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The effect of osmolarity on metabolism and morphology in adhesion and suspension chinese hamster ovary cells producing tissue plasminogen activator

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Abstract

The effects of constant osmolarity, between 300 and 500 mOsm/kg, on the metabolism of Chinese Hamster Ovary (CHO) cells producing tissue plasminogen activator (tPA) were compared between adhesion and suspension cultures. In both suspension and adhesion culture, the specific rates of glucose consumption (ν_G), lactate production (q_L), and tPA production (q_{tPA}) increased as osmolarity increased, while these rates decreased when osmolarity was higher than the respective critical levels. However, specific growth rate (μ) decreased with increase in osmolarity and this slope grew steeper in the osmolarity range higher than the critical level. The decrease in μ in the adhesion culture was more rapid than that in the suspension culture. The critical osmolarity for adhesion culture (400 mOsm/kg) was lower than that for suspension culture (450 mOsm/kg). These results indicated that the adhesion culture was more sensitive to increase of osmolarity than the suspension culture, while the specific rates obtained from the adhesion cultures were in general 1.5- to 3-fold higher than those obtained from the suspension cultures. Cell volume increased as osmolarity increased in both the suspension and adhesion cultures, as reported previously for suspension culture of hybridoma cells, but there was no morphological change in the suspension culture. In contrast, cell height decreased and cell adhesion area markedly increased as osmolarity increased in the adhesion culture. This morphological change in adhesion cultures may be one reason for the higher sensitivity of adherent cells to the increase of osmolarity than suspended cells.

Introduction

Osmolarity is one of the most important variables affecting the metabolism of mammalian cells and almost all reports with respect to the effects of osmolarity have used suspension cultures of hybridoma cells producing monoclonal antibodies (MAb). In these studies, the increase of osmolarity has been proved to stimulate the uptake of amino acids, increase the intracellular content of mRNA and protein, improve the specific production rate of MAb, and decrease the specific cell growth rate (Oh et al., 1995; Oyaas et al., 1994a, b; Ozturk et al., 1991). It was also reported that suspended hybridoma cell volume increased during the culture under higher osmolarity (Ozturk et al., 1991). However, there have been few studies investigating the effects of osmolarity on the metabolism of other kinds of mammalian cells other than hybridoma cells.

Generally, mammalian cells, other than blood cells, adhere to each other *in vivo* using an extracellular matrix such as collagen. Cells have been reported to function by means of signal transduction through adhesion with other cells, especially in the liver (Tong et al., 1990), thymus (Fernandez et al., 1994; Hardin et al., 1995), and bone marrow (Tsuji et al., 1996) and the cell morphology of adhered cells may affect the adhesion area and inner structure of cells. Many kinds of mammalian cells have also exhibited adhesion dependency during *in vitro* cell cultivation to produce

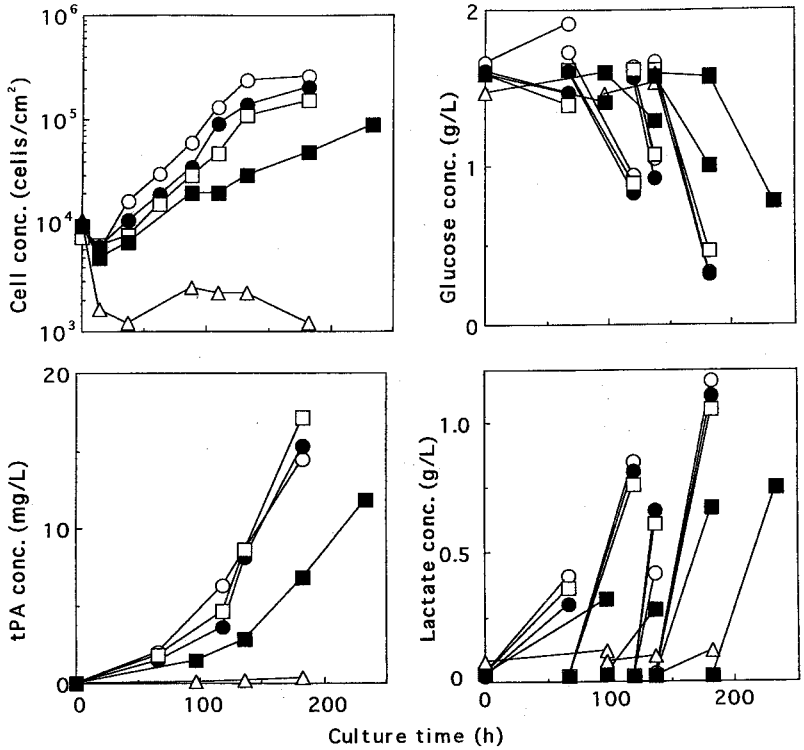

Figure 1. Adhesion culture of CHO cells under various osmolarities. CHO cells were adhesive cultured in 6-well multiplates with the medium which osmolarity was adjusted to 300–500 mOsm/kg by additions of NaCl solution (100 g/L). The culture medium was replaced with fresh medium several times during cultivation to avoid glucose depletion. Symbols: ○, 300; ●, 350; □, 400; ■, 450; △, 500 mOsm/kg.

useful materials such as pharmaceuticals. So, it can be supposed that cell morphological change caused by the change of culture condition affect the cell function. Although adhesion is an important function for mammalian cells, little research has been performed with respect to the effects of osmolarity on cell metabolism in adhesion culture. Chinese hamster ovary (CHO) cells, which are one of the most useful cell lines for the production of pharmaceuticals, have been determined to grow in suspension after some adaptation, despite being originally adhesion-dependent (Hooker et al., 1995).

Consequently, we investigated the effect of osmolarity not only on the metabolism but also on the morphology of CHO cells in adhesion and suspension cultures.

Materials and methods

Cells and media

Tissue plasminogen activator (tPA)-producing CHO 1-15500 cells (ATCC CRL-9606) were used. Ham's F-12 medium (Dainippon Seiyaku Co., Ltd., Japan) supplemented with 10% NBS (Gibco), streptomycin (0.1 mg/L), penicillin (100 u/L) and 500 nM of methotrexate (Sigma) was employed for cell growth. Medium osmolarity was adjusted to 300–500 mOsm/kg by additions of NaCl solution (100 g/L). Medium osmolarity was determined by the measurement of the depression of the freezing point using an osmometer (model OM-801, Vogel, Germany).

Adhesion culture

Two milliliters of the medium in a 6-well multiplate (Corning Costar Japan Co.) was inoculated with 1.88×10^5 cells and incubated in a CO_2 incubator (37 °C, 5% CO_2). The culture medium was replaced with fresh medium several times during cultivation to avoid glucose depletion.

Suspension culture

Thirty milliliters of the medium in a spinner bottle (100 ml, Shibata Hario Co., Japan) was inoculated

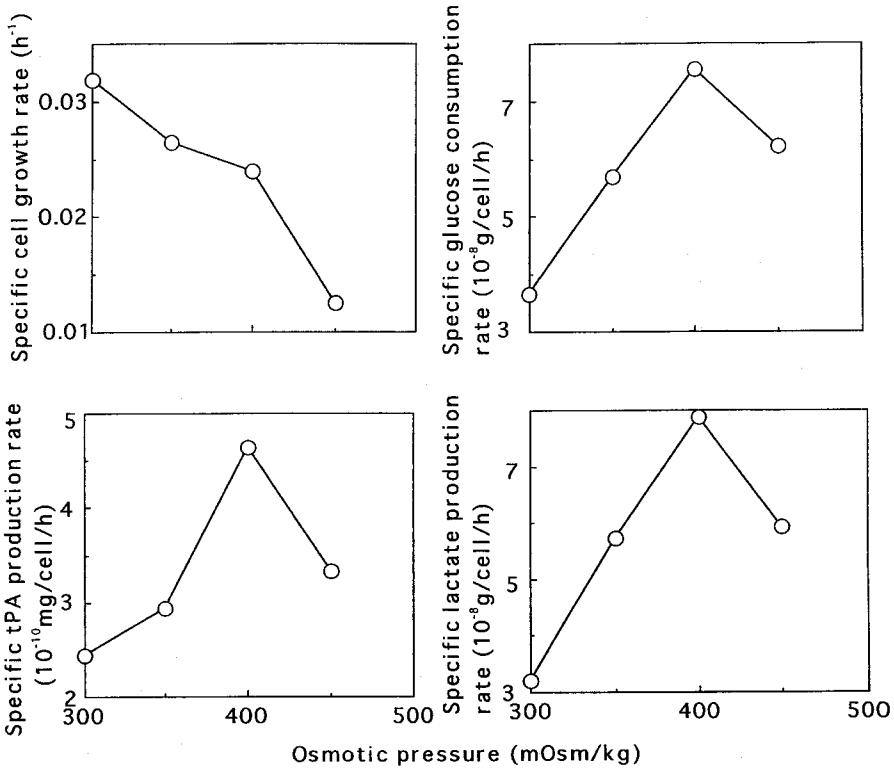

Figure 2. Effect of osmolarity on the specific metabolic rates of adhesive CHO cells. Average specific rates were calculated using the data shown in Figure 1.

Table 1. The specific metabolic rates of adhesive and suspended CHO cells

Osmolarity	Specific rates			
(mOsm/kg)	μ (10^{-2}/h)	ν_G (mg/10^8cells/h)	q_L (mg/10^8cells/h)	q_{tPA} (mg/10^{10}cells/h)
Adhesive cells				
300	3.18	3.66	3.19	2.44
350	2.64	5.70	5.73	2.94
400	2.39	7.56	7.88	4.64
450	1.25	6.21	5.94	3.33
Suspended cells				
300	1.90	1.65	1.49	1.19
350	1.82	2.56	2.07	1.52
400	1.56	3.05	2.80	1.68
450	1.44	3.36	2.74	2.82
500	0.62	3.14	2.77	1.66

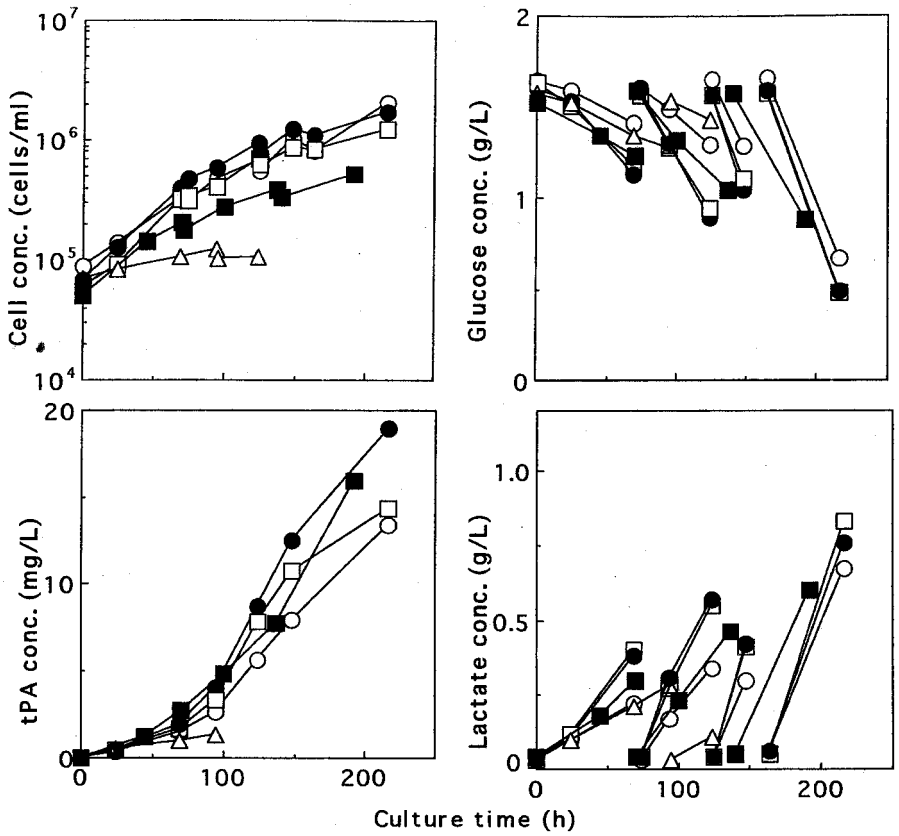

Figure 3. Suspension culture of CHO cells under various osmolarities. CHO cells were cultured in suspension using spinner bottles with medium in which osmolarity was adjusted to 300–500 mOsm/kg by additions of NaCl solution (100 g/L). The culture medium was replaced with fresh medium several times during cultivation to avoid glucose depletion. Symbols are same as those in Figure 1.

with 3×10^6 cells which were harvested from the surface of a culture dish by trypsinization and incubated in a water bath (37 °C, 5% CO_2, 70 rpm). Cell concentration decreased to one fourth of inoculum during initial 24 h and then the cells which adapted to suspension culture, grew to approximately 1×10^6 cells/ml, were inoculated into other spinner bottles in the same manner as mentioned above starting the cultures under each osmotic pressure. Because cells in the suspension culture could be subcultivated several times in suspension maintaining specific growth rate (data not shown), one step adaptation culture was considered to be enough. The culture medium was replaced with fresh medium several times during the cultivation by centrifugation (1000 rpm (186 × g), 5 min) to avoid glucose depletion. Cell specific growth rate in suspension under 300 mOsm/kg (0.025 1/h) was comparable to that in adhesion culture (0.032 1/h). All cultures were performed in duplicate; consistency was confirmed between each duplicate.

General analysis

The cell concentrations in the adhesion and suspension cultures were determined using the nuclei staining (Sanford et al., 1950) ,in which adhered cells were incubated with the solution (citrate 21 g/L, crystal violet 1 g/L) and nucleus were counted under microscope, and trypan-blue dye exclusion method, respectively. The glucose and lactate concentrations in the culture media were analyzed using the glucose oxidase-peroxidase and lactic acid oxidase-peroxidase methods, respectively, with an auto-analyzer (Biochemistry Analyzer 2700; YSI Inc., Ohio, USA). The tPA concentration was determined using an enzyme-linked immunosorbent assay kit (Imulyse tPA, Biopool AB, Sweden).

Cell morphology analysis

The volume of suspension cells was analyzed using a Coulter counter (Type ZM, Coulter Electronics Co.

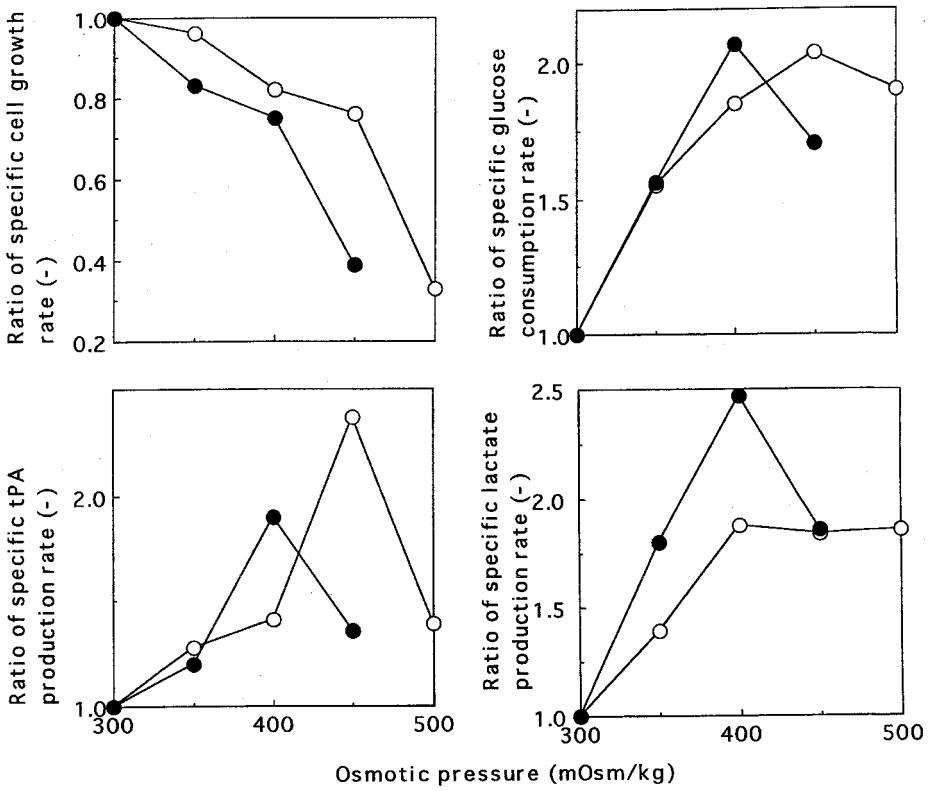

Figure 4. Comparison of the effects of osmolarity on the specific metabolic rates of CHO cell between adhesion and suspension cultures. Average specific rates were calculated for suspension culture in Figure 3 in the same manner as the case of adhesion culture. Both specific rates for suspension and adhesion cultures were indicated as a ratio to the value under 300 mOsm/kg for each culture. ○, suspension culture; ●, adhesion culture.

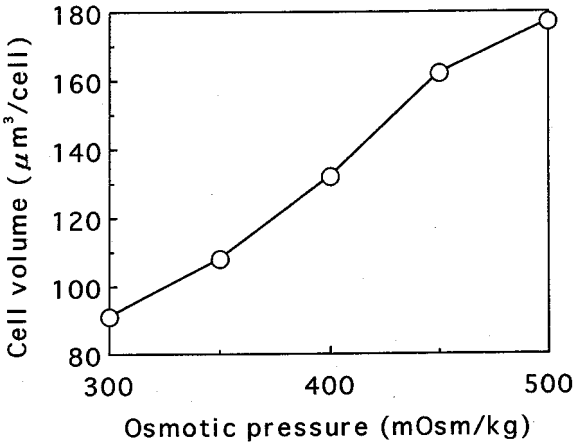

Figure 5. Effect of osmolarity on CHO cell volume in suspension culture. Cell volume was analyzed using a Coulter counter for the cells at the end of suspension culture in Figure 3.

Ltd.). The adhesive cells on the culture dish were washed with PBS having the same osmolarity as each culture medium, fixed using glutaraldehyde (4%), washed with distilled water and dried at room temperature. The height, adhesion area and volume of the fixed cells were analyzed using an atomic force microscope (AFM)(SPM-9500, Shimadzu, Japan). Namely, the height was scanned for all part of each cell and the cell volume was determined by the integral calculation of the heights. The closed area showing positive height was defined as the cell adhesive area(Butt et al., 1990). The average values of 10 cells were shown. Analysis of all terms mentioned was performed in duplicate and the average value was shown in the results.

Results

Effect of osmolarity on adhesion culture

In order to study the effect of constant osmolarity on the metabolism of adhered cells, the adhesion cultures were performed under various constant osmolarities ranging from 300 to 500 mOsm/kg. The cell growth

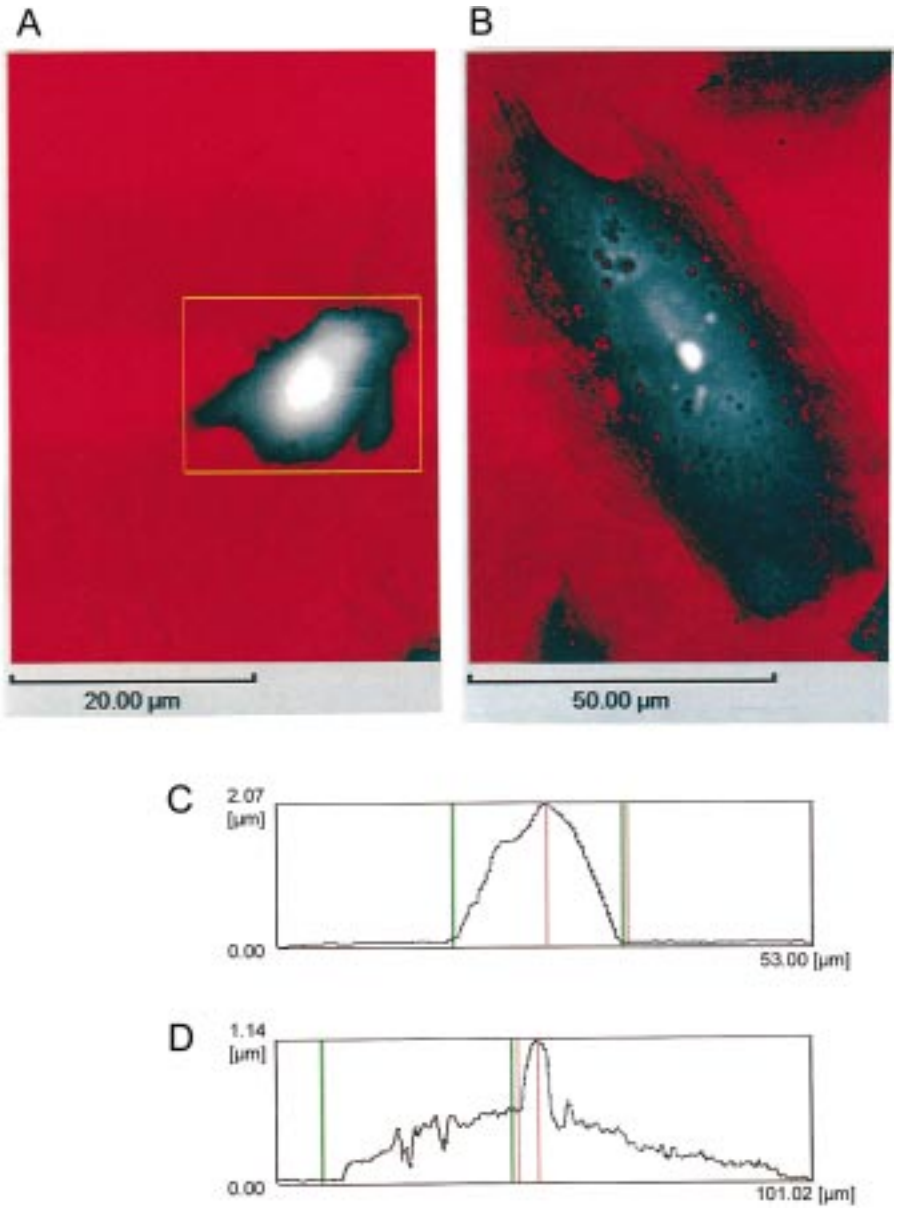

Figure 6. Observation of CHO cells grown in adhesion culture under 300 and 450 mOsm/kg using AFM. Observation using AFM (A, B) and longitudinal cross sections (C, D) were shown for adherent cells grown under 300 and 450 mOsm/kg, respectively. In A and B, red, black and white colors indicated relatively the basement, the middle part, and the highest part, respectively.

was determined to be suppressed under high osmolarities compared to that under 300 mOsm/kg (physiological osmolarity) and no growth was observed when pressure was at 500 mOsm/kg (Figure 1). The total amount of tPA produced was highest under a pressure of 400 mOsm/kg and cells did not produce any tPA under a pressure of 500 mOsm/kg. There was no depletion in glucose during cultivation, whereas glucose was consumed and lactate was produced.

The specific rates of growth (μ), glucose consumption (ν_G), lactate production (q_L) and tPA production (q_{tPA}) were calculated using the data shown in Figure 1 for each culture period separated by medium change and average values were plotted against osmolarity (Figure 2). μ decreased with an increase of osmolarity. However, ν_G, q_L, and q_{tPA} increased as osmolarity increased, although these rates decreased under osmolarity higher than the critical level (400 mOsm/kg).

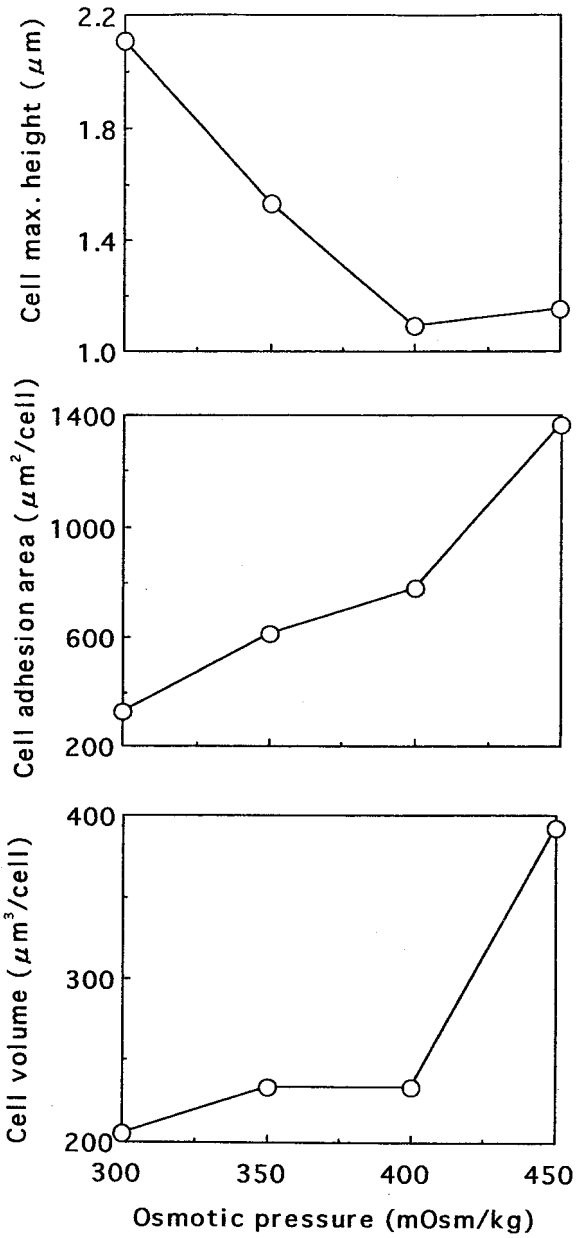

Figure 7. Effect of osmolarity on CHO cell morphology in adhesion culture. Cell volume, cell adhesion area and cell maximum height were analyzed using an atomic force microscope (AFM) for the cells at the end of adhesion culture in Figure 1.

Comparison of the effect of osmolarity between adhesion and suspension culture

In order to compare the effects of osmolarity on cell metabolism in adhesion culture to that in suspension culture, suspension cultures were performed under various osmolarities from 300 to 500 mOsm/kg. The inhibitory effect of high osmolarities in the range from 300 to 400 mOsm/kg on cell growth in suspension culture (Figure 3) was less than that in adhesion culture. Moreover, the cells in suspension could grow and produce tPA under even a high osmolarity of 500 mOsm/kg, in contrast to cell growth and tPA production under an osmolarity of 500 mOsm/kg in the adhesion culture.

The ratios of average values of μ,ν_G, q_L and q_{tPA} under high osmolarity to the values under 300 mOsm/kg are plotted for both adhesion and suspension cultures in Figure 4. The critical osmolarity for ν_G, q_L and q_{tPA} in adhesion culture (400 mOsm/kg) under which osmolarity the specific rates showed maximum level was lower than that in suspension culture (450 mOsm/kg). The curves of μ, shown in Figure 4, had two phases in both cultures; the slope of decrease was small in the lower ranges of osmolarity and large in the higher ranges. The critical osmolarity between the two phases for adhesion culture (400 mOsm/kg) was also lower than that for suspension culture (450 mOsm/kg).

Cell morphology under high osmolarity

The volume of cells in suspension culture under various osmolarities increased with increasing osmolarity (Figure 5) and the cell volume at 500 mOsm/kg was almost 2-fold higher than under 300 mOsm/kg. However, there was no morphological change and cells were globular under every osmolarity.

Figure 6 shows the AFM observations for the adhered cells cultured under 300 and 450 mOsm/kg. In each part (A, B), red, black and white colors indicated the basement, the middle part, and the highest part, respectively. Only a small section shows the highest part within one cell cultured under 450 mOsm/kg, while the highest area was wide in the cell cultured under 300 mOsm/kg. Longitudinal cross sections of the observations show that the shape of cells cultured under 450 mOsm/kg was distorted, whereas under 300 mOsm/kg the shape was smooth. Cells cultured under the higher osmolarity were very flat and thin, less than 0.5 μm, and only few points retain high within one adhesive cell.

The maximum height within one cell, adhesion area and volume of cells in adhesion culture under various osmolarities were measured using AFM (Figure 7). The cell volume increased with increasing osmolarity. At 450 mOsm/kg, cell volume was almost 2-fold higher than at 300 mOsm/kg. The cell

adhesion area increased even more sharply with increasing osmolarity and was more than 4-fold higher at 450 mOsm/kg than that at 300 mOsm/kg. The maximum height of each cell was observed to decrease as osmolarity increased.

Discussion

There were several reports about the effect of osmotic pressure on cell metabolism, volume, intracellular pH, in which additions of NaCl, KCl, and sucrose to same osmolarity had similar effect each other (Oyaas et al., 1994; Reusch et al., 1995). So, the change in cell activity and morphology observed in this study might be due to the change in osmolarity. The results showed that μ decreased and ν_G, q_L and q_{tPA} increased as osmolarity increased to some degree, while all specific rates decreased under osmolarities higher than the critical level in the suspension cultures (Figure 4). This dependency of CHO cells metabolism on osmolarity is similar to those previously reported in suspension cultures of hybridoma cells (Oh et al., 1995; Oyaas et al., 1994a, b; Ozturk et al., 1991). The effect of osmolarity on cell metabolism of adherent cells was investigated in comparison with suspended cells in this study. The qualitative dependency of adhered cell metabolism on osmolarity was identical to that for suspension cultures mentioned above (Figure 4).

However, the critical osmolarity of 400 mOsm/kg over which μ decreased more rapidly with the increase of osmolarity in the adhesion cultures was apparently lower than that in the suspension culture (450 mOsm/kg). The critical osmolarity at which the specific rates of ν_G, q_L and q_{tPA} showed respective maximum (400 mOsm/kg) in the adhesion culture was also lower than that in the suspension culture (500 mOsm/kg). These results indicated that the adhered cells were more sensitive to high osmolaritys than suspended cells.

The absolute values of the specific rates in the adhesion cultures were markedly higher than those in the suspension cultures (Table 1). It suggests that the metabolic activities of the adhered CHO cells were higher than those of the suspended CHO cells.

The increase of suspension cell volume under high osmolarity shown in Figure 5 is consistent with the report in the case of suspension culture of hybridoma cells (Ozturk et al., 1991; Oh et al., 1995). Moreover, the rate ($\times$ 1.52) of cell volume increase at 450 mOsm/kg compared to that at 300 mOsm/kg was almost the same as the rate ($\times$ 1.77) previously reported (Ozturk et al., 1991). In this study, the change of cell morphology was not observed and cell shape was globular under every osmolarity. This increase of cell volume was considered to be one of the reason for the increase of specific metabolic rates under high osmolarity (Ozturk et al., 1991).

On the other hand, morphological change of adhesive cells was observed under high osmolarity. The increase of adhesive area with the increase of osmolarity was observed not only under AFM but also under conventional light inverted microscope (data not shown). The maximum height within each adhesive CHO cell decreased with increasing osmolarity. Moreover, the height of the planar part other than the highest point (summit) of the adhered cell was very small, less than 0.5 μm, under high osmolarity. This two-phase structure (plane and summit) was observed also under a light inverted microscope utilizing the difference of refractive index between the two phases. The two phase structures of the adhered cells under high osmolarity looks to be caused by some external pressure such as high static pressure. It was speculated that there may not be enough physical space in height for metabolism in the planar part of cells cultured under 450 mOsm/kg and endocytosis may be inhibited in these cells. This morphological change of adhered cells under high osmotic pressure may be caused by change in receptor sites and membrane characteristics which results in cells become more susceptible to adverse effect of high osmolarity, i.e. less adaptable to survive in high osmotic pressure stress.

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