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The role of microenvironment in aggregation of the 293-human embryonic Kidney cells

Liang Zhao, Li Fan, Xu Zhang, Minglong Zhu and Wensong Tan†

State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China
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Abstract–The microenvironment severely affects aggregation and growth of human embryonic kidney 293-HEK cells. Key factors such as calcium, magnesium and shear stress were investigated in detail and cell aggregate size control was applied to facilitate cell retention improvement. It was found that the concentration of calcium ion affected the aggregation of 293-HEK cells drastically and exhibited direct proportion to the average diameter of 293-HEK cell aggregates. Similar effect was also discovered in magnesium but to a lower extend. Results also showed the growth of 293-HEK cells was influenced when the concentrations of calcium or magnesium ions were below 0.1 mmol/L, and that was more significant with magnesium. Furthermore, aggregation as well as growth was affected by hard intensive mechanical agitation. According to above results, the 293-HEK cell aggregates were successfully well controlled to proper size as anticipated and the average sedimentation speed of aggregated cells increased about 20 times to single cells. This is highly advantageous to cell retention improvement either in perfusion cultures or media exchange before adenovirus infection by proper control of the cell aggregate size, and thus a high cell concentration and adenovirus production potentially can be achieved.

Key words: 293-HEK Cells, Aggregation, Microenvironment, Divalent Cation, Shear Stress

INTRODUCTION

As a result of advances in gene therapy in recent years, there has been an increasing interest in recombinant viral vectors for *in vivo* gene delivery. Since 1990, adenovirus vectors have been the promising vectors of choice for gene therapy applications [1-3]. To date, over 600 gene therapy clinical protocols have been reported (Wiley web site: www.wiley.co.uk/genetherapy/clinical/), 27% of which used adenovirus vectors to deliver therapeutic or marker genes. These developments have fueled the interest in the 293-human embryonic kidney (293-HEK) cell line that has traditionally been used in the production of E1-deficient adenoviruses [4].

Like other epithelial cells, 293-HEK cells form aggregates readily when cultured in suspension. In some serum-free mediums, the cell aggregates even reached up to 3 mm in diameter [5]. Development of high cell density culture and large-scale adenovirus propagation were baffled by severe cell aggregation [6]. Many methods were attempted to alleviate cell aggregation in Schoofs's study, but took little effect [7]. However, the bottlenecks in the cell retention process for perfusion culture or media exchange before adenovirus infection, such as serious cell adhesion, poor efficiency and throughput etc., could be solved by aggregate culture. An approach was adopted to cultivate some transformed cells as aggregates in order to improve the efficiency of cell retention in perfusion [8]. With its ease of cell retention, aggregate cultures offered an alternative choice for large-scale operations. Therefore, characteristics of 293-HEK cell aggregation should be investigated in detail to direct cell aggregate size control. It has been reported that higher calcium ion concentration induced larger cell aggregation and good viability was observed inside the cell aggregates [5]. But few systematic investigations on microenvironment affecting 293-HEK cell aggregation and cell aggregate size control have been documented. In this study, the key microenvironmental factors, such as calcium, magnesium ion and shear stress, were focused on and the subsequent effects of these factors on 293-HEK cell aggregation and growth have been investigated in detail. The cell aggregate size control experiment was also carried out to test the positive effects on facilitating cell retention improvement.

†To whom correspondence should be addressed.
E-mail: wstan@ecust.edu.cn

MATERIALS AND METHODS

1. Cell Line and Medium

Human embryonic kidney (HEK) 293-HEK cell line obtained from Shanghai Tumor Hospital and adapted in serum-free medium. Serum-free medium was prepared from calcium and magnesium free basal medium DMEM/F12 (1 : 1). The medium was completed with insulin, transferrin, BSA and Pluronic F-68 etc. The calcium and magnesium ion concentration was determined according to respective experiments by adding additional calcium chloride (Sigma) and magnesium chloride (Sigma).

2. Cell Culture Conditions

Cells were cultivated at 37 °C in a 5% CO_2 incubator. All experiments were performed in 150-ml spinner bottles equipped with a magnetic stir bar and having a maximal working volume of 60 ml. Cells from stock culture were centrifuged, resuspended and dispersed before inoculation into the spinner bottles at a concentration of approximately 3×10^5 cells/ml. The spinner bottles were agitated at 60 rpm except for agitating experiments.

3. Analytical Methods

In normal conditions of culture, due to the small and loose cell aggregates, cell concentration was determined by counting the number of cells by using a hemocytometer after a-few-minutes incubation of the samples with 0.02% EDTA solution in phosphate buffer

saline (PBS). The viability was assessed by dye exclusion method using trypan blue. In later stages when cultured in high concentration calcium or magnesium ion medium, due to the large size of the cell aggregates, the cell concentration was determined by nuclei count after overnight incubation of the sample at 37 °C with 0.1% crystal violet solution in PBS containing 0.1 mol/L citric acid. The average size of the aggregates was determined by using a laser particle size analyzer (MALVERN). Flow fields were simulated and quantitatively analyzed by CFD (Computational Fluid Dynamics) methods using commercial CFD code FLUENT (Version 6.2, Shanghai Supercomputer Center).

RESULTS AND DISCUSSION

Pre-experiment phenomena implied that cell aggregation was influenced drastically by the microenvironment of a culture system. It included biochemical circumstances such as some critical medium components and engineering aspects. Cell aggregate size was ultimately determined by a number of concerned factors' multi-effects. Results indicated that calcium, magnesium ion and shear stress played important roles in cell aggregation. Therefore, researches on these key factors were performed in detail with the results shown below.

1. Effects of Microenvironment on Cell Aggregation

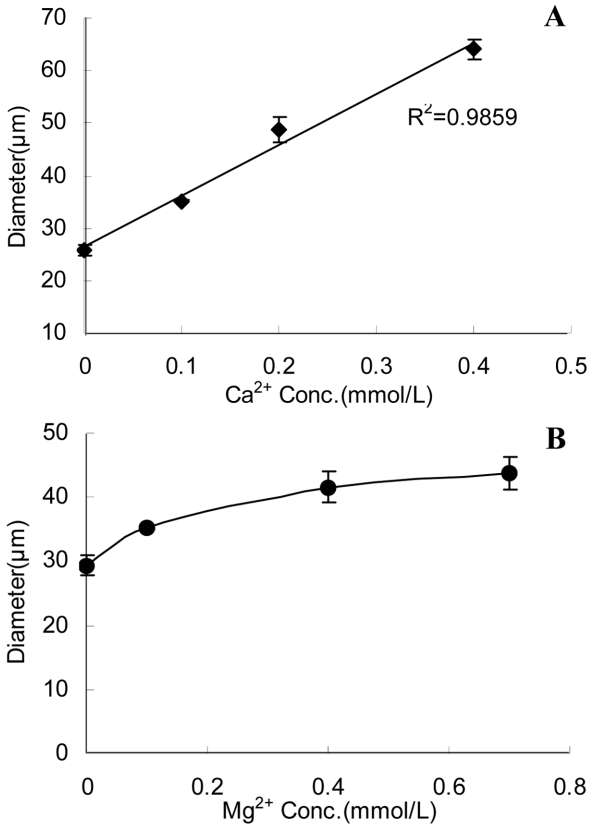

Fig. 1. Effect of calcium and magnesium ion concentrations on the aggregation of 293-HEK cells. Cells were seeded at 3×10^5 cells/ml in spinner bottles. Samples were taken 36 hours after inoculation. (A) cultivated at Ca^{2+} of 0.1 mmol/L and Mg^{2+} of 0.1 mmol/L; (B) cultivated at Ca^{2+} of 0.4 mmol/L and Mg^{2+} of 0.1 mmol/L.

Cells previously cultivated in 0.1 mmol/L Ca^{2+} as suspension culture were inoculated into spinner bottles at 0, 0.1, 0.2 and 0.4 mmol/L of Ca^{2+}. No significant increase in aggregates size at each concentration of Ca^{2+} was observed since 12 hours after inoculation. Average aggregate diameters were analyzed and shown in Fig. 1A. It was found that the concentrations of calcium ion, in the range of 0.1 to 0.4 mmol/L, affected aggregation of 293-HEK cells severely. Cells formed small and loose aggregates at 0 or 0.1 mmol/L of Ca^{2+}. Whereas, they tended to form much larger and tighter clumps at 0.4 mmol/L of Ca^{2+} (Fig. 2). The average diameter of 293-HEK cell aggregates exhibited direct proportion to calcium concentrations in suspension culture. After regression a linear equation was given by

$$D=97.7c+26.4 \quad (1)$$

in which D was the average diameter (μm) and c was the concentration of Ca^{2+} (mmol/L). It depicted the basic relationship between Ca^{2+} concentration and aggregate size in specific conditions and implied cell aggregation could be well regulated by a similar method.

Similar experiments were carried on at 0, 0.1 0.4 and 0.7 mmol/L of Mg^{2+}. It was found that the size of cell aggregates in cultures

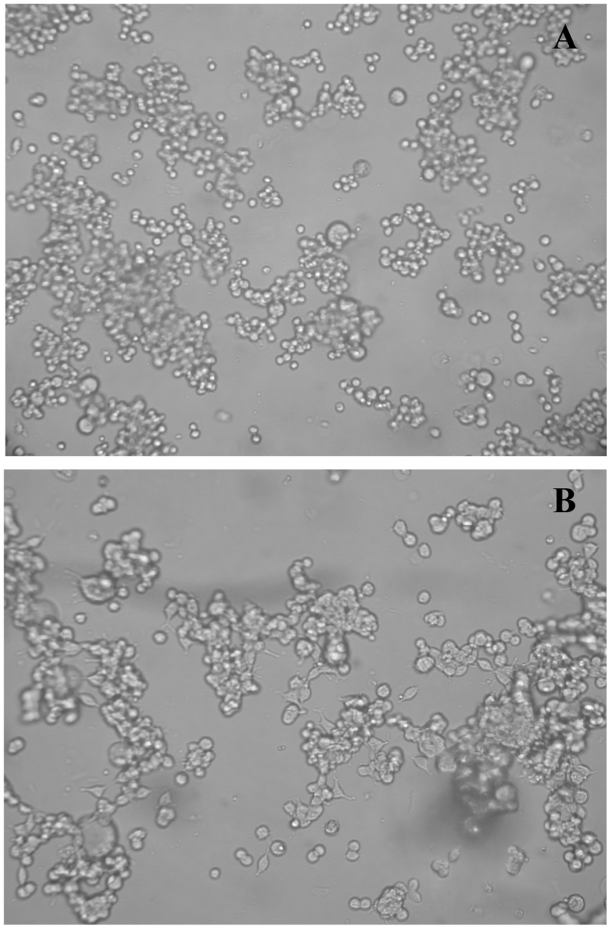

Fig. 2. The morphology of 293-HEK cells in suspension culture. Cells were seeded at 3×10^5 cells/ml at Ca^{2+} of 0.2 mmol/L and Mg^{2+} of 0.1 mmol/L in spinner bottles. Samples were taken 36 hours after inoculation. Each experiment at different agitation speeds was repeated three times. (A) Calculated shear stresses under different agitation speeds; (B) Average diameters of aggregates at different shear stresses.

with higher Mg^{2+} concentrations was a little larger than that in the lower Mg^{2+} cultures (Fig. 1B).

So the size of aggregates was affected by Ca^{2+} and Mg^{2+}. This effect was more profound with Ca^{2+}. Documents indicate that many epithelial cells form tight junctions between neighboring cells. The conglutinations between cells are induced by cadherins, one specific adhesion factor, etc. Anchor conjunctions between cells need adhesion factors, such as cadherins and integrins etc. These adhesion factors at least can be divided into five or more families, such as cadherin, lectin and integrin etc. They are all integrated membrane proteins combining to cytoskeleton and mostly Ca^{2+} or Mg^{2+}-dependent. Cadherin molecular composes of 720-750 amino acid residues which has approximately 50-60% identical primary structure and four Ca^{2+} dependent high homologous structure regions among five extra cellular N-terminal structure regions. Integrin is a heterogeneous dimeric glycoprotein composed of α and β subunit. These subunits conjugate into various dimeric integrins to combine with different ligands which lead to adhesion between neighboring cells, or cell and substrate. It has been reported that at higher calcium concentrations the number of these junctional complex protein assemblies increases [9,10]. It is likely that the effect of calcium and magnesium on the aggregation of 293-HEK cells is caused by a similar mechanism.

Shear stress was also one of the most important microenvironmental factors that affected cell aggregation in suspension culture. It was investigated through cultivating cells in spinner bottles under different agitation speed at Ca^{2+} of 0.2 mmol/L and Mg^{2+} of 0.1 mmol/L. The flow fields were simulated by CFD to calculate the shear stresses under different agitation speeds (Fig. 3A). The results showed that average diameters of cell aggregates were invariable when the maximal shear stresses were below 0.65 Pa, whereas they decreased at higher Shear stresses (Fig. 3B). This effect was mainly due to high liquid shear stress caused by hard intensive mechanical agitation. A single cell's average diameter ranged from 10 to 15 μm and cell aggregates usually reached several decades μm or even larger. The cell aggregates were entirely enveloped in much larger size's vortex and accelerated to the same speed as the big vortex. This was a whole different process. Kinetic energy dissipated on cell aggregates when the vortex size was similar to aggregates and several neighboring vortexes acted on one cell aggregate because they could not envelop it entirely. So high liquid shear stresses above

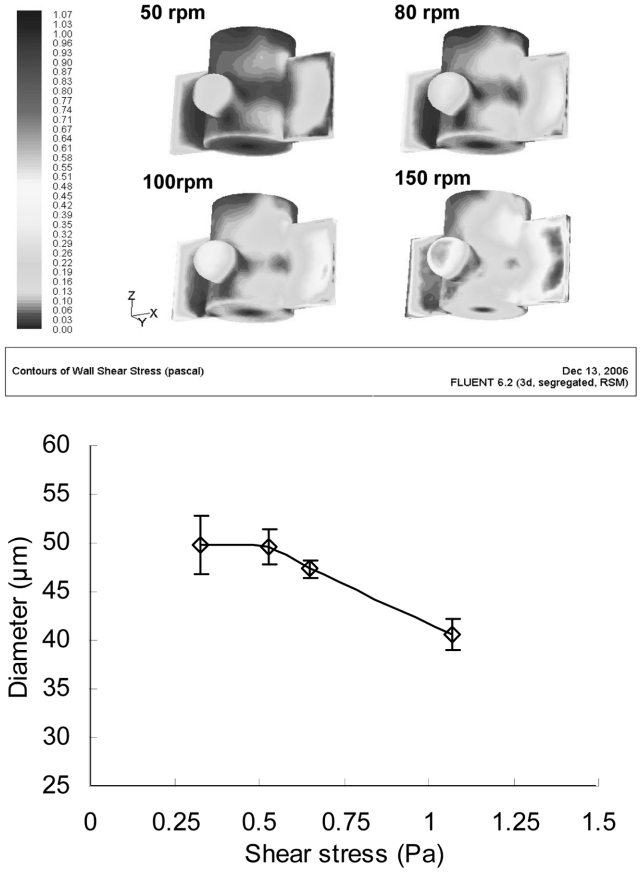

Fig. 3. Effect of shear stress on the aggregation of 293-HEK cells. Cells were seeded at 3×10^5 cells/ml in spinner bottles. Samples were taken 36 hours after inoculation. Each experiment at different ion concentrations was repeated three times. (A) calcium ion concentrations: 0, 0.1, 0.2 and 0.4 mmol/L, magnesium ion concentration: 0.1 mmol/L; (B) calcium ion concentration: 0.1 mmol/L, magnesium ion concentrations: 0, 0.1, 0.4 and 0.7 mmol/L.

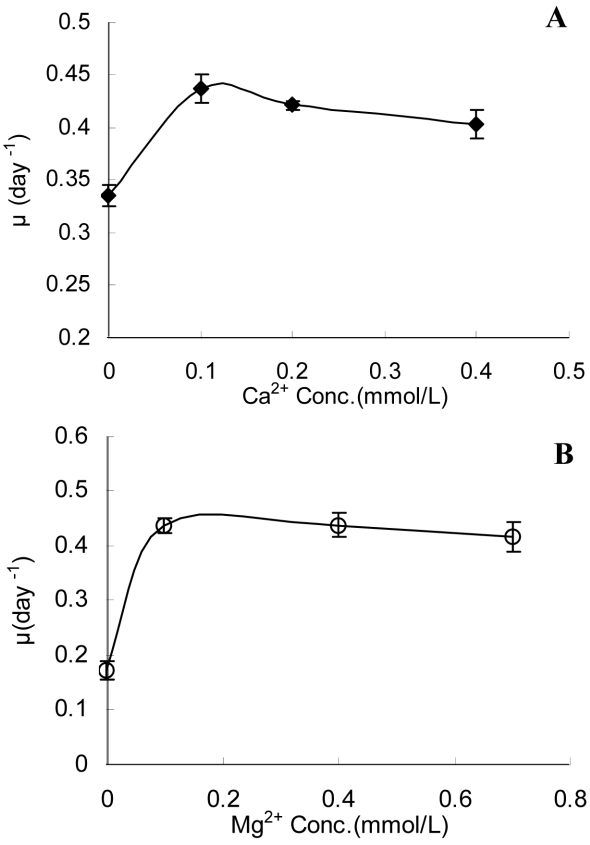

Fig. 4. Effect of calcium and magnesium ion concentrations on the growth of 293-HEK cells. Cells were seeded at 3×10^5 cells/ml in spinner bottles. Samples were taken 36 hours after inoculation. Each experiment at different ions concentrations was repeated three times. (A) calcium ion concentrations: 0, 0.1, 0.2 and 0.4 mmol/L, magnesium ion concentration: 0.1 mmol/L; (B) calcium ion concentration: 0.1 mmol/L, magnesium ion concentrations: 0, 0.1, 0.4 and 0.7 mmol/L.

0.65 Pa broke big aggregates into smaller ones, according to our experiments.

2. Effects of Microenvironment on Cell Growth

The effects of the foregoing relative factors on growth of 293-HEK cells were investigated in order to regulate cell aggregate size on the condition of natural growth. Fig. 4A shows the effect of Ca^{2+} on the growth of 293-HEK cells as presented. The cell specific growth rate was smaller than natural instance without Ca^{2+} and it reached over 0.4 day^{-1} at Ca^{2+} above 0.1 mmol/L.

Ca^{2+} is usually the important accessory factor of some nucleases in cells. It relates to some ion tunnels as well. So cell growth will be influenced if Ca^{2+} is absent. The effect of Mg^{2+} on cell growth was also investigated. The specific cell growth rate drastically declined when the Mg^{2+} concentration was below 0.1 mmol/L, whereas no significant influences on cell growth were observed when Mg^{2+} concentration was above 0.1 mmol/L (Fig. 4B). It is mainly that Mg^{2+} is the key cofactor of many enzyme reactions in cells and it stabilizes nucleoproteins and cell membrane. So the absence of Mg^{2+} influenced cell growth seriously.

The effect of flow field on cell growth was investigated and results are shown in Fig. 5. No significant negative effect on cell growth was observed when the maximal shear stresses were below 0.65 Pa, but specific growth rate and viability decreased dramatically at higher shear stresses. Samples were observed through an optical microscope. It was observed that slick-exterior cells decreased and rough-exterior cells increased on the conditions of high shear stresses. Unshaped cells were surrounded by dead cells and cell debris suspended in medium. This phenomenon indicated that cells were cut by hard intensive mechanical agitation and partly distorted. Therefore, exorbitant agitation would bring a negative effect on cell growth.

3. Control of Cell Aggregation and its Potential Application

According to the above results, cell aggregate size could be controlled easily. Experiments were performed to demonstrate it and test the positive effects on cell retention improvement.

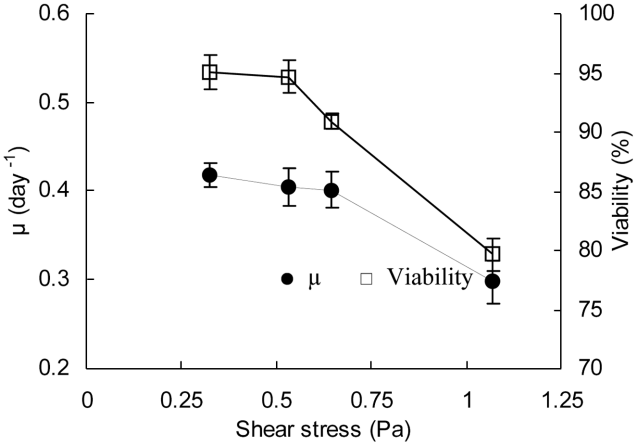

Fig. 5. Effect of shear stress on the growth of 293-HEK cells. Cells were seeded at 3×10^5 cells/ml at Ca^{2+} of 0.2 mmol/L and Mg^{2+} of 0.1 mmol/L in spinner bottles. Samples were taken 36 hours after inoculation. Each experiment at different agitation speeds was repeated three times.

Cells were cultivated at Ca^{2+} of 0.2 mmol/L, Mg^{2+} of 0.1 mmol/L and agitation speed of 50 rpm. Analytic result showed the average cell aggregate size was 49.2 μm, which was greatly similar to the calculated value of 45.9 μm according to Eq. (1). The average sedimentation speed of aggregated cells reached 331 mm/hr, which increased about 20 times to 15 mm/hr of single cells. No abnormality of cell growth was observed during cultivation as anticipated. The much greater sedimentation speed greatly facilitates the improvement of cell retention device performance. And a sedimentary separation experiment of aggregated cells using an inclined settler (developed by our group) was performed to test the gravitational settlement device. Distinct improvement of cell retention efficiency was exhibited.

CONCLUSION

293-HEK cells tended to aggregate severely in suspension culture. In this study, effects of the key microenvironmental factors on cell aggregation and growth have been investigated. Results showed the concentration of calcium ion drastically affected the aggregation of 293-HEK cells. The average diameter of 293-HEK cell aggregates exhibited direct proportion to calcium concentrations. And the size of aggregates was also affected by magnesium to a lower extent. The growth of 293-HEK cells was influenced when the concentrations of calcium or magnesium ions was below 0.1 mmol/L. This effect of magnesium ion concentrations was more significant than that of calcium ion. However, both aggregation and growth were affected by high shear stresses. Therefore, proper control of cell aggregates size could be applied as anticipated to eliminate the negative effects brought by mass transfer resistance or facilitate the improvement of cell retention process. The efficiency of this method had been confirmed in practice. And further research work on its applications in either perfusion cultures or medium exchange before virus infection may be investigated in future.

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