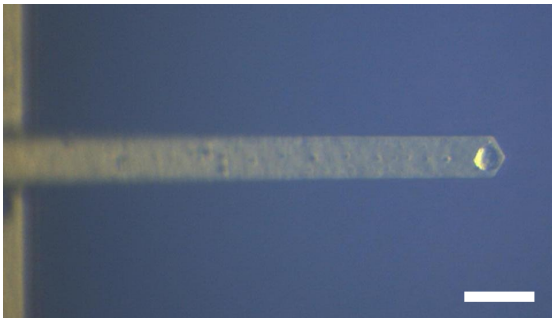

Figure 3 Bottom view of a tumor cell immobilized at the end of an AFM cantilever (scale bar is 50 µm).

Confluent endothelial areas of 90x90µm^2 were selected and divided into 32x32 points. Using this cell–decorated probe, at each point a force-distance curve was recorded.

Pseudo colored maps were reconstructed from the 32 points by 32 lines recorded with the decorated probe. Figure 4 shows the reconstructed three dimensional topography of the confluent endothelial layer. The individual cells, mostly the nuclei, can be clearly distinguished.

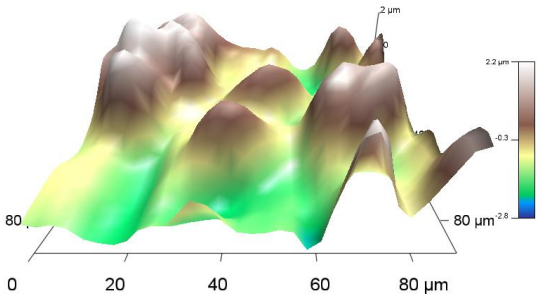

Figure 4 Topography map, reconstructed from contact point of individual force curves measured on a confluent layer of cerebral endothelial cells with a melanoma cell decorated cantilever.

Probably the most ubiquitous intracellular parameter linked strongly to cell behaviour is the elasticity and plasticity. In our case, it is challenging to interpret and to extract elasticity in a classical way, since most models deal with indentation of an elastic material with a hard indenter. Unfortunately, no specific model can be found to extract elastic properties from the obtained curves in case when two living (arbitrary shaped) cells are pushed against each other.

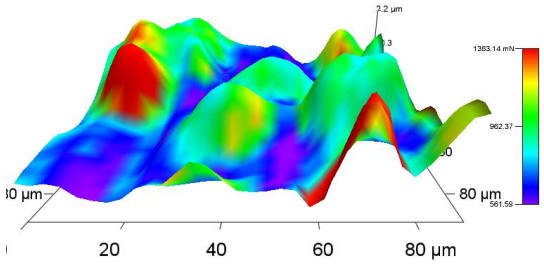

Figure 5 Total elastic work colored topography.

Therefore, in order to characterize the two cells in contact we have decided to calculate the energy needed to push the two cells against each other by a preset force of 2nN. The elastic work parameter represents the deformation of the cell by the applied force. This is shown in Figure 2 with the sum of yellow + cyan colored area between the contact point and the maximal deflection point. The resulted elasticity (elastic work) colored topography map is shown in Figure 5. Peripheral regions appear to be more rigid, which might result partly due to a denser cytoskeletal network at these areas. Even more interestingly, neighbour cells might exhibit different characteristics, ranging from harder (light blue color) to two-fold softer cells (red areas).

After reaching the maximal deflection, the cantilever is pulled back and the applied force shrinks, (blue line on Figure 2) the cells in contact regain their quasi original shape. This might imply presence of

reorganization of cellular organelles and cytoskeletal elements for both cells as well. The ability of reorganization can be quantified by calculating the amount of work the two cells can still exhibit during they are pulled away. This is represented by the plastic work parameter (colored by cyan area, on Figure 2).

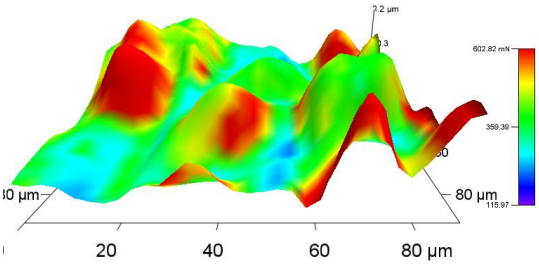

Figure 6 Plasticity (ElastW - PlastW) colored topograpy.

The calculated plasticity would be the elastic minus the plastic work, represented as yellow area on Figure 2. The resulted plasticity map is shown in Figure 6. The value of plasticity close to zero is associated with a perfectly elastic material, while higher values indicate more plastic material.

Although, the resulted difference map (Figure 6) is similar to the elastic map on Figure 5, remarkable alterations can be observed. Not only individual cells show different plasticity values, but even alterations within a single cell can be observed. This indicates that regions of the same cell might behave differently.

In order to characterize the connection strength between the cerebral endothelial layer and the tumor cell, the most widespread parameter is the calculation of the total adhesion force (considered as the difference between the maximal downward deflection of the cantilever compared to the initial non-contact level, see Figure 2).

The pseudo colored adhesion force map of the recorded topography is shown in Figure 7. It is noteworthy, that elastic and plastic behaviour might influence adhesive properties as well. This would imply that apparent elasticity and plasticity would be strongly related to adhesive properties which would result a very similar color distribution in case of adhesion force as well. In contrary, the adhesion force colored topography (Figure 7) shows completely different distribution.

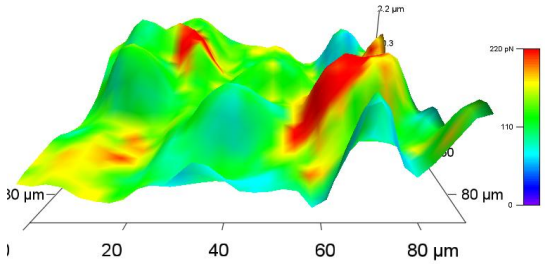

Figure 7 Total adhesion force colored topography.

The most adhesive areas (red zones on Figure 7) are not the most elastic (red zones on Figure 5) or even the most plastic zones (red zones on Figure 6).

Compared to total adhesion force, there is an even more remarkable difference in the adhesion work parameter (Figure 8), which is the total energy needed to separate the two cells in contact. Adhesion work is represented in Figure 2 as the sum of light and dark green areas.

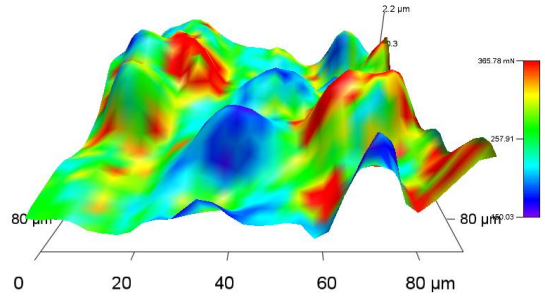

Figure 8 Total adhesion work colored topography.

The total separation energy denoted with adhesion work can be separated into two different areas: adhesion work1 (dark green

are on Figure 2) which is prior to maximal adhesion occurred and adhesion work2 (light green on Figure 2) as the area where the de-adhesion events take place. First is related to deformation dependent work, while the second sums up the contribution of each active bond which builds up the linkage strength.

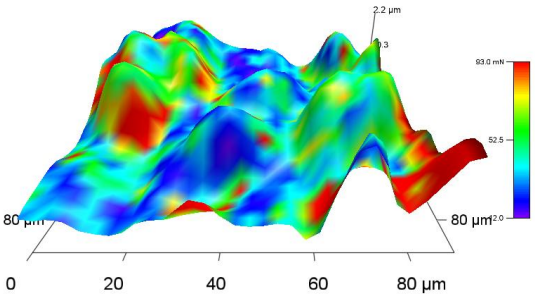

Figure 9 Adhesion work1 colored topography.

The reconstructed topography colored by adhesion work1 is showed on Figure 9. If we compare with the elastic map, remarkable similarities can be found, which indicates that adhesion work1 is truly elastic deformation dependent.

Quantitatively the adhesion work2 (Figure 10) overcomes the work1, which indicates that in most cases long distance rupture events dominate. These events represent the hallmark of the linkage establishment formation, indicating the number of newly formed active focal contacts between the two cells.

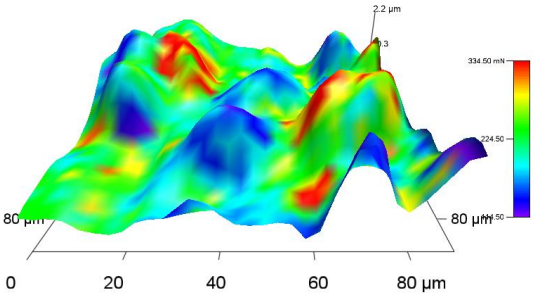

Figure 10 Adhesion work2 colored topography.

Qualitatively, work2 is not directly linked to work1, moreover some times shows exactly opposite behaviour. Similarities of work2 distribution (Figure 10) to those of adhesion force (Figure 7) indicates, that differences in linkage establishment between the neighbour cells can be attributed predominantly to higher number of active cell adhesion molecule bounds. Quantified pondering of work1 and work2 in the total adhesion establishment requires further carefully designed experiments and properly performed analysis.

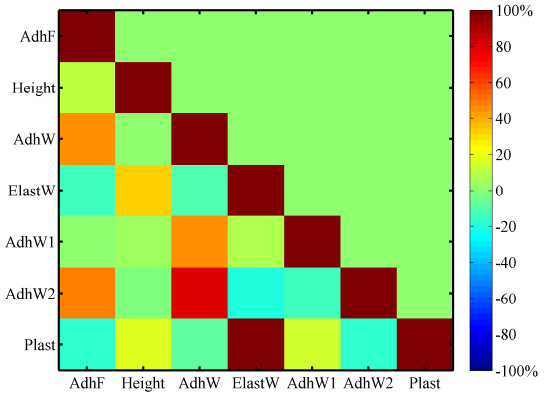

Figure 11 Cross correlation table of the calculated parameters based on Pearson's correlation coefficient.

In order to visualize the dependence of each calculated parameter to the others, a cross correlation plot was constructed. For each parameter pair, a Pearson's correlation coefficient was calculated and scaled from perfect anti-correlation -100% (purple on Figure 11) to perfect correlation as 100% (brown on Figure 11).

Discussion & Summary

Many papers were published with cell to cell adhesion measurements as a subject: starting from bacterial adhesion to eukaryotic cells [18], quantification of metastatic cell adhesion to each other [19], or to endothelium [20]. Spatial mapping for elasticity or adhesiveness of a living cell layer with another living cell has not yet been reported.
In this study, we modeled the initial step towards brain metastasis formation, namely the short term adhesion of a human melanoma cell to a confluent layer of human CECs. It is important to note that during interaction of the two cells, not only the cancerous cell suffers deformation but the endothelial cell as well. As a result, in most of the cases the recorded and analyzed parameters can be regarded as characteristics of the two cells pushed together as a „whole" system.

We have shown previously, that inhibition of the Rho-kinase has increased the number of both the adhered and transmigrated cells. This observation was supported by single-cell force spectroscopy as well. Distribution of the number and average size of adhesion force rupture events were followed. Noteworthy, upon administration of Rho-kinase inhibitor, the enlargement of average rupture size has occurred prior to increase of rupture number [21]. These alterations might point towards surface-based adhesion increase which might be due to a morphological change. This underlines the importance of a constant probe cell shape during data collection. In our case, the shape of the probe cell was checked prior and after recording the force maps. No considerable difference was found.

Elasticity maps on CECs were published previously, revealing their heterogeneity within a single cell: the area of the nucleus being more elastic than the peripheral regions [22]. As the immobilized melanoma cell (Figure 3) has much larger dimensions compared to a few nanometer of a tip apex on a cantilever, the intra cellular inhomogeneities are practically averaged out. Hence, the differences seen in the presented maps can be associated mostly with individual cells, or with their largest organelle, the nuclei [23]. Comparing the elastic map (Figure 5) with the adhesive map (Figure 7) it can be clearly seen that not the cells with apparently lower elasticity exhibit the highest adhesiveness. This is true for comparing plasticity map (Figure 6) to adhesion force map (Figure 7) as well. As elastic and plastic properties are strongly linked to cytoskeletal network organization and reorganizing ability [24], they may reflect endothelial functionality including permeability. Locally increased permeability might be linked to putative transmigration sites for invading cancer cells. Adhesive "hot spots" observed in adhesive maps (Figure 7 and similarly on Figure 8) show stronger co-localization with adhesion work2 presented in Figure 10. This suggests that active cell adhesion molecule based linkage (ruptures) is responsible for higher adhesive properties. Taking into account, that these are represented by long range ruptures, tether formation might be crucial in melanoma-endothelial interaction. Here we have to note, that all these results are deducted by simple mechanical measurements and calculations, and no invasive labeling or staining was involved in the experiments.

Quantification of the correlation between the measured parameters is presented on Figure 11, showing the color coded Pearson's correlation coefficient for each parameter pair. As we can see on Figure 11, no perfectly independent parameter can be found. The most independent one is apparently the height, which underlines that the spatial distribution of elasticity of the endothelial cell layer contributes only moderately to the obtained results. The total adhesion work similarly to total adhesion force is more rupture

(work2) than deformation dependent (work1), which suggests the importance of membrane tether formation and breakage during cell-cell interaction.

As a summary, we can say that successful direct mapping of a confluent layer of CECs is reported with a melanoma cell as a probe. Topography based elastic, plastic and adhesive maps were reconstructed from the recorded force-distance curves. The reconstructed maps reveal elastic, plastic and adhesive heterogeneity of the endothelial layer, but not directly linking these parameters. All these data point towards, that the invading melanoma cell might somehow "screen" for the best places prior to start the transmigration process over the endothelial layer.

These results highlight the importance of cellular mechanics in brain metastasis formation and emphasize the enormous potential towards exploration of intercellular dynamics related processes.

Acknowledgement

This work was supported by the National Science Fund of Hungary OTKA K116158, PD115697 and the GINOP-2.3.2-15-2016-00001 program. Imola Wilhelm and Attila G. Végh were supported by the János Bolyai Fellowship of the Hungarian Academy of Sciences B/O00334/16/8 and BO/00598/14/8 respectively.

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