Article

Compressive Stress Inhibits Proliferation in Tumor Spheroids through a Volume Limitation

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ABSTRACT In most instances, the growth of solid tumors occurs in constrained environments and requires a competition for space. A mechanical crosstalk can arise from this competition. In this article, we dissect the biomechanical sequence caused by a controlled compressive stress on multicellular spheroids (MCSs) used as a tumor model system. On timescales of minutes, we show that a compressive stress causes a reduction of the MCS volume, linked to a reduction of the cell volume in the core of the MCS. On timescales of hours, we observe a reversible induction of the proliferation inhibitor, $p27^{Kip1}$, from the center to the periphery of the spheroid. On timescales of days, we observe that cells are blocked in the cell cycle at the late G1 checkpoint, the restriction point. We show that the effect of pressure on the proliferation can be antagonized by silencing $p27^{Kip1}$. Finally, we quantify a clear correlation between the pressure-induced volume change and the growth rate of the spheroid. The compression-induced proliferation arrest that we studied is conserved for five cell lines, and is completely reversible. It demonstrates a generic crosstalk between mechanical stresses and the key players of cell cycle regulation. Our results suggest a role of volume change in the sensitivity to pressure, and that $p27^{Kip1}$ is strongly influenced by this change.

INTRODUCTION

The importance of the interactions between a tumor and its microenvironment, the stroma, has been recognized for more than a century (1). In most instances, the growth of solid tumors occurring in constrained environments entails a competition for space. The pathways of communication between a tumor and its microenvironment are diverse, but they can broadly be separated into biochemical and mechanical signals. Although the former have been extensively studied (see, for instance, Mueller and Fusenig (2) and Roussos et al. (3)), much less is known about the latter.

The competition for space results in a bidirectional mechanical coupling between the tumor and the stroma: on the one hand, the expanding neoplastic tissue compresses the stroma and thus builds up and stores an internal stress; on the other hand, an active stroma containing contractile myofibroblasts can exert a mechanical stress on the growing tumor (4).

However, given the complexity of these systems, decoupling the effect of biochemical and mechanical interactions is a daunting challenge. A good candidate for such studies is the multicellular spheroid (MCS), introduced by Sutherland et al. (5) as a tumor model system: three-dimensional cellular aggregates that remarkably mimic the relevant in vivo physiological gradients of mitogens, oxygen, or glucose. They have been extensively used (see Hirschhaeuser et al. (6) for a review) as model systems for the study of drug delivery (7), three-dimensional cell proliferation (8), invasion (9), or even angiogenesis (10). Although their mechanical properties might differ from those of tumors, for many purposes, MCSs can be viewed as a tumor subunit. Because they do not have any biochemical crosstalk with their environment, MCSs are ideal to evaluate the impact of mechanical stress on tumor growth (11,12). It has been shown, for example, that the growth of a multicellular spheroid in a confined rigid environment inhibits its own growth (13).

In a previous work, we have studied the influence of a compressive stress applied on MCSs (14–16). We have shown that a compressive stress applied on MCSs grown from the mouse colon carcinoma cell line CT26 drastically and reversibly reduce their growth rate (14,15), and that this reduction is linked to a decrease of cell division in the center of the MCS rather than to an increase of cell apoptosis.

In this article, we dissect the biomechanical sequence caused by a controlled compressive stress. We first show on five different cell lines the generality of the effect observed at the MCS level. On timescales of minutes, we show that a compressive stress causes a reduction of the MCS volume, linked to a reduction of the cell volume in the core of the MCS. On timescales of hours, we observe a reversible induction of the proliferation inhibitor, $p27^{Kip1}$, from the center to the periphery of the spheroid.

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On the timescales of days, we observe that the cell cycle is blocked at the restriction point. We show that the effect of pressure on the proliferation can be antagonized by silencing $p27^{Kip1}$. Finally, we quantify a clear correlation between the pressure-induced volume change and the growth rate of the spheroid. The temporal sequence of events that we study is completely reversible. It demonstrates a generic crosstalk between mechanical stresses and the key players of cell-cycle regulation. It suggests a role of volume change in the sensitivity to pressure.

MATERIALS AND METHODS

Cell culture, MCSs formation, and mechanical stress

HT29 cells (human colon carcinoma cells, ATCC HTB-38; American Type Culture Collection, Manassas, VA), CT26 (mouse colon adenocarcinoma cells, ATCC CRL-2638; American Type Culture Collection), BC52 (human breast cancer cells, given by D. Lallemand, Institut Curie, Paris, France) and AB6 (mouse sarcoma cells, given by Yeh-Shiu Chu, Institute of Molecular and Cellular Biology, Singapore) are cultured under 37°C, 10% CO_2 in DMEM supplemented with 10% calf serum and 1% antibiotic/antimycotic (culture medium). FHI (murine Schwann cells, obtained from immortalization of primary murine Schwann cells, given by D. Lallemand, Institut Curie) are cultured under the same conditions but with F12:DMEM (1:1) medium, supplemented with 10% calf serum and 1% antibiotic/antimycotic. MCSs are formed in 48-well plates using a classical agarose cushion protocol. When the MCS is formed, Dextran (molecular mass = 100 kDa; Sigma-Aldrich, St. Louis, MO) is added to the culture medium to exert mechanical stress, as previously described in Montel et al. (14,15), at a concentration of 55 g/L to exert 5 kPa, and 80 g/L to exert 10 kPa.

Flow cytometry experiments

Fifteen MCSs are gathered together in 0.9 mL of Trypsin and placed in the incubator for 10 min. Cells are mechanically separated by agitation during 5 min. Trypan Blue measurements have been done to ensure the viability of the cells after dissociation, which is >95%. Trypsin activity is inhibited by addition of 100 μL of calf serum. Ice-cold ethanol (70%) is added dropwise to fix the cells, kept at 4°C for at least 12 h. Extracts are centrifuged at 600*g* for 5 min, then rinsed with ice-cold PBS, centrifuged at 300*g* for 5 min. Cells are resuspended in 300 μL of a propidium iodide (Sigma-Aldrich) solution in PBS at a concentration of 50 μg/mL, and 10 μL of RNase A (1 mg/mL; Sigma-Aldrich) is added. DNA histograms are recorded on a FACSort (BD Bioscience, San Jose, CA) and histogram analyses are performed with MODFIT (Verity Software House, Topsham, ME).

siRNA experiments and Western blots

Approximately 200,000 cells were seeded on 6-well plates the day before the experiment. The cells were transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA), with or without siRNA. We used four different conditions: no siRNA, a scrambled siRNA, and two different siRNAs targeted against $p27^{Kip1}$ (Dharmacon, Lafayette, CO). The cells were in the transfection medium for 6 h, then resuspended and spheroids were formed out of them.

To perform Western blots, 50 MCSs are placed in 70 μL of RadioImmunoPrecipitation Assay buffer. They are agitated at room temperature for 20 min, then heated at 100°C for 5 min. Laemmli buffer is then added and the solution is heated at 100°C for 5 min. Polyacrylamide gels (NuPAGE 4–12% Bis-Tris gels; Life Technologies, Carlsbad, CA) are run and transferred onto a nitrocellulose membrane. The membrane is rinsed three times in PBST (PBS + 0.05% TWEEN), blocked 1 h with 10% milk and rinsed three times in PBST. Primary antibodies are incubated overnight at 4°C at a typical concentration of 1/2000. Membranes are further rinsed three times in PBST, blocked 30 min, and HRP-secondary antibodies (1/5000) are incubated for 1 h at room temperature. Membranes are rinsed three times and then revealed. To quantify Western blots, we subtract the background and calculate the sum of the intensity for every band of interest. We normalize it by the level of the α-tubulin band. We estimate the minimum and maximum intensity per band, and found that they differ on an average of 10% of the mean value. We take 0.1 × value for the error bar measurement.

Cryosections, immunofluorescence staining, and density profiles

Cryosections, immunofluorescence, and density profiles are performed using the protocol described in Montel et al. (15). Fluorescent images are recorded on an Eclipse microscope (Nikon, Melville, NY), with a Luca S camera (ANDOR Technology, Belfast, UK). Image analysis are performed with a home-made software employing MATLAB (The MathWorks, Natick, MA). Briefly, we localize the position of each nucleus positive for the staining by thresholding the second derivative of the image. Density profiles are obtained by normalizing the radial distribution of the nuclei positive for the staining against the protein of interest by the radial distribution of every nuclei, obtained using a DAPI staining. To have access to the number of nuclei positive for the staining, we sum this density profile over the radius. By tuning the threshold applied on the second derivative, we are able to plot density profiles with no false positive nucleus detected, and with the maximum of nuclei detected, which gives us a minimal and a maximal value for the integral. On average, the error made on the measurement is then taken to be 0.2 × value.

Cell to cell distance

DAPI stainings are obtained as described in the previous section, and images are recorded on a confocal microscope to ensure the same thickness imaged for each sample. To obtain an estimation of the local cell-to-cell distance inside a MCS, we choose an autocorrelation approach, explained in detail in the Supporting Material. Briefly, a small circular region of interest (ROI) is taken randomly inside the DAPI image, at a distance r from the center of the MCS. The autocorrelation of this ROI is done using a Fourier transform approach. We then plot the radial normalized autocorrelation function. Each maximum corresponds to the correlation of a nucleus with one of its neighbors: the first one with itself, the second one with its first neighbor, the third one with its second neighbor, etc. We take an estimation of the cell-to-cell distance $d(r)$ by the average distance with the first neighbor, at the distance r from the center of the spheroid. Eventually, each $(r,d(r))$ point are gathered in a histogram, obtained with bins of the size of the ROI. Each cell-to-cell distance is the median of all the $d(r)$ in the bins, and the error bars correspond to the error on the median of the distribution. Finally, the mean distance corresponds to the mean value of the cell-to-cell distance inside the MCS, and the error bar to the average of the error made for each measurement.

RESULTS

Mechanical stress reduces growth rate of multicellular spheroids on various cell lines

We first check the robustness of the growth-rate reduction of MCSs due to a compressive stress found in Montel et al. (14). We form MCSs from different cell lines: mouse colon