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The Role of Protein Synthesis in the Adhesion of Chinese Hamster Lung Cells

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Abstract: In order to determine the effect of endogenous protein synthesis on CHL cell adhesion, the initial attachment of these cells to tissue culture dishes was measured in the presence of 1µgml^{-1} emetine, in which protein synthesis was inhibited by 97% as determined by labeling the cells with [^{35}S]-methionine. In the presence of this drug, 80% of cells were able to adhere after 2 hours of incubation at 37^{o}C, while, during the same period, 97% of the control cells became attached. Cells strengthen their adhesion following initial attachment, spreading and growth. Hence, the adhesion strengths of CHL cells that were grown for 24 hours in the presence of cycloheximide, or emetine, or both were measured quantitatively using a converging laminar flow chamber. The inhibition of protein synthesis by the above drug(s) reduced cell adhesion strength. Hence, the levels of critical shear stress of detachment of the cells were 8.18±1.03Nm^{-2}, 6.70±0.30Nm^{-2}, and 5.66±.0.36Nm^{-2}, respectively, while that of the control cells was 10.48±0.78Nm^{-2}. Before subculturing CHL cells in the presence of these drug(s), pre-treatment of the cells with the same protein synthesis inhibitor further reduced the cell adhesion strength. Thus, with a pre-treatment of cycloheximide, emetine and both emetine and cycloheximide the levels of critical shear stress of the detachment of cells were 4.84±0.54Nm^{-2}, 4.20±0.2Nm^{-2}, and 3.4±0.7Nm^{-2}, respectively. These results indicate that cells not require protein synthesis only for initial cell attachment, but also to gain the maximum possible adhesion strength.

Key Words: Protein synthesis, emetine, cell adhesion.

Introduction

Cell substrate adhesion is a very complex process involving extracellular matrix (ECM) proteins, cell surface receptors for these proteins, and intricate interplay between extracellular proteins, membrane proteins, and cytosolic proteins (1 - 5).

When a cell suspension in a serum containing medium is poured into a culture dish the first reaction is that the serum proteins bind and denature onto the dish surface (6 - 8). Such adsorption of proteins to surfaces is largely irreversible and much more rapid than contact of the cell on surfaces. Therefore, cells interact with an interface of previously adsorbed proteins rather than the original form of substrate (9-12). Following cell contact with the protein coated substratum, if there are receptors for these proteins on the cell surface and if the conformation of the adsorbed proteins is not altered by adsorption so as to destroy the high ligand-receptor affinity, then cell attachment will take place (13-15). After adhesion, the cell secretes its own proteins, which mix together with the preadsorbed serum proteins to form an extracellular matrix. This matrix forms the foundation for cell spreading and adhesion strengthening (16-18)

The role of endogenous proteins in cell adhesion could be studied using inhibitors of protein synthesis such as cycloheximide or emetine (19). Although the role of endogenous proteins in various cells has been investigated in the past, most published studies deal solely with initial cell attachment (20) or cell morphology (i.e. whether cells are spreading or not) (21). However, the present study describes the use of a Microflow chamber to investigate not only the relationship between endogenous proteins and initial cell attachment, but also the role of endogenous proteins in cell adhesion strength.

Materials and Methods

Cell Culture

Chinese Hamster Lung (CHL) Cells which were obtained from Flow Laboratories were routinely cultured in minimum essential medium Eagles (modified with earls salt) MEM (Flow Laboratories). To obtain a ten-fold final dilution of culture medium, MEM (x10 concentration) was diluted in sterile HEPES (BDH Chemicals) buffer. These diluted media were supplemented with 10% (v/v) foetal calf serum (Globepharm limited Surrey), 200I.U penicillin/l, 200mg/l streptomycin, 2mM Glutamine and 2% (w/v) non essential amino acids (22).

Determination of Protein Synthesis Inhibition

CHL cells were placed in a 24-well tissue culture plate in the presence or absence of emetine or cycloheximide (Sigma; 0 to 1 µg/ml). The cells were allowed to attach to the wells for 2 hours and were metabolically labeled by the addition of 0.5mCi of L-[^{35}S]- methionine (Dupont Chemicals) to each well. The incorporation was followed over a period of 6 hours. At times ranging from 0 to 6 hours, the labeled medium was carefully removed and each well was washed twice with PBS. The cells were then dissolved in 0.5 ml of 0.1M NaOH, which instantly digested the cells. To this mixture, 2 to 3 ml of ice cold 10% TCA was added and the reaction was left to proceed overnight at 4^{o}C. The precipitated samples were then filtered through a GF/C disc (previously washed with 2 ml ice cold 5% TCA and finally with 2 ml of 95% ethanol). The discs were placed in a scintillation vial and dried at 60^{o}C. After drying, 3 ml of scintillant (Optiphase) was added to each vial. The samples were evaluated in a Packard Tri-carb liquid scintillation counter (23).

Measurement of Cell Attachment

Sub-confluent cells were trypsinized after which trypsin was inhibited by the addition of 2ml (for each 25 cm^2 flask) of the growth medium. The density of the cells was determined by means of a Neubauer hemocytometer. Then a stock cell suspension which contained 5x10^5 cells/ml was prepared by diluting the above cell suspension with growth medium. Following this, 2ml of the latter cell suspension was placed in 35 mm round tissue culture dishes, which were incubated at 37^{o}C. Finally, after the incubation period, the culture medium was transferred into a tube and all unattached cells were removed by gentle washing with serum-free medium. Any cell not removed by this series of gentle washes was considered to be attached. The numbers of attached and non-attached cells were counted in an haemocytometer. In the case of inhibition of protein synthesis, the attachment assay was performed in growth medium supplemented with 1µgml^{-1} emetine.

Measurement of Cell Adhesion Strength

Cell adhesion strength was measured as described previously (24). In brief, a sub-confluent monolayer of cells was trypsinized with 0.05% (v/v) trypsin in EDTA-PBS, after which the trypsin was inhibited with serum-containing culture medium. Then, 20 ml of the resulting cell suspension (2x10^5 cells/ml) was transferred into 90mm tissue culture grade polystyrene dishes. Afterward, the cells were allowed to grow for 24 hours in the conditions defined above. The adhesion strength of the cells was measured by inserting this cell-growing substratum on the convergent Microflow chamber (a detailed diagram is given in a previous study, 24) and after passing the running medium, which was culture medium minus serum, for 10 minutes over the test substratum at a defined flow rate, the critical distance (the distance from the beginning of the test section to the point at which cells start to come off) was measured. By inserting this value and the flow rate in the equation:

$$\tau = \frac{13.15 \times V}{73\text{-}L} \qquad (24)$$

where:

τ= Shear stress (N/m^2) ;

V= Flow rate (ml/s);

L= Critical distance (mm)

The equation below is obtained by modifying the equation of shear stress in the flow channel

$$\tau = \mu x \frac{du}{dy} \qquad (25)$$

where:

τ = shear stress;

μ = viscosity of fluid;

du = the velocity of fluid;

dy = the depth of the channel.

Results

The Determination of Effective Dose of Emetine or Cycloheximide for Protein Synthesis Inhibition

The inhibition of cellular protein synthesis by emetine or cycloheximide was determined by labeling the cells with [^{35}S]-methionine. It was observed that protein

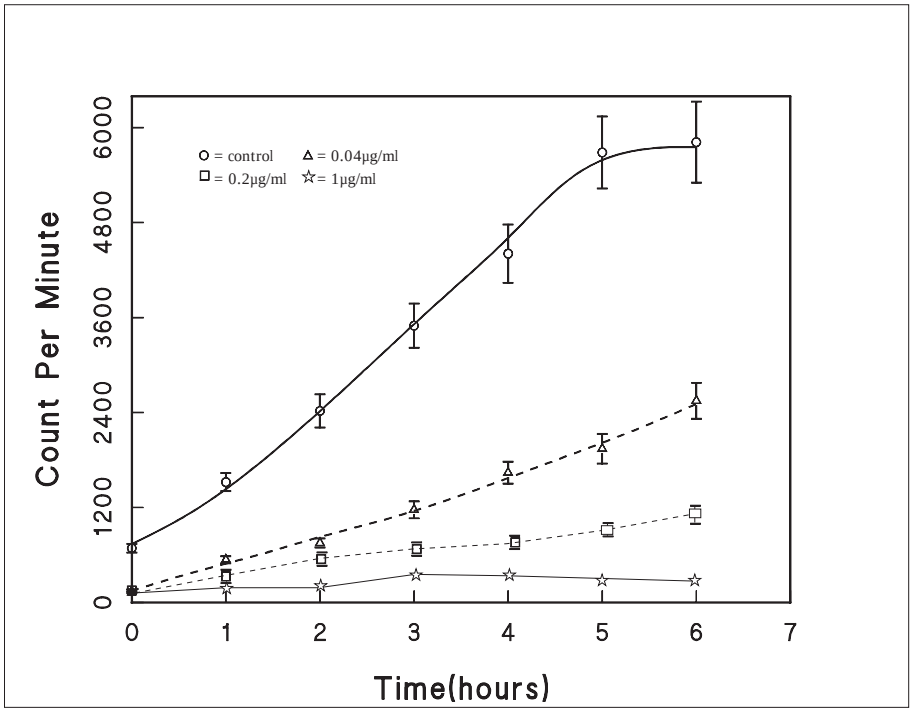

Figure 1. Protein Synthesis in CHL Cells in Response to Cycloheximide.

Adherent cultured CHL cells were plated at a density of $5x10^5$ cells ml^{-1} in the presence or absence of indicated drug and allowed to attach to the 24-well tissue culture plate for 2 hours. At this stage the cells were metabolically labeled with [^{35}S]-methionine, incorporation being followed over a period of 6 hours. Each data point represents five different experiments; in each experiment the effects of different concentrations of drug were examined in triplicate. Each error bar indicates the standard error of the mean.

synthesis was inhibited by 97% and 95% in the presence of 1µg ml^{-1} emetine and cycloheximide, respectively (Figures 1 and 2). The different counts per minute observed in the control cells in Figures 1 and 2 are due to the fact that the experiment with emetine was done as soon as [^{35}S]-methionine was received, while in the case of cyloheximide experiment was performed after a period of time of obtaining [^{35}S]-methionine. It was interesting to observe that even after five hours, residual protein synthesis was maintained response to the above drugs (3% and 5% of the original protein synthesis in the presence of emetine and cycloheximide, respectively). In addition, it was determined that in the presence of 1µg ml^{-1} of either drug in the growth medium, the growth of the CHL cells was completely halted (data not shown). By combining the results from the growth experiments with those from the biosynthetic labeling study, it was concluded that 1µgml^{-1} of either drug was an appropriate concentration with which to inhibit protein synthesis.

The Role of Protein Synthesis in CHL Cell Attachment

To evaluate the role of protein synthesis in CHL cell attachment, emetine, a specific protein synthesis inhibitor, was used (26,27). As stated above, 1µg ml^{-1} emetine inhibits 97%±2% of CHL cell protein synthesis within 5 hours (Figure 2). However, it has been reported that cultured cells contain protein pools, so that even if protein synthesis is totally inhibited, a cell may continue to secrete proteins from these pools (28). Hence, to be able to remove proteins secreted even in the presence of emetine, cells were grown in normal complete medium until the mid-log phase and this medium was then replaced with fresh complete medium that also contained 1µgml^{-1} emetine. Incubation was continued for a further 6 hours at 37^{o}C, after which the cells were trypsinized and the attachment assay performed in 1µgml^{-1} emetine-containing medium.

There was a marked reduction in cell attachment in

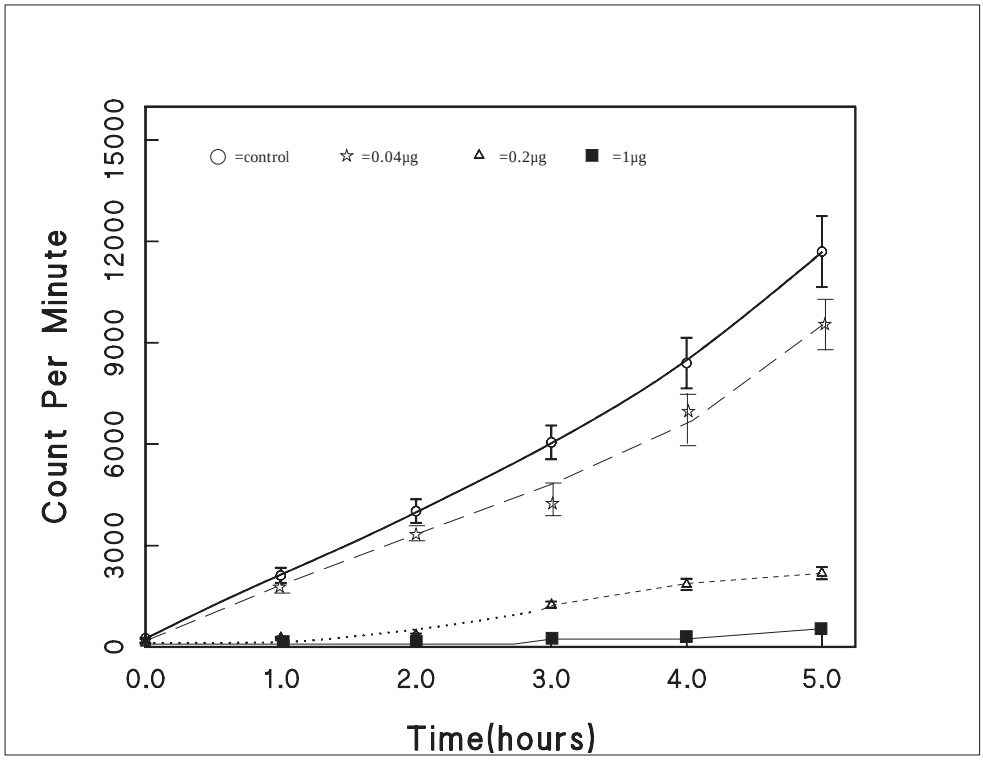

Figure 2. Protein Synthesis in CHL Cells in Response to Emetine.

Adherent cultured CHL cells were plated at a density of $5x10^5$ cells ml^{-1} in the presence or absence of the indicated drug and allowed to attach to the 24-well tissue culture plate for 2 hours. At this stage the cells were metabolically labeled with [^{35}S]-methionine, incorporation being followed over a period of 5 hours. Each data point represents five different experiments; in each experiment the effects of different concentrations of drug were examined in triplicate. Each error bar indicates the standard error of the mean.

the presence of emetine. In 20 minutes only 8.5±2% of the cells attached, whereas 67±6.5% of the control cells attached during the same period. After one hour of incubation, although there was a statistically significant difference ($p=0.0005$) between the amount of attachment in the presence and absence of emetine (75±4.5% and 93±3%, respectively), the difference was not as large as that observed after a 20-or 30-minute incubation period. Nevertheless, after 2 hours, 80±5.6% of the emetine treated cells attached, while the percentage of attached control cells was 97±2%. From the above results it could be said that although inhibition of protein synthesis delayed cell attachment in the initial period of incubation, most of the cells were able to attach within 2 hours of incubation.

The Effect of Emetine and Cycloheximide on the Adhesion Strength of CHL Cells.

In order to determine the role of protein synthesis on the adhesion strength of CHL cells, two approaches were taken:

1. Sub-confluent cells were trypsinized, and the trypsin then inhibited using a serum-containing medium with 1 µg ml^{-1} emetine, cycloheximide or both of these drugs added. The cells were then seeded on tissue culture surfaces in identical media and the adhesion strength of these cells measured after 24 hours incubation using a Microflow chamber.

2. In order to avoid the possible secretion of cellular proteins from protein pools, the medium of sub-confluent cells was replaced with medium containing 1µgml^{-1} emetine, cycloheximide or both of these drugs, and after six hours of incubation (chosen because, as demonstrated using [^{35}S]-methionine labeling, 1µgml^{-1} emetine or cycloheximide inhibits protein synthesis within 5 hours) with the drug-containing medium, the cells were trypsinized and treated as described above. The inhibition of protein synthesis by cycloheximide or emetine reduced the adhesion strength of the CHL cells, the critical shear stress of detachment for the cells being 8.18±1.03 N m^{-2} and 6.70±0.30 N m^{-2}, respectively, while that of the

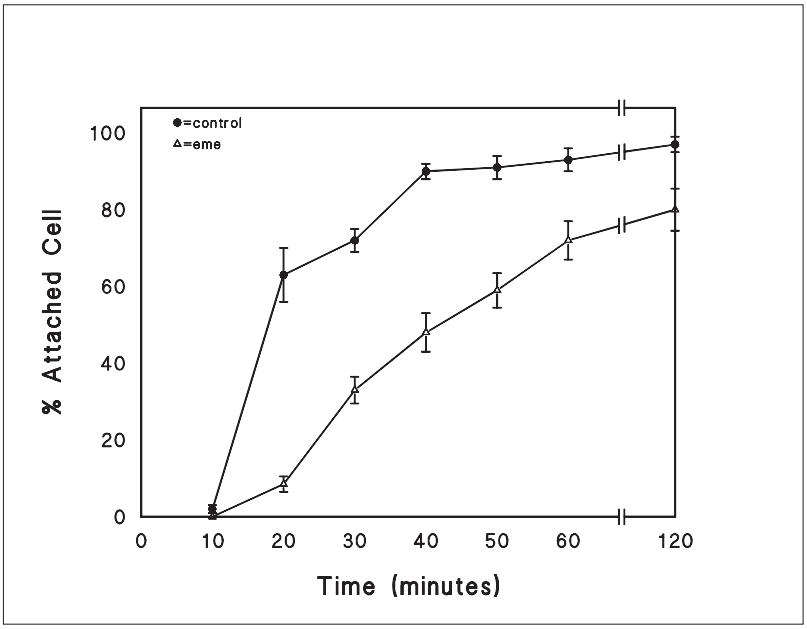

Figure 3. The Effect Of Emetine on the Attachment of CHL cells

Sub-confluent CHL cells were pre-treated with 1µgml^{-1} emetine for 6 hours before trypsinization. After trypsinization, the cells were seeded on 35mm tissue culture grade dishes in medium containing 10% fetal calf serum in the presence of 1µgml^{-1} emetine. Control cells were seeded in the absence of emetine without pretreatment. At the time points indicated, the cell attachment was measured. Each data point represents the mean of five different experiments.

control cells was 10.48±0.78 N m^{-2}. In other words, cycloheximide treatment reduced the cell adhesion strength by 21%, while it was reduced by 36% in the presence of 1µg ml^{-1} emetine. It was significant that the presence of 1µg ml-1 of both emetine and cycloheximide further reduced cell adhesion strength: the c.s.s was 5.66±0.36 Nm^{-2} (45% inhibition). The pre-treatment of CHL cells with the above drugs resulted in a further reduction in adhesion strength. For example, cycloheximide reduced adhesion by 53% when the cells were pre-treated for 6 hours before trypsinization and seeded in a medium containing 1 mg ml^{-1} cycloheximide. Without pre-treatment, as indicated above, cell adhesion strength was reduced by 21% in the presence of cycloheximide. The c.s.s. of detachment of the CHL cells was 4.84±0.54 Nm^{-2} and 8±1.03 Nm^{-2} with and without cycloheximide pre-treatment, respectively - a statistically significant difference (P=0.0007). A similar pre-treatment effect, i.e. further reduction in cell adhesion strength, was observed for media containing emetine alone or both emetine and cycloheximide (Figure 4). (Statistical calculations were carried out using Minitab software version 8.2)

Discussion

The results of this study indicate that not only initial cell attachment, but also strengthening of cell adhesion require cellular protein synthesis. In this study, we were able to show in quantitative terms the role of protein synthesis in the adhesion strength of cells.

From the attachment results it may be said that although inhibition of protein synthesis delayed cell attachment in the initial period of incubation, most of the cells were able to attach within 2 hours of incubation. This was surprising because it is commonly believed that cells adhere to the substratum via cell surface proteins, adhesion receptors (13,14,15,29). This poses the question: how do cells attach to a surface if they are unable to synthesize the necessary proteins? The answer could be that since protein synthesis is not completely inhibited (97%), a residual amount of protein synthesis is sufficient for cells to carry out attachment. An alternative explanation could be that only a few of the adhesion proteins are involved in adhesion at any one time, and although many may be broken upon trypsinization, many others are still available, either whole or in subunit form

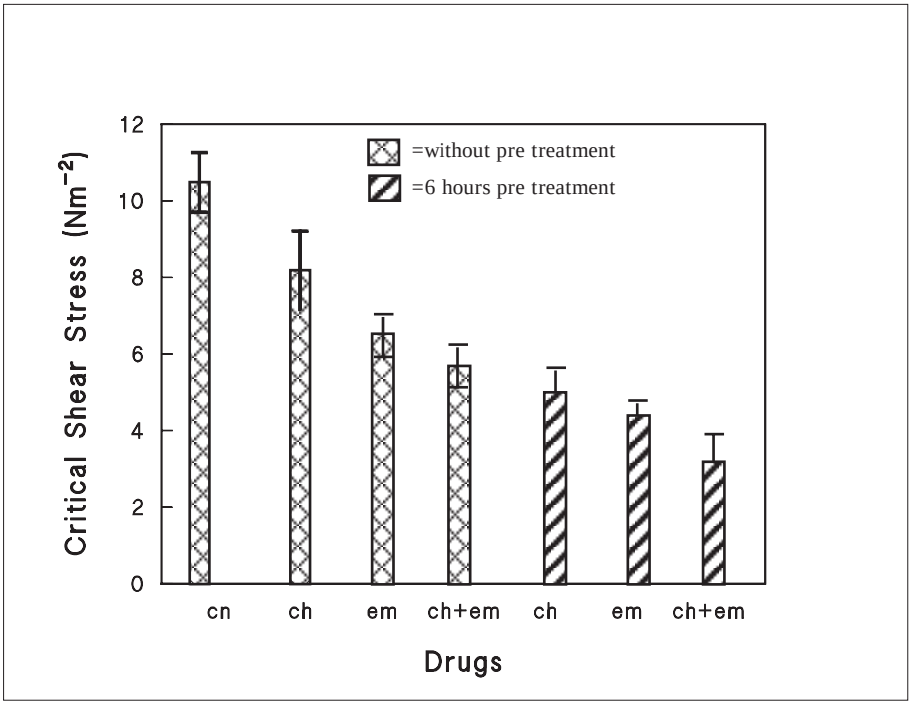

Figure 4. Adhesion Strength Of CHL Cells on Plastic in Response to Cycloheximide (ch) or Emetine (em) or cycloheximide plus emetine (ch+em); cn = control.

CHL cells (either pre-treated with the indicated drug for 6 hours or with no pre-treatment) were inoculated in the culture medium containing 1µgml^{-1} drugs or without drugs. After 24 hours of incubation the adhesion strength of the cells was measured in terms of the critical shear stress (c.s.s.) of detachment. The error bars indicate the standard error of five different experiments in each of which ten measurements were made. Each error bar represents the standard error of the mean.

(30). A final explanation could be that a cell can attach to a substratum without the need for specific cell surface proteins (31).

Although the explanations above are speculative, we found that the effect of protein synthesis inhibition on CHL cell attachment was less than might have been expected. However, it has been reported that inhibition of protein synthesis in 3T3 cells with emetine did not affect cell attachment, suggesting that the initial process of the adhesion did not require protein synthesis (31). In contrast to our results and those of Kolodony (30) it has been reported that inhibition of protein synthesis, by cycloheximide, actually increases adhesion of Ehrlich-Lettre hyperdiploid ascites carcinoma (EAT) cells to plastic surfaces (32). A similar observation was also made by Antoni et al. (33) using emetine and thymic cells. Both reports suggested that the increased adhesion was due to the inhibition of anti-adhesion protein synthesis by these drugs. Unlike adhesive cellular proteins, for example fibronectin (34), anti-adhesive proteins (e.g. tenascin, thrompospondin and SPARC) can interfere with cell-substrate adhesion (35). These anti-adhesion proteins may exert their effects in different ways. For example, they may interfere with the interaction between integrin receptors and adhesive proteins (36,37). Another possible mechanism for anti-adhesion proteins is a simple steric interference: these proteins adsorb to the surface and in doing so prevent the subsequent adsorption of adhesive proteins (38). However, recently it has been reported that SPARC acts via interactions with cell surface molecules rather than by steric or physical distruction of integrin-extracellular matrix ligands (39).

In the present study, 97% of protein synthesis was inhibited and, in contrast to the findings of Antoni et al. (33), the adhesion of CHL cells was reduced in the presence of emetine. These contrasting results may be due to the different cell systems used, which possibly behave differently during the attachment process. In the present study, protein synthesis inhibition reduced initial cell attachment but did not prevent it completely.

Inhibition of cellular protein synthesis also reduced

adhesion strength. In the presence of both emetine and cycloheximide, further reduction in the adhesion strength might suggest that these two drugs act synergistically to inhibit protein synthesis. In fact, it is known that these two agents inhibit different stages of protein synthesis: cycloheximide primarily acts on the initiation stage of synthesis, while the elongation stage is most sensitive to inhibition by emetine (27). Hence, one might expect a mixture of these two drugs to be more effective in reducing the strength of cell adhesion. The pre-treatment of CHL cells with the above drugs resulted in an even further reduction in adhesion strength. These results support the theory that the cell contains protein pools which are used in the absence of cellular protein synthesis (28). It is therefore possible to suggest that when protein synthesis is inhibited, the cell uses these proteins to perform its limited adhesive function. In the case of pre-treatment, the cell might use up most of its stored proteins during the treatment period. Hence, in the latter case, cell adhesion strength was significantly lower than in the former case. This point was reinforced by Flickinger and Culp (20), who reported that the spreading of human fibroblasts on collagen was inhibited after 18 hours of pre-treatment. It was suggested that, after this long period of incubation, cells could deplete collagen receptors. Nevertheless, as indicated above, not all cellular proteins promote cell adhesion. Some of these proteins have negative effects on cell adhesion (36) and it may be that, during inhibition of protein synthesis, the inhibitory effect of anti-adhesive proteins is more pronounced. In fact, Hasselaar et al. (40) reported that the anti-adhesive effect of SPARC (secreted protein acidic rich in cysteine), an anti-adhesive protein, is not blocked by cycloheximide in bovine aortic endothelial (BAE) cells. Therefore, cell spreading was still inhibited by SPARC.

The present study could suggest that, although cells are able to attach under the inhibition of synthesis of cellular proteins, due to a lack of cellular adhesive proteins and adhesive receptors, they are unable to perform required functions such as signaling, response to these signals, reorganization of cytoskeletal proteins and the formation of focal adhesions. Therefore cell adhesion strength is significantly reduced by these drugs.

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