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Effect of an osmotic stress on multicellular aggregates

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ABSTRACT

There is increasing evidence that multicellular structures respond to mechanical cues, such as the confinement and compression exerted by the surrounding environment. In order to understand the response of tissues to stress, we investigate the effect of an isotropic stress on different biological systems. The stress is generated using the osmotic pressure induced by a biocompatible polymer.

We compare the response of multicellular spheroids, individual cells and matrigel to the same *osmotic* perturbation. Our findings indicate that the osmotic pressure occasioned by polymers acts on these systems like an isotropic mechanical stress. When submitted to this pressure, the volume of multicellular spheroids decreases much more than one could expect from the behavior of individual cells.

1. Introduction

Mammalian cells constantly interact with their mechanical environment [1]. Each cell of connective tissues is embedded in an elastic and remodelable environment, the Extracellular Matrix (ECM). Moreover, the whole tissue is confined and compressed by its surrounding. At the single cell level it is admitted that the mechanical environment can determine cell fate. The stiffness and the geometry of the substrate on which cells grow has indeed a strong impact on cell division [2], migration [3] and differentiation [4]. There are increasing evidences that the mechanical environment also determines the behavior of supra-cellular structures. For instance, tumor multicellular aggregates growth can be inhibited when embedded in an elastic agarose gel [5]. In particular, the stiffer the agarose gel, the stronger the inhibition. Similarly, Alessandri et al. [6] showed that a multicellular spheroid (MCS) confined inside an elastic shell grows more slowly when its peripheral cells touch the shell wall. Both agarose gels and shells are deformed by the growing aggregate. As a consequence, they mechanically react by applying an increasing stress to the spheroid. Above a certain stress, spheroid growth stalls.

Mechanical stress may have a different effect if, like a stationary isotropic pressure, it is kept constant over time. In that case, the growing aggregate can progressively adapt to the external stress on the long timescale (longer than cell division time). In order to explore this possibility and grow multicellular spheroids under a constant compressive stress, we culture them in a medium supplemented with dextran polymer [7]. Unlike more physiological osmolytes, such as glucose or ions (Na^+, Mg^{2+}, etc), dextran macromolecules are not internalized by cells, and do not infiltrate the intercellular space of spheroids [8]. Thus, they exert a persistent osmotic stress onto the whole spheroid and induce a water efflux from the spheroid, similar to what would do a permeable piston squeezing a spongy aggregate. The stress would not persist if physiological osmolytes (e.g. glucose, NaCl or $MgCl_2$) were used instead of dextran. Indeed, their concentration gradients through the plasma membrane would be progressively balanced by ions pumps and channels activity or endocytosis.

In our previous works, we showed that a 5 kPa osmotic stress strongly hampers spheroid growth, by inhibiting cell proliferation in its core. It is worth noticing that 5 kPa is a rather small stress corresponding to the osmotic pressure exerted by 1 mM NaCl. Moreover, it has been observed that such a small osmotic pressure has no long term effect on proliferation or death for cells cultured in 2D (Petri dish). This raises the fundamental question as to why 3D spheroids respond to osmotic pressure while individual cells do not.

In this article we focus on the short timescale response of a spheroid to an osmotic stress, and evaluate the impact of such stress. In particular, we measure the volume decrease of a MCS in the first minutes after the injection of dextran, when the adaptive response is not activated yet and the cell cycle is still

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unaffected. Complementarily, we correlate the volume decrease to the change of the cell-to-cell distance, depending on the position inside the spheroids. In order to rationalize the effect of the osmotic stress on a semipermeable system, we submit individual cells on 2D substrate to the same stress and record the corresponding volume change. We also compare the response of living cells and spheroids to the passive contraction of matrigel beads and of polyacrylamide gels, when submitted to similar pressures.

2. Materials and methods

2.1. Osmotic pressure

The osmotic pressure is exerted by supplementing the buffers and culture media with a well-defined amount of dextran. This biocompatible polymer is not internalized by eukaryotic cells and the relationship between its concentration and the osmotic pressure has been previously calibrated [9,10] (see Supplementary Fig. 1). Dextran molecules (Sigma–Aldrich, St. Louis, MO) must be large enough to penetrate neither the polyacrylamide (PA) gel nor the MCS. We observed that dextran molecules with a molecular weight of 100 kDa enter neither the cells nor the MCS [8]. Conversely, their hydrodynamic radius [11] (about 8 nm) is small enough to allow them to penetrate into soft PA gels (1–5 kPa), whose pore size is around 15 nm [12], and Matrigel. In this case, the experiments are performed using larger polymers (2 MDa), with a hydrodynamic diameter of roughly 27 nm.

2.2. Cell culture and multicellular spheroid preparation

Cells are grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 1% antibiotic–antimycotic at 37 °C under 90% air/10% CO_2 atmosphere. We used mouse colon carcinoma cells (CT26 cell line, ATCC CRL-2638). Due to the intestinal transit, colon cells are constantly submitted to shearing and compression and represent a relevant candidate for this study. Nevertheless, similar results are observed on other cell lines, including human carcinoma (HT29), mouse sarcoma (AB6), Mouse schwann (HEI), human breast cancer (BC52), and mouse fibroblasts [14].

Spheroids are assembled for 48–72 h on an agarose cushion [13] (Ultrapure agarose, Invitrogen, Carlsbad, CA) from cell suspensions with roughly 20,000 cells per sample, yielding to an initial radius of about 200 μm.

2.3. Multicellular spheroid size and cellular volume measurement

We previously demonstrated that multicellular spheroids grown on agarose cushions only slightly deviate from the spherical shape [8]. MCS volume is thus inferred by the optical measurement of its radius, with a relative precision of about 1%. In order to evaluate the cellular volume, we perform cryosections, about 3 μm thick, at the level of the equatorial plane. The nuclei of the slices are further stained with DAPI and imaged by confocal microscopy. The relative distance between the nuclei of adjacent cells is measured as a function of their distance from the center of the MCS as described in Ref. [14].

2.4. Polyacrylamide gel preparation

32 mm round glass coverslips are silanized using pure ethanol solution containing 0.36% (v/v) Bind-Silane (GE Healthcare life science) and 3.1% (v/v) 10% acetic acid for 5 min. The coverslips are then gently wiped before being stored in a Petri dish. A high quality quartz mask (Toppan) is first cleaned with iso-propanol and eventually smeared with n-hexane prior to use, in order to maintain a hydrophobic surface [15]. Then, according to the desired stiffnesses [16], pre-mixed acrylamide solutions containing 4–10% acrylamide (Fluka) and 1–2.5% bis-acrylamide (Fluka) are prepared. Drops of 50 μL are polymerized for 45 min between the mask and a silanized coverslip, leading to a typical thickness of 70 μm. Tetramethylethylenediamine (Sigma) is used with ammonium persulfate (Sigma) to catalyze the polymerization. The coverslip, with gel, is carefully removed from the mask and stored in DPBS. PA films are prepared in PBS buffer, with three different stiffnesses. According to the protocol described by Tse and Engler [17], the expected Young's moduli of the PA are respectively 1.1 kPa (3% acrylamide/0.1% bis-acrylamide), 2.0 kPa (4%/0.10%) and 4.5 kPa (5%/0.15%) in water.

2.5. Measurement of gel thickness

The coverslip supporting the gel is mounted in a home-made support and covered with a DPBS solution containing typically 20 nM of Sulforhodamine G (SRG). This low concentration do not perturb the gel and make it possible to perform Fluorescence Correlation Spectroscopy using a 488 laser line, to control the state of the gel (diffusion within the gel is lower than in the solution located above). SRG slightly interacts with the gel which then appears more fluorescent than the surrounding solution (see the confocal signal in Fig. 2A). A motorized focus drive (Marzhauser) is used to vertically scan the gel and determine the gel thickness, from the position of the two transitions (coverslip–gel and gel–solution interfaces). Absolute precision is around 1 μm, which is sufficient to measure a compression of 10% on a gel of 50 μm.

2.6. Measurement of single cells volume

To measure the evolution of the cellular volume in real time, we use the fluorescence exclusion principle recently re-adapted by Bottier et al. [18]. Cells are cultured in a microfluidic device in Polydimethylsiloxane (PDMS), with a constant height of 20.9 μm. The culture medium is supplemented with 1 mM FITC-dextran (10 kDa, LifeTechnologies), a fluorescent dye that is not internalized by the cells, thus cells appear dark while the surrounding medium is fluorescent (see Figs. 4A and 3B). The fluorescence excluded by the cell is then proportional to its volume. Micropillars of 6, 13 and 20 μm high are used to calibrate the measurement (see Supplementary Fig. 2A). Images are acquired with a low numerical aperture objective (10X, NA0.3) in order to get the fluorescence over the height of the chamber. A brass master is fabricated using micromilling machine (MiniMill/3 (Minitech)) with a 100 μm diameter milling cutter to produce the PDMS chambers. This microfabrication technique easily allows producing structures of various heights i.e. the pillars for calibration. Osmotic shocks are generated by simply flowing fluorescent medium (FITC-dextran 10 kDa, LifeTechnologies) as described above, supplemented with the desired amount of 100 kDa dextran to generate osmotic pressure (Sigma–Aldrich, St. Louis, MO). For volume measurements, fluorescence is integrated over a fixed area around the cells of interest. Eventually, the flow arises from the hydrostatic pressure difference originating from the level difference between the inlet and outlet of the chamber.

3. Results

3.1. Multicellular spheroids

We measure the volume change due to the osmotic stress, both at the level of the whole spheroid and at the cellular scale. These