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Cell Shape Description as a Tool for Assessing Cell Change after Drug Treatments

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ABSTRACT

Cell shape determines cell activities and behavior. Quantitative analysis of cell shape can be performed by computational image-based analysis. Therefore, the aim of this study was to characterize cell-shape change using various cell-shape descriptions. Pre-osteoblasts were treated with the osteoporosis drugs, alendronate and geranylgeraniol. Cell shapes were defined via ImageJ analysis. Parameters, including perimeter, area/perimeter, circularity, roundness and solidity, were quantified. Alendronate and geranylgeraniol treatments generated changes in cell shape, which were compared between control cells and treated cells. Cell perimeter was increased when geranylgeraniol was added to alendronate-treated cells. Area/perimeter and solidity showed the same trend after treatments. Alendronate decreased both area/perimeter and solidity, while geranylgeraniol increased these parameters toward the baseline level. Circularity was reduced by alendronate and geranylgeraniol. No difference in roundness was observed in either alendronate or geranylgeraniol treatments. Taken together, these parameter measurements demonstrated changes in the cell shape and the cell surface. Each parameter gave distinctive and overlapping information on cell-shape analysis. Evaluation of cells using combined parameters provided a better insight into the cell changes. These data could be applied for the screening of cell changes after treating with drugs.

Keywords: Area/perimeter; Cell shape; Circularity; Shape description; Solidity

1. Introduction

Cell shape regulates biological states such as proliferation and differentiation [1, 2]. In osteoblasts, cell shape and area correlate to the phases of cells [2, 3]. Large spreading area and geometric shape influence the lineage commitment of stem cells. Mesenchymal stem cells with a high aspect ratio or that possess a star shape are inclined toward an osteogenic fate [2]. Apoptotic osteoblasts demonstrate a reduction in cell size and become round in shape [4].

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Furthermore, the circular cell shape increases the percentage of apoptotic osteoblasts compared with the branched cell shape [3].

Traditionally, the study of cell morphology is usually done by visual inspection, which is qualitative and subjective. Slight differences in morphology are difficult to detect by this method. Computational image-based analysis has been developed to identify alterations in cell shapes and phases in several cell types. Cells increasing in size during the differentiation process from monocytes to osteoclasts can be evaluated using digital image analysis [5]. The images of white blood cells from peripheral blood and bone marrow samples are quantitatively assessed to help diagnose blood disorders such as acute leukemia [6]. In addition, computational methods are able to differentiate morphological changes in epithelial cervix adenocarcinoma cells treated with actin polymerization inhibitor [7].

In the dental and medical research fields, several studies rely on image analysis for evaluation of cell change after stimuli addition. Thus, precise measurements of cell changes should be obtained. A simple method for quantification of cell-shape change using an affordable crystal violet staining [8] and ImageJ program have been reported [5, 8]. However, a few parameters including cell size and aspect ratio are commonly used for analysis of cell changes [5, 8]. More parameters should be studied on this matter.

Alendronate (ALN) and geranylgeraniol (GGOH) are drugs known to be able to induce changes in osteoblast behaviors via cytoskeleton alteration [4]. ALN decreases cell viability, cell differentiation, and bone formation. Furthermore, cell morphology is also altered [9]. GGOH partially reverses these negative effects of ALN on osteoblasts [4]. In this study, ALN and GGOH were added to osteoblasts. After the induction, additional parameters were analyzed to improve understanding of cell behavior. This provided more detailed morphology information, which could be applied for future *in vitro* cell culture studies.

2. Materials and Methods

2.1 Cell culture, treatments, and staining

MC3T3 cells are mouse osteoblast precursors (ATCC). These cells were plated at a density of 8,000 cells/cm^2 and cultured in α-minimum essential medium containing 10% fetal bovine serum and 1% penicillin/streptomycin (all from Gibco) at 37°C and 5% CO_2 humidified atmosphere. These cells were treated with 10 µM ALN (A10) and 100 µM GGOH (G100) for 3 days (Fig. 1A). Cells which received no treatment are abbreviated as A0G0. Cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature. Fixed cells were stained with crystal violet (Life Science Dynamic Division, Arnaporn Co., LTD) for 5 min and rinsed thoroughly to remove excess dye (Fig. 1B).

2.2 Digital image processing

Images of cell appearance from 8 random fields were recorded at 200x magnification under a light microscope (Nikon Eclipse TS100) and Nikon Digital sight DS-L2 software. Imaging was performed using ImageJ software version 1.52a Java 1.8.0 (National Institute of Health). The backgrounds of images were eliminated. Images were converted to 8-bit grayscale. Images were auto-thresholded and subjected to Watershed command. The clustered cells were segmented into individual cells and analyzed for cell perimeter, circularity, roundness, and solidity (Fig. 1C). Area/perimeter (A/P) ratio was also calculated. Descriptions and/or formulas of these parameters, according to ImageJ, are as follows:

(1) Perimeter is the length of the outside boundary of an object.

(2) Circularity is a value that indicates a shape of an object. A value of 0.0 reveals an

elongated shape, whereas a value of 1.0 indicates a perfect circle. The formula is as shown in Eq. 2.1.

$$4\pi \times \frac{[Area]}{[Perimeter]^2}. \quad (2.1)$$

(3) Roundness is defined as the ratio of the area of an object relative to the aspect ratio. The formula is as shown in Eq. 2.2.

$$4 \times \frac{[Area]}{\pi \times [Major\ axis]^2}. \quad (2.2)$$

(4) Solidity describes the stiffness and deformability of an object. It can be calculated by obtaining the area of the *object* and dividing it by the area of the convex hull. The formula is as shown in Eq. 2.3.

$$\frac{[Area]}{[Convex\ area]^2}. \quad (2.3)$$

2.3 Statistical analysis

Normality was measured by performing the Shapiro-Wilk test using GraphPad Prism version 5 (GraphPad software). Data were then analyzed by One-way analysis of variance followed by Tukey comparison test. Data are expressed as mean ± SD. Significance was assigned as $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1 Qualitative cell shapes

Fig. 1D depicts the outline of cells after ImageJ segmentation. Most untreated cells were polygonal in shape with cell protrusions. Some A10G0 cells were smaller and appeared as elongated or round in shape. A10G100 cells were enlarged and well spread (Fig. 1D).

3.2 Perimeter

The cell counts for A0G0, A10G0, and A10G100 were 518, 71, and 280, respectively. Cell perimeter of A10G100 cells was significantly larger than those of A0G0 and A10G0 cells (Fig. 2A). Cell perimeter data is also presented by histogram. The pattern of distribution for A0G0 and A10G100 cells was similar. The peak frequency was seen in 100-150 µm range (Figs. 2B and 2D). The distribution of A10G0 cells showed a different distribution pattern, which was spread out evenly among 50-100, 100-150, and 150-200 µm range (Fig. 2C).

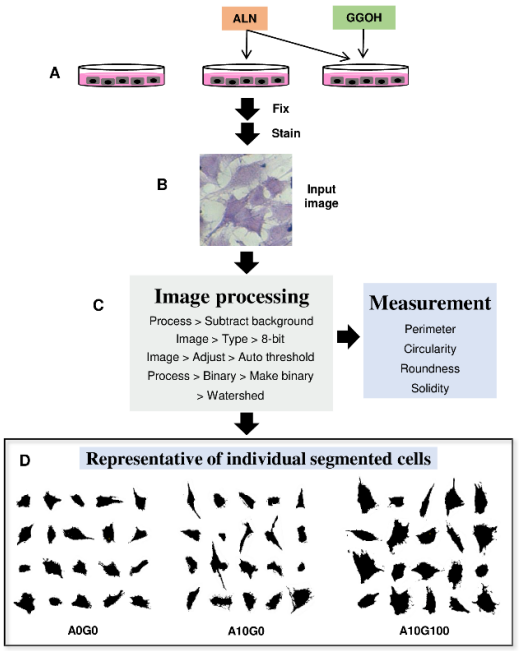

Fig. 1. Overview of experimental design, image processing, and data extraction. (A) Pre-osteoblasts treated with ALN and GGOH. (B) Image of cells stained with crystal violet. (C) Image processing and measurement. (D) Individual cells after cell segmentation.

3.3 Area/perimeter (A/P) ratio

According to Pasqualato [10], A/P parameter is a dimensionless parameter that measures modifications to cell membrane dimension [9]. In this study, A/P ratio was utilized to monitor changes in the cell surface and the correlation between cell area and perimeter. A/P ratio significantly decreased after treatment with A10. The addition of G100 to A10 increased the A/P ratio (Fig. 3A). Overall, perimeter was highly correlated

with cell area. The correlation of perimeter and area in A0G0 had the lowest *r* values. The *r* value of this group was 0.6247 (Fig. 3B), whereas the *r* values of A10G0 and A10 G100 were 0.9229 and 0.9330, respectively (Figs. 3C-3D).

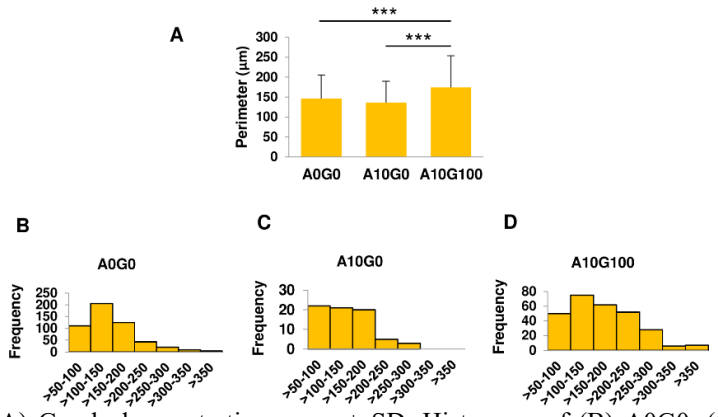

Fig. 2. Perimeter. (A) Graph demonstrating mean ± SD. Histogram of (B) A0G0, (C) A10G0, and (D) A10G100. A; alendronate and G; geranylgeraniol.

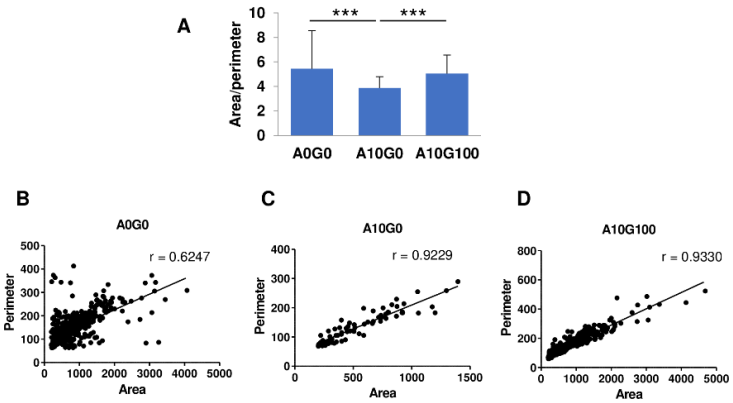

Fig. 3. Area/perimeter. (A) Graph demonstrating mean ± SD. Graphs illustrating the correlation between perimeter and area (B) A0G0, (C) A10G0, and (D) A10G100. A; alendronate and G; geranylgeraniol.

3.4 Circularity

The circularity of A0G0 cells was significantly higher than those of A10G0 and A10G100 (Fig. 4A). The histogram of circularity data shows that the peak frequency of A0G0 was in the range of 0.4-0.6 (Fig. 4B). The peak distribution of A10G0 and A10G100 was in a lower range (0.2-0.4) (Figs. 4C-4D).

3.5 Roundness

Roundness values remained unchanged after treatment (Fig. 5A). The pattern of distribution was similar among A0G0, A10G0, and A10G100 cells (Figs. 5B-5D).

3.6 Solidity

A mild decrease in solidity was seen in A10G0 cells relative to A0G0 cells. The addition of G100 to A10 cells increased solidity to the basal level of A0G0 (Fig. 6A). The distribution patterns of A0G0 and A10G100 cells were similar with peak frequency in the range of 0.8-0.9 (Figs. 6B and 6D). The distribution pattern of A10G0 cells was different with the peak frequency within 0.7-0.8 (Fig. 6C).

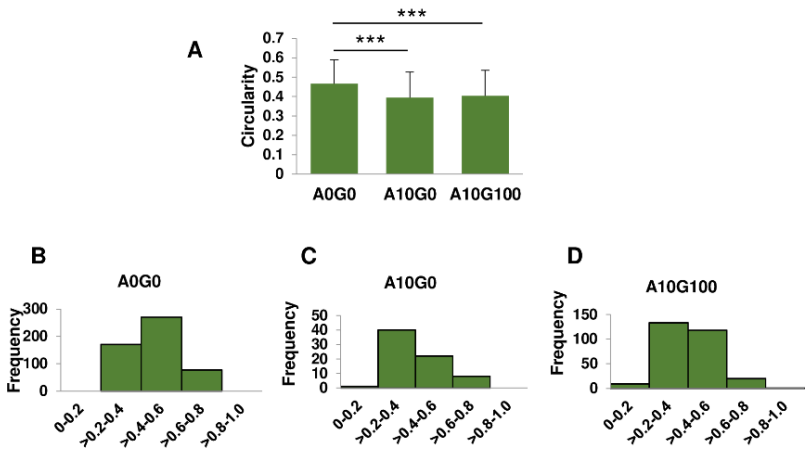

Fig. 4. Circularity. (A) Graph demonstrating mean ± SD. Histogram of (B) A0G0, (C) A10G0, and (D) A10G100. A; alendronate and G; geranylgeraniol.

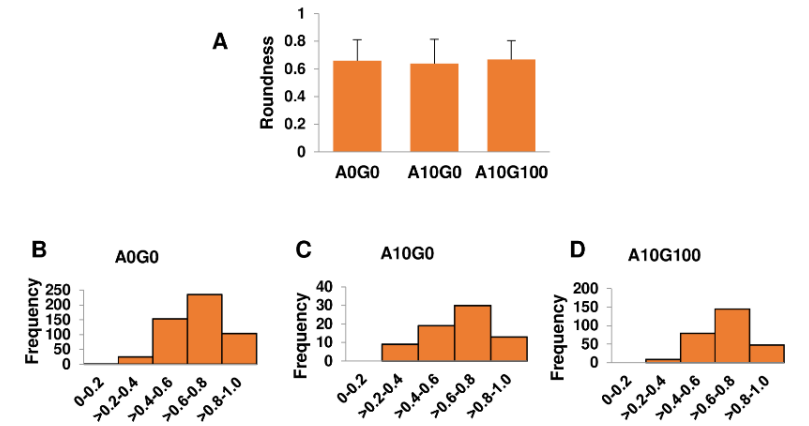

Fig. 5. Roundness. (A) Graph demonstrating mean ± SD. Histogram of (B) A0G0, (C) A10G0, and (D) A10G100. A; alendronate and G; geranylgeraniol.

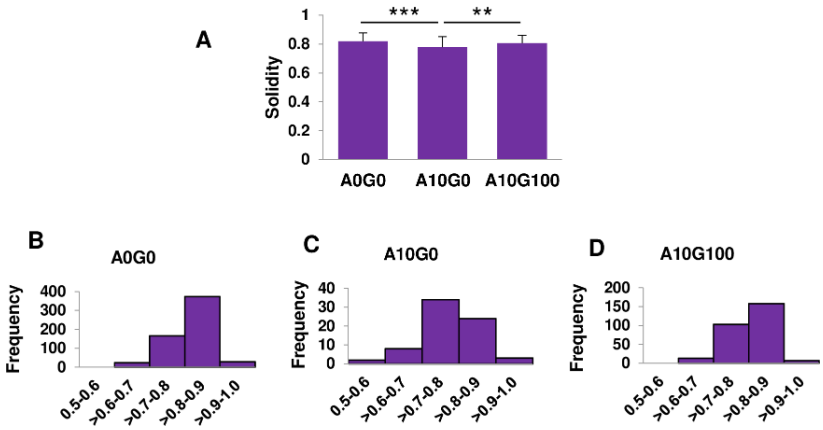

Fig. 6. Solidity. (A) Graph demonstrating mean ± SD. Histogram of (B) A0G0, (C) A10G0, (D) A10G100. A; alendronate and G; geranylgeraniol.

4. Discussion

Cell morphology is known to reflect cell conditions and activities such as viability, proliferation, differentiation, and migration [4, 10, 11]. The conventional approach of cell shape measurements has relied on visual observation. Recently however, quantitative measurements of cell shape has become available. This allows for the interpretation of cell states as physiological or pathological. The key quantitative parameters need to be precise, biologically relevant, and represent actual cell shape. The numeric descriptors of cell morphology mostly focus on cell area and cell dimension [8, 11]. In the present study, perimeter, A/P ratio, circularity, roundness, and solidity were further analyzed. The results are summarized in table 1.

It should be noted that ALN, used in this study, is an established drug known to disrupt the reorganization of actin in the cytoskeleton [4, 12]. When the normal organization of actin is disrupted, osteoblasts lose their viability [4, 13]. This interference alters cell shape, subsequently leading to cell death and detachment from the culture well [4]. On the contrary, GGOH converts cell morphology toward the basal shape seen in untreated cells [4, 12]. Therefore, treatments with these substances indeed altered pre-osteoblast morphologies, allowing quantitative measurement and analysis of cell parameters.

The A/P value of A10G0 cells was substantially lower than the values of A0G0 and A10G100 cells. This was due to a decrease in cell area but not in cell perimeter, resulting in the reduction of A/P ratio. The correlation between perimeter and area was varied with less correlation in the A0G0 group. Since A/P signifies change in the cell surface caused by a modification in the cell membrane dimension [10], this data elucidated the change in the cell surface of pre-osteoblasts after treatments.

Circularity and solidity indicate cell deformability and changes in cell function, which affect cell adhesion and migration [10, 14]. Circularity is a normalized ratio of area to perimeter. A value of 1 means that the object is a perfect circular shape. Solidity distinguishes the convex cell area and the concave cell area, which describes the presence of membrane protrusions including lamellipodia, filopodia, and blebbing [14]. High values of circularity and solidity reflect fewer protrusions [4] and lower cell deformability [10]. These shape descriptors can characterize the morphological changes in mesenchymal stem cells under hyper-gravity and/or osteoinductive nanoparticle applications [15]. In this study, solidity differed between ALN- and GGOH-treated cells, while circularity differed between both ALN- and GGOH-treated cells from untreated control cells. Solidity appears to be the most reliable parameter and is a more informative morphologic descriptor since it clearly differentiates fibroblast morphologies grown on various substrates [16] as well as expresses the distinction between chemoresistant and wild-type cells [10].

Roundness describes the level of cellular area and reflects how close the shape of an object is to a perfect circle. This parameter has been shown to suitably reflect the morphological changes seen during the epithelial to mesenchymal transition [17]. Roundness value may not considerably differ from circularity value [10]. However, in this study roundness and circularity values did not have the same trend. This could be because the measurements of roundness and circularity are corresponding to different factors. Roundness depends on cell dimension (major axis), while circularity depends on perimeter.

Analysis of all parameters pointed to the events preceding cell death. Most cells experience cell deformation and become more circular in shape. In fact, cell death or detachment should result in a circularity value of 1. However, circularity, solidity, and A/P ratio of A10G0 cells were reduced, implying that the cells were at the stage during which they begin to shrink but are still attached to the culture plate. Cell shrinkage is one of the hallmarks of apoptosis. It has been shown that apoptosis is increased, even in branched-shape

osteoblasts, when actin polymerization is disrupted [3]. A reduction in cell volume induces a sequence of events that eventually leads to apoptosis [18]. Most cells undergoing apoptosis from drug treatment were completely round, had lost contact with the well surface, and were dislodged from thorough washing [4]. Therefore, it should be noted that the values of A10G0 were mainly calculated from cells still left on the culture plate. Some detached cells were not included in the analysis. This issue was the limitation of this study. In the future, this issue should be addressed in order to give a more complete picture of changes in cell shape resulting from drug treatment.

Cell spreading area, length, and aspect ratio emphasize the general aspects of cell shape [7, 8, 11], which describe the changes in cell size and dimension. It is essential that the various parameters are used in combination in order to provide more detailed information on other cell aspects. This information is useful for observing how cells react to drug treatments.

Table 1. Summary of all parameters measured from untreated, ALN-treated, and GGOH-treated pre-osteoblasts.

Treatment	None	ALN	ALN GGOH
			
Perimeter	0	-	↑
A/P ratio	0	↓	↑(0)
Circularity	0	↓	↓
Roundness	0	-	-
Solidity	0	↓	↑(0)

0 = baseline

5. Conclusion

Interpretation of the shape-dependent cell activity requires more than one parameter. Each parameter measured different aspects of cell state and some of the parameters provided overlapping information. While using just a single parameter may partially reflect the state of a cell and its activity, measuring multiple parameters in combination provides a more complete understanding of how cells react to drug treatment and how they differ from untreated control cells. This information should be helpful for evaluating cell appearance, which might be useful for other applications in the field of biology and medical sciences.

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