Vitronectin enhances adhesion force and t-PA production of weakly adherent 293 cells exposed to a shear stress

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Abstract

The effects of shear stress on the adhesion and productivity of 293 cells were studied quantitatively and compared with those of Vero and human liver cells. These cells were cultured in polystyrene dishes by using shear stress exposing equipment. 50% of 293 cells cultured in 2% FBS supplemented medium detached from the dish after 29 h of exposure to a shear stress of 0.10 Pa. On the other hand, 90% of Vero and human liver cells remained on the dish under the same condition. Observations with scanning electron microscopy about cell adhesion plaques on the surface of the dish showed that the area covered with lamellipodia and the number of microspikes for 293 cells were found to be less than those of the other cell lines. Several attachment factors, especially vitronectin, were found to enhance the number of microspikes and the adhesion force of 293 cells. Almost 100% of 293 cells remained on the vitronectin-coated dish after 40 h under 0.10 Pa of shear stress. A higher shear stress (greater than 0.10 Pa) caused a decrease in tissue plasminogen activator (t-PA) productivity of 293 cells. But 0.03 Pa stimulated the t-PA secretion on the non-coated dish. Vitronectin also enhanced the t-PA secretion even under 0.10 Pa. These results indicate that the adhesion force of 293 cells is obviously weaker than that of the other cell lines, and vitronectin enhances the adhesion force and the productivity of 293 cells exposed to a shear stress.

Abbreviations: t-PA, tissue plasminogen activator; SEM, scanning electron microscopy; CMF-PBS, Ca^{2+} , Mg^{2+} -free phosphate-buffered saline solution

Introduction

Various useful biologicals such as interferon, plasminogen activator and viral vectors have been produced by many kinds of animal cells. Some of these cells, including anchorage-dependent cells, are cultivated in agitated bioreactor with microcarriers as a culture substratum for large-scale culture (Smiley et al., 1989). The adhesion force of the anchorage-dependent cells to the substratum varies according to cell lines and some cell lines have relatively weak adhesion force and detached from microcarriers due to a high shear stress caused by the impeller in the reactor. Among these weakly adherent cells, recently a human embryonic kidney 293 cell attracts one's at-

tention about the production of useful proteins and adenovirus vector for gene therapy, because an efficient 293/EBNA transient expression system was developed (Langle-Rouault et al., 1998) and 293 cell has the E1A region of adenovirus genome, in which a replication-defective adenovirus vector lacking E1 region can propagate (Lochmuller et al., 1994). To achieve the large-scale culture of 293 cells, it is necessary to know the effect of shear stress on the adhesion of the cells quantitatively and the optimum culture condition like a kind of attachment factor to increase the adhesion force to a culture substratum

There are many reports describing the effects of shear stress on endothelial cells under the physiological conditions in the blood vessel, but few reports

Figure 1. Shear stress exposing system. (1) Upper medium reservoir, (2) lower medium reservoir, (3) flow chamber, (4) flow meter, (5) warm plate, (6) microscope objective lens, (7) vacuum, (8) medium sampling port, (9) roller pump.

about the quantitative studies of the shear stress effects on the animal cell lines including weakly adherent cells utilized for biotechnology can be found. Crough et al. (1985) investigated the effect of the shear stress on anchorage-dependent cells using a 'cell adhesion measurement module'. This device produced shear stress in the radial direction on a loading disc. For BHK, Vero and MRC-5 cells, the reported values of the critical shear stress against detachment from the plastic dish were 4.47, 3.51 and 2.94 Pa, respectively. Stathopoulos and Hellums (1985) reported the effects of shear stress on cell productivity using human embryonic kidney cells. Shear stresses less than 2.6 Pa had no apparent effect on cell viability. The largest amount of urokinase was released from the cells exposed to shear stresses under 0.65 Pa. Frangos et al. (1985) suggested the production of PGI_2 by a vascular endothelium under physiological flow conditions might be significantly higher than that under static conditions.

Polycarbonate plate

Silicone gasket

Culture dish with cell monolayer

Figure 2. Details of flow chamber.

In this paper, we mainly focused on the weakly adherent 293 cells and studied the effect of shear stress and attachment factors on the adhesion and productivity of the cells quantitatively by using shear stress exposing equipment. We also observed the morphologies of the adhesion plaques formed by 293 cells through scanning electron microscopy and compared the characteristics of 293 cells with those of strongly adherent animal cells such as Vero and human liver cells (PLC/PRF/5).

Materials and methods

Cell line and culture conditions

Three anchorage-dependent cell lines were used in these experiments. Human embryonic kidney cells (293: CRL-1573) were obtained from Flow Laboratories (McLean, VA, USA). Monkey kidney cells (Vero: JCRB0111) was obtained from Japanese Cancer Research Bank, and human liver cells (PLC/PRF/5) was

kindly gifted by Dr. N. Koide of the Okayama University school of medicine. 293 and Vero cells were grown in Eagle's minimum essential medium (MEM). PLC/PRF/5 cells were grown in Williams' medium E (WE). Both media were supplemented with 2 to 10% (v/v) fetal bovine serum (FBS, Hazleton Research Products, Lenexa, KS, USA). All cells were grown on 9 cm diameter polystyrene dish (LUX, Sanko Purechemical Co., Tokyo, Japan) for 400 to 600 h in static culture under 5% CO₂ and 95% air at 37 °C. After growing to the confluent monolayer, the culture dishes were used in the experiments. 24 x 30 mm coverslips (LUX, THERMANOX™ 5412) made by polystyrene were used to take scanning electron microscope images. The surface of the coverslip has the same characteristics to that of the culture dish.

Attachment factors

The attachment factors used in this experiment were collagen type-I (Nitta Gelatin Co., Osaka, Japan), fibronectin (Iwaki Glass Co., Tokyo, Japan), vitronectin (Iwaki Glass Co.) and gelatin (Sigma). The coating concentration of the factors to the culture dishes and coverslips were 2, 10 and 50 $\mu\text{g cm}^{-2}$ for collagen; 6.3, 31, 63 and 630 ng cm^{-2} for fibronectin; 16 ng cm^{-2} for vitronectin, and 10 $\mu\text{g cm}^{-2}$ of gelatin. The concentrations of fibronectin and vitronectin used were based to the method described by Bodary et al. (1990). 1.2 ml of each solution described above was added to culture dishes and the coverslips resting in the bottom of the dish. The coated culture substrate was dried for 8 to 12 h in clean bench. The 293 cells suspended in MEM medium supplemented with 2% FBS were then inoculated into the dishes.

Shear stress exposing system and flow chamber

The shear stress exposing equipment designed by Frangos et al. (1985) was adjusted to fit a 9 cm diameter culture dish. Figure 1 shows that the shear exposing system consisting of two reservoirs situated one above the other, and a chamber where the cells are subjected to shear stress placed between them. The hydrostatic pressure head created by the vertical distance between the upper and lower reservoirs drove medium flow. The flow rate was controlled by adjusting the relative distance between the two reservoirs. The medium in the lower reservoir is circulated to the upper reservoir by a roller pump to maintain a constant pressure head. During the experiments, the culture dish was maintained at 37 °C by a warm plate (Kitazato

Figure 3. Microphotographs of 293 cells exposed to the shear stress. A: before exposure to shear stress, B: after exposure to 0.1 Pa for 16 hr, C: after exposure to 0.1 Pa for 24 hr.

Supply Co., Shizuoka, Japan) and the medium pH was maintained at the optimum level by gassing with a humidified mixture of 95% air and 5% CO₂. Total volume of media in the system was 70 ml.

The flow chamber consists of a machine-milled polycarbonate plate, a rectangular silastic gasket of 500 μm in thickness, and the culture dish as shown

Figure 4. Changes in adhesion ratio of various cell lines with 2% FBS under 0.10 Pa shear stress. (●) 293 cell, (Δ) Vero cell, (□) PLC/PRF/5 cell.

Figure 5. Effect of FBS concentration on the adhesion of 293 cells exposed to 0.10 Pa shear stress. (■) 2%, (○) 4%, (□) 10%, (Δ) 10% → 2%.

Figure 6. Effect of attachment factors on the adhesion of 293 cells exposed to 0.10 Pa shear stress and 2% FBS. (■) No coating, (□) Vitronectin 16 ng cm^{-2} , (Δ) Collagen $2 \text{ } \mu\text{g cm}^{-2}$, (●) Gelatin $10 \text{ } \mu\text{g cm}^{-2}$.

in Figure 2. The depth of the channel formed was $500 \text{ } \mu\text{m}$, and the shear exposing area was 9.12 cm^2 . These shapes were kept together by a vacuum, forming a channel of parallel plate geometry. This chamber was placed on the stage of a microscope in order to observe cell morphology and cell number.

The calculation of shear stress exposed on the surface of cells was carried out by using momentum balance for Newtonian fluid and assuming parallel-plate geometry:

$$\tau = \frac{6\mu Q}{bh^2}$$

where Q is medium flow rate [$\text{m}^3 \text{ s}^{-1}$], μ is the medium viscosity at 37°C [$7.4 \times 10^{-4} \text{ Pa}\cdot\text{s}$], b is shear exposing channel width [$1.9 \times 10^{-2} \text{ m}$] and τ is wall shear stress [Pa]. Cell adhesion ratio was defined by the following equation.

Cell adhesion ratio [%] =

$$\frac{\text{Cell number after exposing shear stress}}{\text{Initial cell number}} \times 100$$

The flow chamber dimensions were $0.5 \times 19 \times 48 \text{ mm}$ (height (H) $\times$ width (W) $\times$ length (L)). The Reynolds number in these experiments was kept under 3.5 to maintain laminar flow. The velocity profile seemed to be fully developed since the entrance length was 0.14% of the length of the channel.

Analyses

During exposing shear stress to the cells, microphotographs were taken at the different five points in the chamber at a certain exposing time. Cell adhesion ratio was estimated by counting the cell number in the photographs. The viable cell concentration was determined by the trypan blue dye exclusion method after trypsinization of the shear-exposed cells. Morphological changes were observed with a microscope and a SEM. The 293 cells secreted t-PA into the medium (Kawakubo et al., 1994) and the t-PA productivity was evaluated by measuring t-PA activity with the chromogenic synthetic substrate S-2251 (H-D-Val-Leu-Lys-pNitroanilin-2HCl, Kabi Vitrum, Stockholm, Sweden) according to the method of Verheijen et al. (1982).

Procedure of a scanning electron microscopy (SEM)

After exposing shear stress to the cell monolayer on the coverslip that was placed on the chamber, the coverslip was taken out of the chamber and rinsed by filtrated CMF-PBS. The rinsed coverslips were soaked in 3% glutaraldehyde solution in CMF-PBS and incubated for 15 h at 37°C to fix the cells, and dehydrated with graded t-butanol solutions (70, 80,

Figure 7. SEM photographs of 293, Vero and PLC/PRF/5 cells. A; 293 cell in static culture for 302 hours, B; Vero cell in static culture for 125 hours, C; PLC/PRF/5 cell in static culture for 125 hours. l; lamellipodia, m; microspike.

90, 100%) on the warm plate at 37 °C. After removing the solution, the slip was kept at 4 °C for 5 to 10 min and treated with suction drying. Finally, the cells were sealed with silver paste on the specimen stub of a SEM and gold-palladium sputtered and subsequently observed in the SEM.

Figure 8. SEM photographs of 293 cell cultured on vitronectin coating dish. B is an expansion of A.

Results

Effect of the shear stress on the each cell lines

Figure 3 shows microphotographs of 293 cells exposed to 0.1 Pa of shear stress. As shown in these photographs, cell number decreased gradually with increase of exposure time. From these photographs, the cell adhesion ratios were calculated as described in Materials and methods. Figure 4 shows the effects of 0.10 Pa shear stress on the adhesion of Vero, PLC/PRF/5, and 293 cells to the polystyrene culture dish with 2% FBS. The adhesion ratio of the 293 cells decreased with the increased exposing time to the shear stress; the adhesion ratio decreased to 50% after 29 h. On the other hand, the adhesion ratio of the other cell lines hardly changed under the same

Figure 9. Changes in cumulative t-PA secretion by 293 cells under different shear stress 293 cells were cultured in MEM medium supplemented with 2% FBS. (●) 0.00 Pa (Static), (○) 0.03 Pa, (□) 0.05 Pa, (■) 0.10 Pa, (△) 0.70 Pa.

conditions. The adhesion force of the 293 cells was obviously weaker than that of PLC/PRF/5 and Vero cells. However, the viability of the 293 cells remaining in the chamber after 40 h of 0.10 Pa shear stress was over 90%.

Effect of the FBS concentration and the attachment factors on the adhesion of 293 cells

The effect of the FBS concentrations on the adhesion of 293 cells was investigated. The adhesion ratio of 293 cells markedly increased with increasing FBS concentration in the medium (Figure 5). When the FBS concentration was decreased from 10% to 2% after 300 h of culture period, the adhesion ratio increased and was higher than the cells maintained in 2% FBS throughout the entire culture period. These results suggest that the attachment factors present in the serum affected the adhesion ratio. Some of these attachment factors (fibronectin, vitronectin, collagen and gelatin) were then directly investigated.

The fibronectin did not increase the attachment of 293 cells since the cells detached from the fibronectin-coated culture dish during the early logarithmic phase of cell growth in the static culture (data not shown). Collagen type-I, vitronectin and gelatin were more effective in increasing cell attachment. Collagen concentrations over $10 \mu\text{g cm}^{-2}$ caused the formation of cell aggregates and the cells didn't grow to conflu-

ence as a monolayer (data not shown). The collagen concentration used for 293 cells was decided to be $2 \mu\text{g cm}^{-2}$. Figure 6 shows the effect of attachment factors on the adhesion ratio of 293 cells exposed to 0.10 Pa shear stress in MEM medium with 2% FBS. The vitronectin (16 ng cm^{-2}) was more efficient in increasing attachment as compared to collagen ($2 \mu\text{g cm}^{-2}$) and gelatin ($10 \mu\text{g cm}^{-2}$). Although the concentration of the vitronectin was about one-hundredth that of collagen, the vitronectin induced an increase in adhesion force.

Observation of cells with SEM

The results described above indicate that the adhesion force of the cells as well as the attachment mechanism between the cell and the culture substratum depend on the cell line. Figure 7A, B and C show the SEM photographs of 293, Vero and PLC/PRF/5 cells in static culture, respectively. The area of the lamellipodia of the Vero and PLC/PRF/5 cells was larger than that in 293 cells. Also, Vero and PLC/PRF/5 cells have many microspikes, while the 293 cells do not have as many. This cellular attachment morphology of 293 cells seems to cause the weakness of the cell adhesion.

The attachment factors, especially vitronectin, increased the adhesion force of the 293 cells (Figure 6). Figure 8 shows the SEM photographs of the 293 cells on vitronectin coating dish. The microspikes from la-

Figure 10. Changes in t-PA secretion rate of 293 cells under different shear stress 293 cells were cultured in MEM medium supplemented with 2% FBS. (●) 0.00 Pa (Static), (○) 0.03 Pa, (□) 0.05 Pa, (■) 0.10 Pa.

mellipodia were well developed and anchored on the surface of the dish compared with non-coated dish (Figure 7A). These microspikes might be the reason for the increase in the adhesion force of the 293 cells.

The morphology of the 293 cells changed during exposure to shear stress. A similar observation was made with Vero cells. The Vero cells, which had strong adhesion force, had a triangular shape in the confluent static monolayer. When the cells were exposed to shear stress, the cells became more spherical in shape. The SEM images of the shear-exposed Vero cells showed a decrease in the number of the microspikes (data not shown).

Effect of shear stress on t-PA production of the 293 cells

Figure 9 shows the changes in cumulative t-PA secretion by the 293 cells under shear stresses less than 0.70 Pa in MEM supplemented with 2% FBS. The 0 Pa indicates the static culture on the polystyrene dish. The t-PA secretion increased in proportion to shear stress exposure time between 0 to 0.10 Pa of shear stress. Above 0.10 Pa, however, the t-PA secretion decreased markedly; for a shear stress of 0.70 Pa, secretion has practically stopped. Interestingly, the t-PA secretion was stimulated most by a low shear stress of 0.03 Pa.

Figure 10 shows t-PA secretion rate of 293 cells under 0 to 0.10 Pa of shear stress. As indicated in

Figure 9, 0.03 Pa shear stress increased the t-PA secretion rate of the cells, however, the stimulation was transient, and the rate decreased gradually and reached at the almost same level with static culture after 20 h of exposure to shear stress.

Effect of the attachment factors on the t-PA secretion of the 293 cells

Figure 11 shows the effect of the attachment factors on the t-PA secretion of the 293 cells exposed to shear stress of 0.10 Pa with 2% FBS. The secretion by the 293 cells grown with attachment factors was obviously higher than without attachment factors. Most importantly, vitronectin stimulated the t-PA secretion twice as much as the secretion without attachment factors under a shear stress of 0.10 Pa.

Figure 12 indicates the t-PA secretion rate of 293 cells cultured on the attachment factors coated dishes under 0.10 Pa of shear stress. As mention above, vitronectin enhanced the rate (4.8 IU/10⁶ cells/day) of 293 cells at 4 times higher level compared with static culture of non-coated dish at 7 h of exposure. After that the rate decreased, but even though after 40 h of exposure, twice higher secretion rate of t-PA was maintained in the case of vitronectin-coated dish compared with that of non-coated dish. We also checked the rate of the cell in static culture on the vitronectin-

Figure 11. Effect of the attachment factors on the cumulative t-PA secretion of 293 cells exposed to shear stress of 0.10 Pa with 2% FBS. (■) No coating, (□) Vitronectin 16 ng cm⁻², (Δ) Collagen 2 μg cm⁻², (○) Gelatin 10 μg cm⁻², (●) No coating and static.

coated dish. The value (2.3 IU/10⁶ cells/day) was lower than that under 0.10 Pa.

Discussion

The adhesion forces of the anchorage-dependent mammalian cells vary according to cell line. The number of cellular adhesion plaques such as lamellipodia and microspikes seems to cause the difference in the adhesion force for various anchorage-dependent cells. In fact the number of lamellipodia and microspike was found to vary in each cell line through SEM observation. The weakly adherent 293 cell does not have many lamellipodia and microspike compared with Vero and PLC/PRF/5 cells. On the vitronectin-coated dish, the lamellipodia and microspikes of the 293 cell were well developed. The vitronectin seems to induce the organization of integrin subunits for the cell attachment, because the integrin which is a superfamily of cell surface receptors provides a transmembrane link between the cell and the extracellular matrix such as vitronectin and collagen (Phillips et al., 1991). Therefore the vitronectin was effective for increasing the adhesion force of the 293 cells.

The cellular products were mentioned to be affected by the shear stress of the medium fluid. Stathopoulos and Hellums (1985) reported the maximum release of the urokinase by the human embryonic kid-

ney cells was obtained at the shear stress of 0.65 Pa. The 293 cells used in this experiment showed the maximum productivity of t-PA to be under 0.03 Pa on the non-coated dish and 0.10 Pa on the vitronectin-coated dish, respectively. The maximum productivity (4.8 IU/10⁶ cells/day) under 0.10 Pa with vitronectin was higher than that (2.3 IU/10⁶ cells/day) in static culture with vitronectin. Although the value of the shear stress was lower than that in Stathopoulos et al., these results indicate the shear stress affects cellular production. The mechanism of the enhancement effect of shear stress on productivity was not clear yet, but cytoskeletal organization connected with integrin and adhesion plaques and membrane fluidity seem to be related to protein secretion process inside the cells. The higher shear, however, decreased the t-PA productivity of the 293 cells. The animal cells must then be protected from shear stress, and the adherent force should be enhanced by using attachment factors to increase the efficiency for the production of useful biologicals, especially for weakly adherent animal cells such as 293 cells.

We have recently developed a simulation technique for 3-dimensional flow in agitated bioreactors by using finite element analysis. We reported that the maximum shear stress of 0.095 Pa was caused at the tip of a two-bladed paddle in a typical 250-ml spinner flask under 60 rpm and the maximum shear stress of 0.108 Pa was also found at the tip of 4 pitched bladed paddle in 6-L

Figure 12. Effect of the attachment factors on the t-PA secretion rate of 293 cells exposed to shear stress of 0.10 Pa with 2% FBS. (□) No coating, (○) vitronectin 16 ng cm⁻², (●) gelatin 10 μg cm⁻², (■) no coating and static.

jar under 60 rpm (Funatsu et al., 1997). These results suggest that 0.10 Pa of shear stress loaded on the cells in this experiment is a representative value for a typical agitated bioreactor.

In this paper, we gathered quantitative data on the effects of shear stress and attachment factor on cell adhesion and productivity. In the future, by using these quantitative data and simulation technique for 3-dimensional flow, we can design a more suitable impeller and bioreactor, and find the best operating conditions such as agitation speed and concentration of attachment factor to enhance production of biologicals from shear stress-sensitive weakly adherent animal cells.

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