Role of nano-biomechanics in brain metastasis formation and amyotrophic lateral sclerosis

Thesis Book

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2018

Introduction and objectives

Brain metastases (BM) formation and amyotrophic lateral sclerosis (ALS) are both severe pathologies accompanied with short survival times. Better understanding of the underlying pathogenic processes is fundamental for the advancement of early diagnostics and improved therapeutics. In our work, Atomic Force Microscopy (AFM)-based nanomechanical methods were directly applied in order to elucidate important questions about the different aspects of these life-threatening diseases.

Nano-biomechanics is an emerging research field that forms a bridge between physical and biological sciences aiming to explore mechanical aspects of biological matter at the nanoscale [1]. The main toolset of this cross-field consists of AFM, magnetic tweezers, optical tweezers/stretchers and cell traction force microscopy. Among them, AFM is one of the most versatile systems serving as a reliable and accurate nanoforce tool.

The objectives of the work:

- Direct application of AFM-based methods in order to elucidate important questions related to severe diseases, namely brain metastasis and amyotrophic lateral sclerosis.
- Gain direct insight into the surface scanning mechanism of tumor cells during extravasation into the brain parenchyma, by quantifying and spatially resolving the nanomechanical parameters of metastatic cancer cell - endothelial layer interactions.
- Compare the mechanical phenotype of melanoma cells of different malignancy levels and examine whether the metastatic potential is identifiable in nanomechanical properties.
- Elucidate the contribution of cytotoxic T cells to the development of ALS, by quantifying short term adhesion forces between CD8+ T cells and motoneurons.
- Monitor the elasticity of ALS derived differentiated skeletal muscle cells and compare them with their healthy derived counterparts, with the aim to assess cellular-level differences.

Applied techniques

AFM is a non-optic microscopy technique that uses an extremely sharp tip at the end of a very flexible cantilever as a mechanical probe in order to scan the sample's surface and build up a highly resolved three-dimensional topographic image [2]. Apart from its high resolution imaging capacity, AFM in force spectroscopy mode can provide quantitative data about a wide variety of the sample's physico-chemical properties [3]. In addition, it requires minimal sample preparation and it allows performing measurements under physiologically relevant conditions.

During force spectroscopy the horizontal (x, y) position of the cantilever is fixed, while its vertical movement (z) and instantaneous deflection, in response to various forces, are recorded in force-distance curves (FD-curves). From these, various information can be extracted that is related to the mechanical properties of the sample or to the interactions between the substrate and the tip, such as elastic (or Young's) modulus, maximal adhesion, but also the number, step-size, or occurring distance of the individual ruptures. By defining a grid over a selected area of the sample, and performing force measurements in every grid node, the spatial distribution of mechanical properties over entire tissues, cells, or sub-cellular structures can be mapped. Another technique is called single-cell force spectroscopy (SCFS) [4], where biological objects could serve as a probe. The tip can be functionalized with various molecules for specific intermolecular binding measurements [5], with bacteria for testing its adhesion to different substrates [6], or even whole living cells can be immobilized on the cantilever allowing the direct measurement of intercellular forces [7].

Results and Discussions

I. Nanomechanics of metastatic melanoma

Being the main cause of cancer patient mortality, the most life-threatening aspect of cancer is metastasis. Among all types, the BM is one with the poorest prognosis [8]. Although melanoma is only 1 to 2% of all cancers, it invades the brain with one of the highest frequencies [9], and is among the most aggressive and therapy-resistant human cancers. Since the central nervous system (CNS) lacks a classical lymphatic system, the only way for cancer cells to reach the brain is via the blood stream. A malignant tumor cell, in order to form a metastasis in the brain, first needs to escape from the primary tumor site, invade nearby tissues, brake through the blood vessel wall and enter into the blood circulation by intravasation. By this it can spread toward distant organs, where leaving the blood vessel by extravasation and invading the brain parenchyma, it can proliferate and form a secondary tumor. A relevant issue concerning brain metastasis formation that was addressed in our work by AFM-based nanomechanics is the adhesion of blood-travelling tumor cells and their successful penetration through the tightly connected endothelial layer, which serves as a first defense line of the blood-brain barrier (BBB).

I.1. Direct mapping of melanoma cell - endothelial cell interactions

To model the "screening" process of metastatic cancer cells during their extravasation into the brain parenchyma, we combined force mapping with SCFS measurements and performed measurements between highly metastatic melanoma cells and brain endothelial cells. Single tumor cell-decorated tip-less cantilevers were used as a probe to directly map adhesive and elastic properties of a confluent cerebral endothelial cell (CEC) layer. Spatial distributions of several nanomechanical parameters were recorded. Our aim was to spatially characterize the elasticity and linkage strength between the melanoma cell and the CEC. As no specific model can be found to extract elastic properties from the obtained curves in the case when two living (arbitrary shaped) cells are put in contact against each other, the different work-like parameters of the interaction could be a good indicator of the underlying processes. Thus we have calculated the total elastic (or deformation) work that is needed to deflect the cantilever and deform the two cells, the

dissipated work during the measurement cycle, the maximal adhesion force, the adhesion (or detachment) work needed to separate the two cells, and its two components, namely the early- and late-detachment works.

Although, cell to cell adhesion measurements are the subject of an increasing number of studies, spatial mapping of elasticity or adhesiveness of a living cell layer with another living cell has not yet been reported. Since in our short-term intercellular adhesion measurements both cells suffer deformation during the interaction, the extracted parameters cannot refer to neither of them separately, but characterize the "whole" system. To better interpret the dependence of each calculated parameter to the others, a cross-correlation plot was constructed. Interestingly, the data showed that adhesive properties are only slightly dependent on elastic characteristics. Examining the cross-correlations between the measured parameters revealed the enhanced contribution of ruptures (late-detachments) against deformation (early-detachment) to maximal adhesion force and total adhesion work, and highlighted the importance of long range tether-like linkages for successful adhesions.

I.2. De-adhesion dynamics of melanoma cells from brain endothelial layer

Since decades has been known that individual cancerous cells show reduced elastic modulus compared to normal ones, as it has been unraveled for most of cancer types [10]. However, the question of how the metastatic potential relates to tumor cell's autonomous and inter-cellular nanomechanical properties has been examined by few [11–14]. In a second series of experiments the relations between the different levels of malignancy and the nanomechanical parameters of tumor - endothelial heterocellular interactions were addressed by SCFS. Three melanoma cell types (WM35, A2058 and A375) with altered invasive characteristics were examined. Besides measuring their intercellular interactions, in each case the very same melanoma cells were tested against a cell free area of the Petri dish as a control. As no proper model exists to obtain elastic or plastic properties when two cells are put in contact, in order to compare the elastic properties of the studied cell types in situ, a new parameter was used, called relative elasticity. This relative dimensionless parameter was defined as the ratio of remnant and total work exerted by the system. Its values range between 0 and 1, where 0 corresponds to a perfectly plastic,

while 1 to a perfectly elastic behavior. The apparent Young's modulus, as the most frequent used parameter to characterize cellular elasticity, was also determined. Other investigated parameters included the maximal adhesion force, as well as the number, step size, and occurring distance of the individual de-adhesive ruptures events.

The performed control measurements against a bare Petri dish surface indicated that the calculated relative elasticity is predominantly a property of melanoma cells, while endothelial cells have only low contribution. In addition, a strong dependence between the relative elasticity and the melanoma cell type have been observed, where WM35 cells appeared to have a more elastic deformation, followed by A2058 and A375 cells revealing a more plastic behavior upon deformation. Similarly to relative elasticity, WM35 cells exhibit the highest elastic modulus values, followed by A2058 and A375 cells being the softest. Although not in the case of a bare Petri dish, but when melanoma cells were pushed against the endothelium, a clear difference in maximal adhesion force was observed, showing increasing values along with increasing level of malignancy. Moreover, this correlated inversely with relative elasticity and at the same time directly with the number of rupture events, indicating the low relative elasticity, high maximal adhesion and high number of individual linkages, as key properties of highly metastatic melanoma cells. The size and occurring distance of the individual de-adhesion events were also quantified, indicating that the role of tether based adhesive properties of invading melanoma cells cannot be neglected in the metastasis formation process. Our adhesion force dynamics data recorded between a confluent brain endothelial layer and three melanoma cell types of different invasiveness underline the importance of mechanical properties in case of intercellular interactions. Moreover, it suggests that in adequate circumstances elastic and adhesive characterizations might be used as relevant biomarkers.

II. Nanomechanical aspects of amyothrophic lateral sclerosis (ALS)

ALS is a fatal neurodegenerative disease, which causes a gradual degradation of the human motor system. The origin of ALS in 5-10% of the cases is familial (fALS), while the rest of the patients diagnosed with ALS have a sporadic disease (sALS) [15]. Mutations in the gene of copper/zinc ion-binding superoxide dismutase (SOD1) is one of the main causing factor for the development of the disease [16], which is responsible for 20% of the familial [17] and 5% of the seemingly sporadic ALS [15]. Transgenic mice expressing mutant human $SOD1^{G93A}$ gene were proven to provide a good model for the human ALS disease [17]. In our *in vitro* experiments primary cultures isolated from this mouse model were addressed by AFM-based nanomechanics to unravel different aspects of ALS development, namely lymphocyte - motor neuron interactions and myotube elasticity.

II.1. T cell and motoneuron interaction forces in an ALS diseased mouse model

T cells, or T lymphocytes, play an important role in cell-mediated immunity and their interaction with other cells is pivotal in the recognition and elimination of potential pathogens. The forces of these interactions and the relating molecular linkages have been extensively studied [18,19], unraveling various aspects of the immune response. The pathogenesis of this fatal neurodegenerative disease is complex, consisting not only in autonomous cell death, but involving the contribution of several cell types and characterized by the presence of inflammatory processes as well. It was found that the early stage ALS is characterized with protective T cell accumulation [20]. However, as the disease progress, cytotoxic CD8+ T cells also infiltrate into the CNS [21]. With the study of such interactions our aim was to quantitatively evaluate the contact-dependent neurotoxic activity of ALS mouse derived CD8+ T cells, supported by preliminary data of our collaborators' in vitro co-culturing experiments [22]. By comparing the adhesive properties of CD8+ cytotoxic T cells isolated from wild-type as well as $SOD1^{G93A}$ mutant mice against wild-type motor neurons, significant differences were observed. Mutant mice derived T cells show an enhanced adhesion strength compared to the healthy mice derived ones. Moreover, after blocking the specific binding between pMHC-I and TCR molecules, which are involved in the recognition process, highly significant reduction in

adhesion force have been observed in case of mutant T cells, while wild-type T cells show only slight or no telling effect for the different contact times. Together these results strongly corroborate the contribution of cytotoxic T cells in the development of ALS, as an active player in neurodegeneration.

II.2. Myotube elasticity of an ALS diseased mouse model

Muscle cell formation is a multistep and highly regulated process that is under constant research. The precursors of the subsequent muscle cells are the myoblasts. Myotubes, formed by the fusion of single myoblasts, are the developing muscle fibers containing multiple centrally located nuclei. Although several studies have examined the mechanical properties of healthy skeletal muscle cells from different origin and in various states of differentiation [23–25], few investigated the effect of diseases on skeletal muscle cell elasticity, out of which the majority of studies address mainly muscular dystrophies [26–28]. Here we report a detailed comparative study on the Young's modulus of healthy and ALS diseased asymptomatic $SOD1^{G93A}$ mice derived differentiated primary skeletal muscle cells. High resolution nanomechanical mapping was performed on single elongated myoblasts and multinuclear myotubes with varying thickness. In case of single myoblasts the measured Young's modulus values were significantly different between their central portion and elongated projections. The contribution of actin and myosin content to the elasticity was also examined and discussed. Immunofluorescence images were reported, presenting the actin rich contact regions prior fusion, as well as the expression level of different actin and myosin coding genes were quantified. Regarding myotubes, differences have been observed between the elasticity of a thin and thick population of wild-type myotubes, indicating the different maturity of the two populations. Interestingly, the same observation was not valid for the case of SOD1 mutant skeletal muscle myotubes. Here the thin, and thus pre-mature, myotubes showed no significantly different elastic modulus values compared to thick and therefore more mature myotubes. This finding suggests an enhanced autonomous hardening of ALS derived myotubes, which could be substantial in deciphering the development of this yet incurable neurodegenerative disease.

In summary, we can say that successful cellular-level mechanical studies have been performed involving various aspects of two different life-threatening pathologies resulting in novel discoveries about their fundamental processes. This work highlights the high impact and the important role of AFM-based nanomechanical methods in providing valuable knowledge about disease pathophysiology, development, diagnostics and progression that could provide new possible target candidates and greatly contribute to the evolvement of future therapies.

Publications directly related to the subject of the thesis:

I. **Varga B.**, Fazakas C., Molnár J., Wilhelm I., Domokos R. A., Krizbai I. A., Szegletes Z., Váró G., Végh A. G. **Direct mapping of melanoma cell - endothelial cell interactions**, *J. Mol. Recognit.* **2017**, 30(6):e2603, DOI: 10.1002/jmr.2603

IF: 2.175

II. **Varga B.**, Domokos R. A., Fazakas C., Wilhelm I., Krizbai I. A., Szegletes Z., Gergely C., Váró G., Végh A. G. **De-adhesion dynamics of melanoma cells from brain endothelial layer**, *BBA Gen. Subjects*, **2018**, 1862(3):745-751, DOI: 10.1016/j.bbagen.2017.10.013

IF: 4.702

III. **Varga B.**, Martin M., Hilaire C., Sanchez-Vicente A., Areias J., Salsac C., Cuisinier F.J.G., Cedric Raoul C., Scamps F., and Gergely C. **Myotube elasticity of an amyotrophic lateral sclerosis mouse model** (Revised manuscript submitted to *Sci.Rep.*)

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