Cell Adhesion Strength on Chemically Modified Substratum

Kimyasal Olarak Modifiye Edilmiş Yüzeylerde Hücre Yapışma Kuvveti

Ali YILDIRIM

Abstract

The adhesion strength of cultured Chinese Hamster Lung (CHL) cells on various concentrations of fibronectin or polylysine coated tissue culture dishes were measured by using the convergent Microflow chamber. The amount of adsorbed fibronectin was about 70% as it was measured by using iodinated fibronectin (^{125}I-Fn).The effect of fibronectin was concentration dependent from 0.1 μg to 25 μg/dish, while above 25 μg, increasing the fibronectin concentration did not significantly increase the critical shear stress (c.s.s) of detachment of CHL cells. Hence the c.s.s. of detachment of CHL cells, which were grown in serum free medium for 24 hours at 37°C, were 7.60±0.90 Nm^{-2}, 9.56±1.62 Nm^{-2}, 15,60±1.75 Nm^{-2} and 16.02±2.48 Nm^{-2} on 0.1 μg, 10 μg, 25 μg and 50 μg fibronectin coated dishes respectively. Preadsorbed fibronectin was also able to increase the cell adhesion strength even in the presence of serum in the growth medium. That is, the c.s.s. of detachment was 16.79±2.48 Nm^{-2} on dishes with 25 μg fibronectin while it was 9.40±0.60 Nm^{-2} on noncoated dishes. Like fibronectin, the poly-D-lysine effect was also concentration dependent. Thus the c.s.s. of detachment of CHL cells (grown in serum free medium) were 2.80±0.30 Nm^{-2}, 4.11±0.54 Nm^{-2}, 6,10±0.82 Nm^{-2} and 9.94±0.58 Nm^{-2} on 0 μg, 5 μg, 10 μg, and 25 μg poly-D-lysine coated dishes respectively.

Key words: cell adhesion, fibronectin, polylysine, CHL cells

Özet

Değişik konsantrasyonlarda fibronektin veya polylysine ile kaplanmış doku kültür kaplarında kültür edilmiş Chinese Hamster Lung (CHL) hücrelerinin yapışma kuvveti düzgün ve giderek hızlanan bir sıvı akış kabiliyetine sahip Microflow Chamber yardımı ile ölçülmüştür. Adsorbe olmuş fibronektin miktarı iyotlanmış fibronektin (^{125}I-Fn) kullanılarak %70 olarak belirlenmiştir. fibronektin etkisi 0.1 μg/kap ile 25 μg/kap arasında konsantrasyona bağlı idi. Bununla beraber 25 μg dan daha fazla miktarda konsantasyon artırma CHL hücrelerinin yüzeyden koparılmaları için uygulanması gerekli kritik kuvveti (c.s.s) önemli ölçüde artırmadı. Bu nedenle serumsuz ortamda ve 0.1 μg, 10 μg, 25 μg ve 50 μg fibronektin kaplı kaplarda 37°C de 24 saat inkübe olmuş hücreleri koparmak için gerekli kuvvetler, 7.60±0.90 Nm^{-2}, 9.56±1.62 Nm^{-2}, 15,60±1.75 Nm^{-2} ve 16.02±2.48 Nm^{-2} idi. Doku kültür kaplarına önceden fibronektin adsorpsiyonu, hücre büyüme ortamında serum olması durumunda da hücre yapışma kuvvetini artırabiliyordu. Dolayısıyla 25 μg fibronektin ile kaplı kaplardaki hücreleri koparmak için uygulanması gerekli c.s.s. 16.79±2.48 Nm^{-2} iken bu değer normal doku kültür kaplarında 9.40±0.60 Nm^{-2} idi. Fibronektinde olduğu gibi, poly-D-lysine etkisi de konsantrasyona bağlı idi. Bundan dolayı serumsuz besi ortamında 0 μg, 5 μg, 10 μg, and 25 μg poly-D-lysine ile kaplanmış doku kaplarında 37°C de 24 saat inkübe olmuş hücrelerin koparılması için gereki c.s.s. değerleri sırasıyla dishes 2.80±0.30 Nm^{-2}, 4.11±0.54 Nm^{-2}, 6,10±0.82 Nm^{-2} and 9.94±0.58 Nm^{-2} idi.

Anahtar sözcükler : hücre adezyonu, fibronektin, polilizin, CHL hücreleri

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INTRODUCTION

Cell-substrate adhesion is a very complex process involving extracellular matrix (ECM) proteins, cell surface receptors for these proteins and a complex interplay of extracellular proteins, membrane proteins and cytosolic proteins (1-8). However, the mechanism of cell substratum adhesion can be subdivided into a number of steps. These are; protein adsorption on the surface from the medium, the contact of the cell with the surface bound proteins and finally, attachment. From this protein the cell spreads and grows until division where it rounds up and divides (9-12). Therefore in the presence of serum in the culture medium, the serum proteins irreversibly bind and denature onto the surface (13-15) and the cell interacts with an interface of previously adsorbed proteins rather than the original form of substrate (10, 16-18).

However, serum contains a mixture of many adhesive and non-adhesive proteins (19-22). In order to further define the role of individual proteins, purified forms of adhesive proteins can be used in adhesion studies. Normal plastic tissue culture dishes were therefore modified with a specific protein, fibronectin, or polylysine and studies undertaken to determine the effect of this modification upon cell adhesion. Cells usually bind to fibronectin and other adhesive proteins such as vitronectin via a receptor-mediated mechanism that confers specificity to cell-protein interactions (22-27). Whereas ,cells are able to bind to polylysine, which is positively charged, mainly via a non-specific interaction (28). Consequently, the effect of a polylysine-coating on cell adhesion strength was also determined by using the convergent Microflow chamber.

MATERIALS AND METHODS

Cell Culture: CHL cells were obtained from Flow lab and were maintained in Eagles minimum essential medium (EMEM), with Earls salt, supplemented with 20mM HEPES buffer, 10% v/v fetal calf serum, 200I.U penicillin, 20µg streptomycin, 2mM glutamine and 2% (w/v) non essential amino acids. Cells were incubated at 37 °C in a 5% CO_2/air (v/v) atmosphere (29).

Detachment Assay: 20 millilitres of cell suspension which contained $5x10^5$ cells/ml were poured into tissue culture grade polystyrene dish and cells were incubated for 24 hours at above conditions Then the cell growing substratum is subjected to a defined hydrodynamic flow in the convergent Microflow chamber for 10 minutes. Therefore the critical distance at which cells start to detach was measured. By inserting this magnitude of the measured critical distance and flow rate in the equation:

$$\pi = \frac{13.15V}{73 - L} \frac{(N)}{(m2)}$$

in where;

π= the shear stress (Nm^{-2}).

V= volumetric flow rate (cm^3s^{-1}), L= critical distance (mm).

the adhesion strength of the cell can be determined as the critical shear stress (css) of detachment (Nm^{-2}) (30).

Fibronectin Coating: Fibronectin coating was performed as described previously (31). Briefly, 1mg of lyophilised bovine plasma fibronectin (Flow lab) was dissolved in 1 ml of sterilised double distilled water at room temperature in a laminar flow cabinet. The required concentrations of fibronectin were dissolved in 10 ml of sterilised double distilled water. The resulting solution was poured into 100 mm diameter tissue culture grade plastic dishes. Fibronectin from this solution was allowed to adsorb on the plastic dishes and water was evaporated overnight. The dried dishes were washed twice with double distilled water and once with PBS immediately before seeding the cells. Control dishes were prepared in an identical manner except that the first incubation was in 10 ml double distilled water without fibronectin.

Quantification of Fibronectin Adsorption: 0.5 ml of 1µCi/ml ^{125}I-fibronectin (5.3µCi/µg) in the form of a solution was obtained from Flow laboratories and the same day this solution was made up to 10 ml with double distilled water to give final concentration 1µci/ml. 0.5 ml of this solution was added per well of a 24 well tissue culture grade dish and allowed to adsorb overnight. The water was evaporated and each well was washed twice with 0.5 ml of double distilled water. Both washings were pooled together.

The coated ^{125}I-fibronectin was extracted from the surface by washing twice with 0.5 ml of 1M NaOH. Each extraction lasted for half an hour. Ex-

tractions and washings were counted separately for 2-10 minutes on the gamma counter (19). Therefore the amount of fibronectin adsorbed onto the dish (%) was determined.

Poly-D-Lysine Coating: 5mg of poly-D-Lysine Mw 300,000 (Sigma) was dissolved in 10 ml of PBS. The required amount was transferred into 10 ml of double distilled water. The resulting solution was poured into 100 mm diameter tissue culture grade plastic dishes. After leaving overnight in a laminar flow cabinet, dried dishes were washed three times with double distilled water and once with PBS immediately before seeding cells (32).

3. RESULTS AND DISCUSSION

The Effect of Pre-adsorbed Fibronectin on the Adhesion strength of CHL Cells.

In order to define the role of fibronectin in adhesion strengthening, CHL cells were subcultured on fibronectin-coated dishes in serum-free medium. As a control, CHL cells were also seeded on non-coated dishes, again in serum-free medium. In all cases, the CHL cells were allowed to grow for 24 hours, before the c.s.s. of detachment was measured.

The effect of fibronectin on the strengthening of CHL cell adhesion was concentration dependent. Nevertheless, even a very low amount of fibronectin significantly increased the strength of cell adhesion. The c.s.s. of CHL cell detachment was 139% higher on dishes coated with 0.1 µg fibronectin, in the absence of serum, than on non-coated dishes; the c.s.s. values were 7.60±0.9 Nm^{-2} and 3.20 0.60 Nm^{-2}, respectively (figure 1). The cell adhesion strength increased with an increasing adsorbed fibronectin concentration. For example, the. c.s.s. of detachment of CHL cells were 9.56±1.62 Nm^{-2} and 15.60±1.75 Nm^{-2} on 10 µg and 25 µg fibronectin-coated dishes, respectively (P=0.000). While increasing the concentration of adsorbed fibronectin above 25 µg did not change cell adhesion strength significantly: the c.s.s. of detachment of CHL cells was 16.02±2.48 Nm^{-2} on 50 µg fibronectin-coated dishes (P=0.74, between 25 µg and 50 µg fibronectin coated dishes). It was interesting to find that the c.s.s of detachment of CHL cells grown in serum-free medium on dishes coated with 25 µg fibronectin was significantly higher than that of cells grown on non-coated dishes in serum-containing medium. Hence the c.s.s. of detachment was 16.79±1.03 Nm^{-2} on dishes coated while it was 9.40±0.60 Nm^{-2} on non-coated dishes.

The results obtained in response to pre-adsorbed fibronectin indicate that even very low amounts of fibronectin (e.g. 0.1 µg/dish) are able to significantly strengthen cell adhesion (see figure 1). However, because the fibronectin was only adsorbed to the plastic,

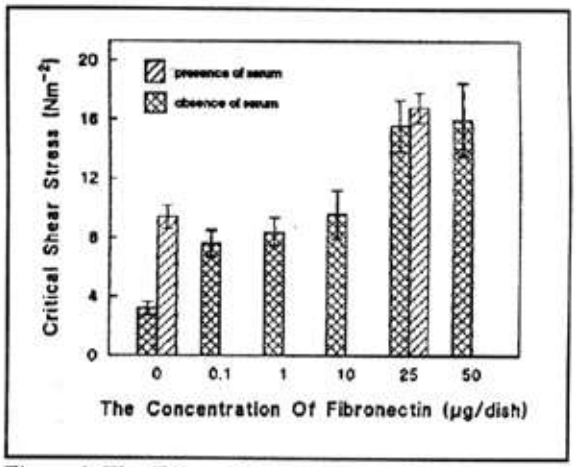

Figure 1: The Effect of Pre-Adsorbed Fibronectin on CHL Cells Adhesion Strength.
20 millilitres of cell suspension which contained $5x10^5$ CHL cells/ml were poured into various concentrations (0.1 to 50µg) of fibronectin-coated plastic tissue culture dishes, each of which had a 72 cm^2 surface area. The cells were allowed to grow for 24 hours, either in a serum-containing or serum-free medium. The adhesion strength of these cells in terms of the critical shear stress (c.s.s.) of detachment were measured in the Micro Flow Chamber. Each data point represents the mean of three different experiments, in each of which ten measurements were made.

it was not known how much of it remained fixed to the surface after extensive washing. Iodinated fibronectin (^{125}I-Fn) was therefore employed to determine the amount of adsorbed fibronectin and it was found that about 70% (68.68%±3.99%) of the added fibronectin was adsorbed to the surface.

The Effect of Pre-adsorption of Poly-D-Lysine on the Adhesion Strength of CHL Cells.

The results indicate that pre-adsorption of polylysine strengthened the adhesion strength of CHL cells significantly. For example, the c.s.s. of detachment of CHL was 2.80±0.30 Nm^{-2} and 4.11±0.54 Nm^{-2} on non coated and 5 µg polylysine coated surfaces , respectively (P=0.0001). The adhesion strength of CHL cells increased as the amount of pre-adsorbed polylysine increased up to 25µg.

However, increasing the concentration of the coated polylysine beyond this value did not increase cell adhesion strength significantly. Hence the c.s.s. of detachment of CHL cells was 9.94±0.57 Nm^{-2} and 10.90±0.88 Nm-2 0n 25 µg and 50 µg polylysine coated surfaces, respectively (P=0.020). Unlike fibronectin, the adhesion strength of CHL cells on polylysine coated surface was not affected significantly by the presence or absence of serum in culture medium. Therefore the c.s.s. of detachment of CHL cells on 25 µg polylysine coated surfaces was 9.94±0.57 Nm^{-2} and 9.82±0.85 Nm^{-2} in the serum free medium and serum containing medium, respectively (P=0.72).

DISCUSSION

It was interesting to find that cells were able to increase their adhesion strengths significantly on even 0.1µg preadsorbed fibronectin. Therefore, it is possible to suggest that fibronectin is acting as an activator rather than a mediator. However, it would be wrong to simply assume that adhesion increases with an increasing number of bonds because the receptor is not a simple passive antenna-like component whose function is restricted to recognising certain molecules. Since cells are bound to ligands through their transmembrane receptors, the binding of a receptor to a ligand will send a message to the interior of cell; the cell would act according to this signal and possibly be able to perform further processes (12,26,33). In fact it has been reported that a signalling mechanism mediates fibronectin-enhanced adhesion strength (34,35). Signals can be generated in adhesion by adhesion receptors. Cell contact with the substratum causes receptor clustering; this clustering at the site of contact generates signals and these signals can regulate adhesion (36,37). There are two different views on the mechanism of integrin-mediated signalling. The first one is that integrins are true receptors capable of giving rise to biochemical signals within the cell (38,39). In this case, the effects on cytoskeleton are mediated by small molecules such as cAMP (40). Increasing cAMP concentration increase protein kinase activity and, consequently protein phosphorylation (41-43) which will strengthen cell adhesion (44,45). The second view is that integrins transmit signals by organising the cytoskeleton, thus regulating the shape and internal cellular architecture of the cell (18,33,37,46). The determination of the signalling mechanism was not an aim of this study, however, from the above reports it is possible to suggest that fibronectin binding via receptors signals the cell to strengthen cell adhesion.

Although the fibronectin effect was concentration dependent, increasing the fibronectin concentration above 25µg did not have a significant effect on the cell adhesion strength. Therefore, it is possible to suggest that at this fibronectin concentration the CHL cells gained a maximum possible adhesion strength and increasing the amount of fibronectin would not make a significant change to the CHL cell adhesion strength. Interestingly, we have obtained similar results with collagen type IV (47). It has also been reported that the adhesion of 3T3 cell was maximal at 10ng fibronectin cm^{-2}, and that above this concentration adhesion was independent of fibronectin concentration Truskey and Pirone (48).

At concentrations of fibronectin of 25 µg or above, almost all of the receptors might be occupied by fibronectin molecules. Therefore, at these higher concentrations (cells were spreading very well, figure 3) cell adhesion strength was even greater due to both increased receptor-ligand bonds and activation. Hence, it could be said that an increase in the concentration of ligands leads to an increase in the number of receptor-ligand bonds that will eventually

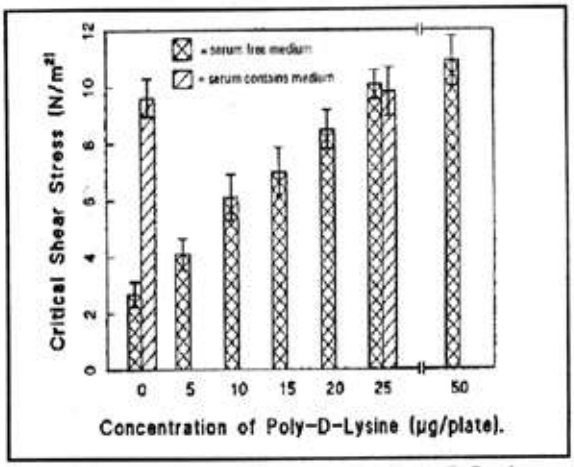

Figure 2: The Effect of Pre-Adsorbed Poly-D-Lysine on CHL Cells Adhesion Strength.
20 millilitres of cell suspension which contained $5x10^5$ CHL cells/ml were poured into various concentrations (5 to 50µg) of polylysine coated plastic tissue culture dishes, each of which had a 72 cm^2 surface area. CHL cells were grown on these polylysine coated dishes for 24 hours. The adhesion strength of cells in terms of the critical shear stress (c.s.s.) was measured by using Microflow Chamber. Each data point represents the mean of 30 different determinations, the error bars indicate the standard error of that mean.

trengthen cell adhesion (49-51). In the detachment process bonds formed between the cell and the substratum will be pulled apart or broken by the applied force (35,52); hence, as the number of bonds increases, the force required to break the cell-substratum linkage will also increase (53).

It was interesting to find that, when the cells were grown in serum-containing medium, cell adhesion strength was significantly higher on dishes pre-adsorbed with fibronectin than on non-coated surfaces. This could be due to that in medium containing 10% foetal calf serum, only 38ng cm^{-2} fibronectin was adsorbed on tissue culture dishes (54), whereas in this study 243ng cm^{-2} (25 µg dish-1) fibronectin was pre-adsorbed. It is therefore probable that cell adhesion was lower on non-coated dishes than on fibronectin-coated dishes because less fibronectin was adsorbed to the former.

The adhesion strength of CHL cells on surfaces pre-adsorbed with 25 µg fibronectin was not significantly affected by the presence or absence of serum in the growth medium (c.s.s. 16.79±1.03 N m^{-2} and 15.50±1.75 N m^{-2}, respectively; P=0.23). This could suggest that once fibronectin-activated events have begun and the sequence of events leading to the final adhesion strength is initiated, then the adhesion strength is independent of the presence of serum.

Although cells were not spreading even on 50 µg polylysine coated surfaces (figure 4), cell adhesion was strengthened by the pre-adsorbed polylysine. However, CHL cells were spreading very well on fibronectin coated dishes (figure 3). Therefore it is possible to suggest that increasing of cell adhesion strength on the above substratums have different

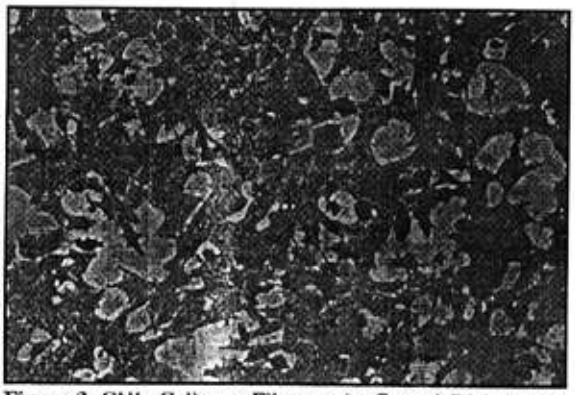

Figure 3: CHL Cells on Fibronectin Coated Dish in the Absence of Serum.

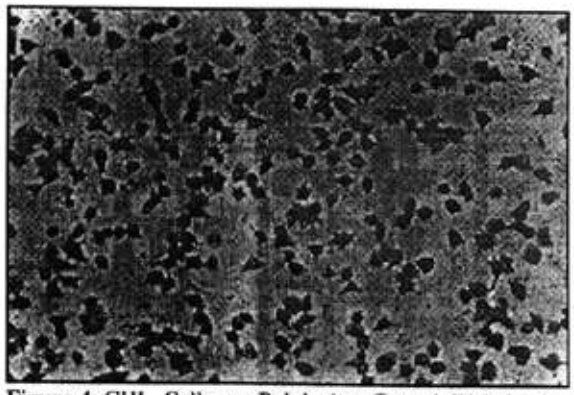

Figure 4: CHL Cells on Polylysine Coated Dish in the Absence of Serum.

mechanisms. On fibronectin case, cells adhere to fibronectin with fibronectin receptors which is followed by tyrosine phosphorylation and focal adhesion (44,55) these could lead to cell spreading and strengthening of cell adhesion (12). Whereas, as polylysine treated surfaces present positively charged surfaces while cell surface has negative charges, cells adhere to polylysine through electrostatic interactions (32,56). Therefore non-specific cell substrate adhesion take place regardless of the availability of the receptors and complementary ligands (28) on which tyrosin phosphorilation and focal adhesion does not take place (55,56). Thus, adhesion strength increases as the density of the adsorbed polylysine increases and hence increases the number of ionic bonds between cell and substratum (57). Thus it seems possible to suggest that most of the cell surface negative charges were occupied on 25 µg polylysine coated surfaces therefore increasing of density of polylysine did not increase cell adhesion strength significantly.

In the present study adhesion strengths of CHL cells on fibronectin coated surfaces were compared with that cells were adhered to the polylysine coated dishes by Microflow chamber. Cells adhere to it with specific integrin receptors which allow cells to perform adhesion process completely. Hence cells were able to spread and strengthen cell adhesion, However, cells adhere to the polylysine via electrostatic interactions on which unlike adhesion to fibronectin is not followed signalling and activation of kinases. Thus cells were not spreading on polylysine and adhesion strength is could be solely dependent the number of electrostatic interactions between negative charges of cells and positive charges of polylysine.

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