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Cell deformation in response to long-term hyperosmotic loading

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Hypertrophic and elongated cells are found in differentiation zones of load-bearing tissues, where tissue is hyperosmotic. In this paper we study if, in response to long-term hyperosmotic loading, cells are affected with hypertrophy or elongation, and whether these responses are cell-specific.

Surface adhesion and elongation of CHO-K1 and C2C12 cells were determined with CLSM, after 2 and 5 days of culture in 380 mOsmol medium.

Results show that both cell types increase an adhesion area ($p < 0.001$ for CHO-K1 cells, $p < 0.01$ for C2C12 cells), independent of the method used to increase osmotic pressure. Despite the differences between cell types (CHO-K1 cells are smaller ($p < 0.001$) and their morphological changes are more pronounced), aspect ratio remains constant for all cell types and experimental conditions ($p > 0.1$).

Conclusively, all cells hypertrophy, but do not elongate under hyperosmotic loading. Quantitatively, CHO-K1 cells respond more than C2C12 cells.

Key words: cell deformation, long-term hyperosmotic loading

1. Introduction

Cell deformation, either by changing its volume or shape, is observed at particular stages of the development of load-bearing tissues. In the embryonic stage as well as during bone fracture healing, cell elongation and hypertrophy of mesenchymal cells precede the mineralisation of bone matrix [1]. Hypertrophied mesenchymal cells are also found in the epiphysis, whereas in the middle and deep zones of hyalin cartilage, cell volume is twice the volume of cells in the superficial zone [2]. Combined cell elongation and hypertrophy is observed during the early development of intervertebral discs [3],

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where it is of ultimate importance for the final structure of the annulus fibrosus. In addition to elongation, the mesenchymal cells in the early disc align directed by stress fibers [4]. Subsequently, the collagen fibers of the annulus develop in the direction coinciding with the orientation of the elongated cells [5]. Thus, cell swelling is observed in the development of load-bearing structures in general, and the combination of cell swelling and elongation is of particular importance for the development of the annulus fibrosus of the intervertebral disc.

Regulation cell volume has widely been studied for all kinds of vertebrate cells [6]. Special attention has been paid to cell volume regulation in response to the changes in the osmotic environment of the cells. It has been known for long that in suspension, cells initially behave as osmometers by undergoing osmotic swelling in hyposmotic media and shrinkage in hyperosmotic environment [6]. The size of nucleus pulposus cell in vivo and in suspension culture is related to medium osmolarity 10 minutes after osmotic shock, independent of the way in which osmolarity change is induced [7]. In cartilagenous tissues, osmolarity is of particular interest. The combination of the absence of blood supply, compressive loading and the electrically-charged proteoglycans results in a hyperosmotic environment and daily fluctuations in osmolarity [7], [8], [9]. For instance, depending on the constitution of the matrix and the loading history of the tissue, osmolarity in intervertebral discs can exceed 400 mOsmol, whereas plasma osmolarity is in the order of 280 mOsmol (the table). In the longer term, cells are able to counter the change in cell volume that is the result of changes in the osmolarity of their environment by controlling active and passive ion channels [6], [10]. Such compensatory mechanism may explain why, in contrast to the expected decrease in cell volume in hyperosmotic environment, TAKAGI recently found that after 24 hours of both suspension and adhesion cultures, CHO (Chinese Hamster Ovary) fibroblasts exhibit higher volumes in hyperosmotic medium [11].

Table. Concentrations and osmolarity in the intervertebral disc compared to plasma. Values in the disc depend on the position in the disc. As a result of fixed negative charges in cartilagenous tissue, ion concentrations and therefore osmolarity differs importantly from other tissues (data adapted from URBAN et al. [20]).

	Plasma	Disc
Fixed charge density (mequiv/dm^3 H_2O)	0	100–230
[Na^+] mM	140	210–320
[K^+] mM	5	7–11
[Ca^{2+}] mM	2	4–15
[Cl^-] mM	140	70–110
Osmolarity (mOsmol)	280–310	350–440

Recent studies have shown that the changes in osmolarity of the extracellular environment not only affect cell volume, but have many other diverse effects on

cell morphology as well. Extension of osmolarity-induced podia is, for instance, observed in hematopoietic cells in response to sustained osmolarity increase [19]. Also, CHO fibroblasts in hyperosmotic adhesion cultures are distorted in height and have larger cell adhesion areas after being cultured for 24 hours [11]. The latter finding and the simultaneously observed increase in cell volume were surprising, as in a hyperosmotic environment cells are expected to shrink rather than swell. As the study [11] involved measurement of the geometry of 10 cells only, verification of the result with a larger number of cells is desired. This is one of the aims of the present study.

Furthermore, morphological changes of cells, in particular cell elongation, have been reported in response to stimuli such as anisotropic topology of the culture plate surface [12], application of shear stress [13], [14] or fluid flow [15], [16], chemotaxis or direct current electrical fields [17], and during cell migration [18]. One would a priori expect that in response to osmotic changes, which is an isotropic condition, the changes in cell geometry would be isotropic. Yet, this has not been shown previously, and it may well be that cytoskeletal restrictions force cells to elongate in hyperosmotic environment. Hence, the present paper questions whether the changes in cell shape in response to an isotropic rise in the osmolarity of culture medium are restricted to cell height and adhesion area, or also involve other geometrical parameters such as cell elongation.

In particular, the present study addresses the following three questions:

1. Do cells hypertrophy in response to long-term hyperosmotic loading?
2. Do cells elongate in response to long-term hyperosmotic loading?
3. Are these responses to long-term hyperosmotic loading cell-specific?

If cells indeed are affected with hypertrophy and elongation in response to hyperosmotic loading, this is a relevant finding for tissue differentiation of load-bearing tissues. Hyperosmotic environments are expected in these zones where negatively charged proteoglycans are deposited, while the tissue is under sustained compression. In these particular zones, hypertrophied and elongated cells are found.

2. Methods

Fibroblasts (CHO-K1 cells), similar to those used by TAKAGI et al. [11], and myoblasts (C2C12 cells) were grown in standard 270 mOsmol Ham's F12 (fibroblasts) or DMEM (myoblasts). Both media contained stabilised L-glutamin and
2 g/dm^3 $NaCHO_3$ (VWR) and were supplemented with 10% FBS (VWR) and 0.1% gentamycin (Sigma). For both cell types, the following protocol was used. Cells were maintained and grown at 37 ^oC humidified atmosphere of 5% CO_2. At confluence, cells were split into three groups. The first group was maintained in the aforementioned, standard 270 mOsmol medium, the second and the third groups in

hyperosmotic 380 mOsmol medium, adjusted by addition of either NaCl or D-glucose to the standard 270 mOsmol medium (figure 1). Each of these conditions was applied in six wells of a 6-well plate.

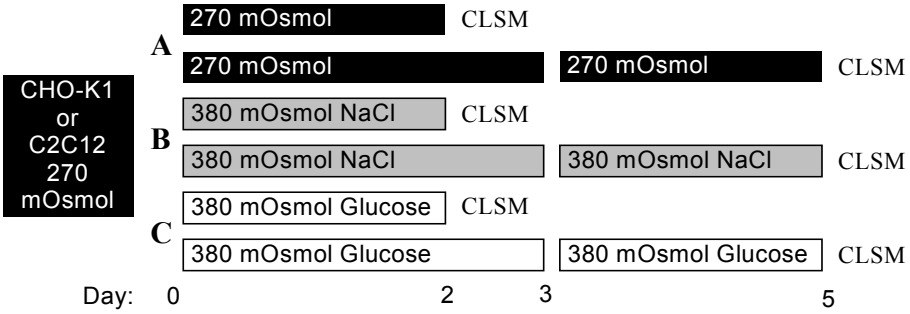

Fig. 1. Timeline of the experimental setup. CHO or C2C12 cells were grown in regular, 270 mOsmol culture medium. On day 0, the cells were split in three groups. Group A was grown in 270 mOsmol medium (dark bars), group B in 380 mOsmol medium after NaCl supplementation (light bars), group C in 380 mOsmol medium after d-glucose supplementation (white bars). Two days after trypsinization, on the 2nd and the 5th days, CLSM images were obtained from each group for examination of cell geometry

Two days thereafter, when cells were approximately 50% confluent, the cytosol of the living cells in three of the wells for each experimental condition was stained by adding calcein (Molecular Probes) to the medium to a 5 μM concentration, and images of the cells were acquired on a confocal laser scanning microscope (CLSM) (Zeiss LSM 510, Carl Zeiss, Jena, Germany). The confocal system was based on Zeiss Axiovert 200 microscope using a Epiplan LD 50×/0.6 corr objective for CHO-K1 cells and a Plan neofluar Ph2 20×/0.5 objective for the larger C2C12 cells. Excitation wavelength was 488 nm, a 505–530 nm bandpass emission filter was used and confocal pin hole aperture was 100 μm. Random images were obtained from the calcein-stained wells. Examples of CLSM images taken from control, NaCl and d-glucose treated CHO-K1 cells are shown in figure 2. The three unstained wells were trypsinised on the third day, and measured in the same way on the fifth day as a control for possible longer-term effects of hyperosmotic culture (figure 1).

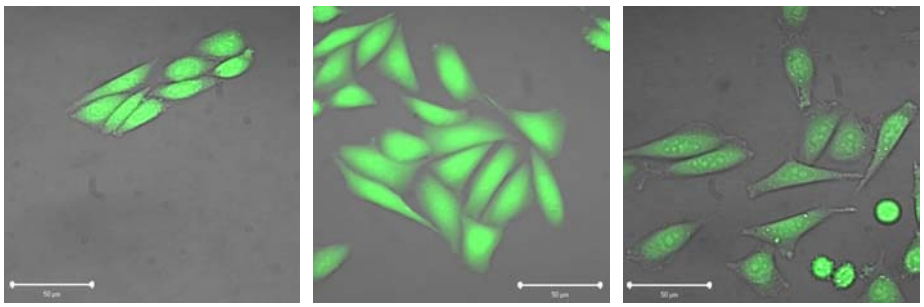

Fig. 2. Visualization of calcein-stained CHO-K1 cells at 500× magnification. (Left) The control cells grown in standard 270 mOsmol medium. (Middle) The cells grown under hyperosmotic 380 mOsmol medium adjusted with NaCl. (Right) The cells grown under hyperosmotic 380 mOsmol medium adjusted with d-glucose. Bars are 50 μm

Using the software associated with the CLSM (Zeiss LSM Image Examiner 2.8, Carl Zeiss, Jena, Germany), cell morphology was analysed afterwards. Three parameters were determined for each cell: total adhesion area, the longest axis of the cell and the longest cell axis perpendicular to the former one. The ratio between these two length quantities was defined as the aspect ratio of the cell.

For the image analysis, the following procedure was used. Cell contours were roughly indicated manually. Subsequently, the mean intensity of the indicated area was computed. All pixels with an intensity below 20% of the mean intensity were removed. The remaining number of pixels was considered the cell adhesion area. Subsequently, the boundaries of the visible contours were used to obtain the cell length and width. If a cell was not completely visible in the image, and in rare cases a cell boundary could not be indicated clearly, the cell was excluded from further analysis.

Statistics

Adhesion area, length, width and aspect ratio of the cells all had skew distributions with outliers. Therefore, a Kruskal–Wallis signed rank test for independent groups was invoked to determine the probability that the groups of cells originated from the same population (two-tailed distributions). For comparison between groups, the probability values are indicated as: * $p < 0.01$; ** $p < 0.001$.

3. Results

All results of measurements carried out on the 2nd and the 5th days were statistically similar for each group ($p > 0.01$). Hence, two-day and five-day groups were pooled to obtain a total number of 149–326 cells per experimental group. Median, the 25^{th} and the 75^{th} percentiles of cell length, cell width, adhesion area and aspect ratio of all groups are shown in figure 3.

Hyperosmotic pressure turns out to increase length, width and adhesion area of the cells. These changes are more apparent in CHO-K1 cells, where differences between control and hyperosmotic conditions are highly significant for all quantities ($p < 0.001$). In C2C12 cells, 3 out of 6 quantities have $p < 0.01$. Addition of NaCl or d-glucose provoked the same effect on all quantities, except for the length of CHO-K1 cells ($p < 0.01$).

Although length, width and adhesion area of C2C12 cells are significantly larger than those values of CHO-K1 cells ($p < 0.001$) in all conditions, the aspect ratio

between cell types and osmotic pressures ($p > 0.1$) is surprisingly consistent (figure 3).

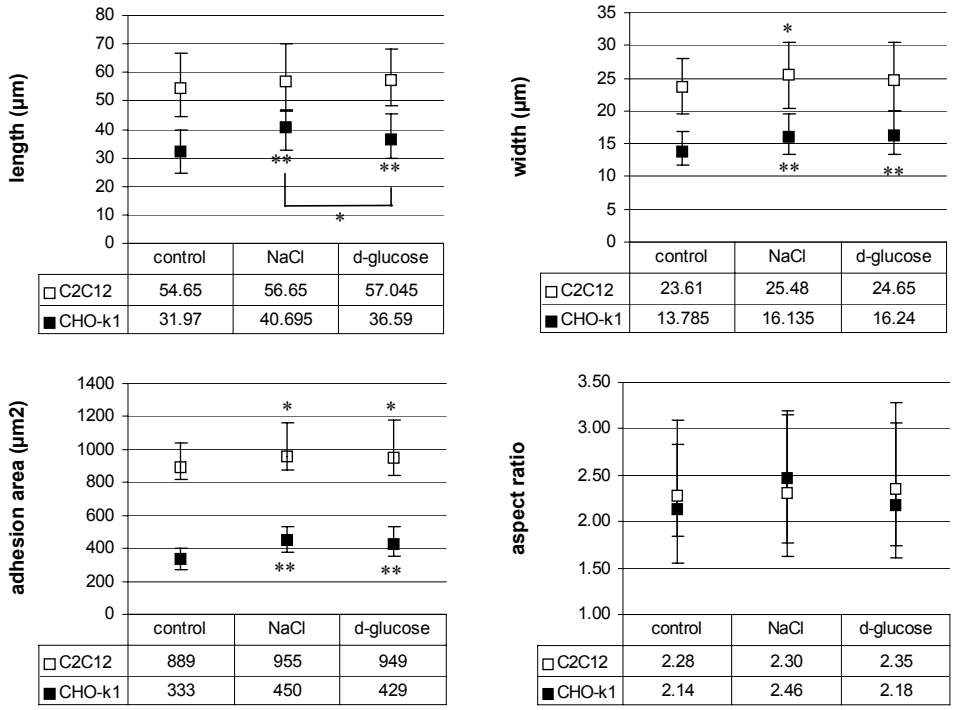

Fig. 3. The median and the 25th and 75th percentiles of cell length (upper left), cell width (upper right), adhesion area (bottom left) and aspect ratio (bottom right) are shown for control and NaCl or d-glucose supplemented hyperosmotic media for both cell types. Significance levels with respect to the control groups are indicated below (CHO-K1) or above (C2C12) the data as follows: * $p < 0.01$, ** $p < 0.001$. Additional statistics reveal that length, width and adhesion area of C2C12 cells are significantly larger than those of CHO-K1 cells ($p < 0.001$). Aspect ratio, however, is independent of cell type and osmolarity in all groups ($p > 0.1$)

4. Discussion

Three highly significant conclusions are derived from the data presented, which reach the research goals of this study.

First, the present study provides significant evidence that the cells which are subjected to a hyperosmotic environment exhibit enlarged adhesion areas, thus confirming the finding of TAKAGI et al. [11]. Quantitatively, CHO-K1 adhesion area that was determined in the control measurement (333 µm^2) agrees very well with the one described by TAKAGI et al. [11] (~350 µm^2). In hyperosmotic culture however, the latter approaches 700 µm^2 in 380 mOsmol medium, while in the present study, median

adhesion area equals 450 μm^2. The difference may stem from different procedures that are being used to determine the adhesion area of cells, i.e. atomic force microscopy of fixed cells [11] vs. CLSM on living cells (present study). Yet, cell sizes perfectly match in the control situation. Normal variability in cell size could also explain the different results, as TAKAGI et al. [11] measured only 10 cells. It should be noted however that an adhesion area of 700 μm^2 or more is found for only 35 out of 649 cells in the CHO-K1 NaCl and d-glucose groups in the present study.

The present study shows that the enlargement is independent of whether NaCl or d-glucose was used to enhance osmolarity in the culture medium, which is in agreement with previous findings reported for disc cells [7]. Also, the changes in cell geometry are present after two days of culture, and sustain after three additional days of culture in hyperosmotic environment and trypsinisation.

The finding that cells extend their adhesion area in response to hyperosmolarity is surprising, as environment with an increased osmotic pressure is expected to result in cell shrinkage rather than in cell swelling [6]. Although it is generally accepted that the initial cell shrinkage can be countered in a longer term [6], [10], the present finding suggests that the cells overcompensate volumetric changes, assuming that cell volume increases with increasing adhesion area under hyperosmotic conditions [11]. It cannot be derived from the present data whether the cells are actually affected with hypertrophy in response to the osmotic loading as their height has not been determined. Although estimates of cell volume can be obtained due to confocal microscopy, such measures are rather inaccurate because of the low resolution that can be obtained in the vertical dimension. Furthermore, determining cell volume for the large number of cells in the present study is not feasible because of the long scanning times involved. In principle, it is possible that cells sag out, increase adhesion area with decreasing height, and thus decrease volume.

The second conclusion of the present study is that the median aspect ratio is constant independent of cell type and osmotic conditions. In spite of the changes in cell length, its width and adhesion area after 48 hours of being cultured in hyperosmotic environment, and in spite of the fact that C2C12 cells are significantly larger than CHO-K1 cells with respect to all these structural variables ($p < 0.001$), the median aspect ratio in all experimental conditions and for both cell types is between 2.14 and 2.46 (figure 3). Thus, although hyperosmotic loading affects cell shape in different ways, cell elongation as such is not affected by increased external osmotic pressure.

The third conclusion is that the response of CHO-K1 fibroblasts to hyperosmolarity is more pronounced than the response of C2C12 myoblasts. The changes observed are larger and more significant. For instance, the adhesion area of CHO-K1 cells increases by 30% (NaCl, $p < 0.001$) or 36% (d-glucose, $p < 0.001$), while the adhesion area of C2C12 increases by 7% only, in both the NaCl and the d-glucose group ($p < 0.01$).

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