Cytoskeletal rearrangement in K562 erythroleukaemic cells forced to grow on a positively charged polymer surface

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We have recently demonstrated that if human K562 erythroleukaemic cells, which normally grow in suspension, are grown on a positively-charged surface composed of polylysine, a transient reorganization of CD54 (ICAM-1), CD58 (LFA-3) and $\alpha v\beta_3$ (vitronecin receptor), three important CAMs located on the cell membrane, takes place. In addition, changes of longer duration in membrane conductivity (ionic transport across the cell membrane) and membrane permittivity (static distribution of charges across the cell membrane), indicating more permanent structural as well as functional alterations in the cell membrane, were also observed [2]. Because of the close interrelationship which exists between the cell membrane, CAMs and the cytoskeleton, changes in this intracellular network as well as in the surface morphology of K562 cells grown on the positively-charged polymer, polylysine, were examined. In particular, actin and tubulin were investigated qualitatively and quantitatively by immunofluorescence microscopy and flow cytometry, respectively, while the cell surface was studied by scanning electron microscopy (SEM). The data indicate that when K562 cells are grown onto polylysine no quantitative changes occurred to the cytoskeletal elements even if these were rearranged and that the cell membrane surface is also greatly altered. These results are discussed in light of the pivotal role played by CAMs and the cell cytoskeleton in transducing environmental stimuli, in this case those provided by a positive charge, from the cell membrane to the inside of the cell.
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1. Introduction

Understanding the effects that the surface charge of a biomaterial may have on cells is of extreme importance if better materials are to be designed for biomedical use. Since the cell membrane is the first point of contact between a cell and a biomaterial, it is especially important to study these charge effects on this membrane. The cell membrane is a complex, dynamic structure composed of lipids, proteins and carbohydrates. However, of the numerous species found on the cell membrane, the cell adhesion molecules (CAMs) are particularly important with regard to charge interactions. It should be recalled that these charged molecules are directly responsible for cell–cell and cell–substratum adhesion and that they take part in the transduction of environmental stimuli into signals which allow the cell to adapt to its ever-changing environment and survive.

We have recently demonstrated that if human K562 erythroleukaemic cells, which normally grow in suspension, are grown on a positively-charged surface composed of polylysine, a transient reorganization (up to 2 h) of CD54 (intracellular adhesion molecule-1; ICAM-1), CD58 (leucocyte function-associated antigen-3; LFA-3) and $\alpha v\beta_3$ (vitronecin receptor), three important CAMs located on the cell membrane, took place [1]. In addition, changes of longer duration (48 h) in membrane conductivity (ionic transport across the cell membrane) and membrane permittivity (static distribution of charges across the cell membrane), indicating more permanent structural as well as functional alterations in the cell membrane, were also observed [2]. From these data, it was hypothesized that the early redistribution of CAMs is indicative of the passage of signals from the outside to the inside of the cells stimulated from growth of K562 cells onto the positively charged polylysine polymer while the variations in membrane electrical parameters represent some of the structural and functional variations necessary for cell adaptation and survival [1].

The cell membrane is intimately associated with the underlying cytoskeleton. In fact, it is this intracellular

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network which mechanically tranduces the stimuli received by the cell membrane to the rest of the cell. In addition, it has been demonstrated that CAMs, together with other molecules, play an active role in transducing environmental signals through the cytoskeleton [3]. Thus, in view of the results obtained previously and with the above considerations in mind, it was the purpose of the present paper to investigate the possible variations occurring in the cytoskeleton of K562 cells grown on the positively charged polylysine surface. Immunofluorescence analyses of both actin and tubulin demonstrate that, in fact, both of these cytoskeletal components underwent important reorganization with respect to controls when K562 cells were grown on a polylysine surface at all the exposure times tested (30 min, 1 h, 2 h, 24 h and 48 h) while flow cytometric analyses revealed that no quantitative variations occurred at all the times tested. In addition, scanning electron microscopy (SEM) evaluation demonstrated that a well-evident reorganization of the cell membrane also occurred in these cells. The results are discussed in light of the consideration that adaptation and survival of K562 cells when forced to grow onto a positively charged polymer may take place through the orchestrated interrelationships between the cell membrane, CAMs and the cytoskeleton.

2. Materials and methods

2.1. Cells

K562 cells (a human erythroleukaemic cell line which normally grows in suspension) were grown in RPMI 1640 supplemented with 10% fetal calf serum, non-essential amino acids, 100 i.u. ml^{-1} penicillin and 100 μg ml^{-1} streptomycin and incubated at 37 °C in a 5% CO_2 atmosphere. Aliquots of the same batch of frozen cells were thawed and used for all experiments at about the same number of passages (between 15 and 18 passages) so as to make certain that no major variations in the cell populations had occurred between each trial.

2.2. Polylysine exposure

For control samples, cells were counted using a Coulter Counter (Coulter Electronics Ltd, Luton, UK) and seeded in Petri dishes at a concentration of 10^6 cells in 2 ml. For polylysine exposure, glass coverslips were washed and sonicated in ethanol, air dryed, sterilized by autoclave, placed in sterile Petri dishes and coated overnight at room temperature with sterile 0.1 mg ml^{-1} polylysine (hydrobromide, lyophilized form; Sigma, St Louis, MO) dissolved in deionized water (pH 7.4). Coating was performed 24 h before use. Cells to be exposed to polylysine were counted by a Coulter Counter, seeded in the Petri dishes containing the polylysine-coated coverslips at a concentration of 10^6 cells in 2 ml and maintained for 30 min, 1 h, 2 h, 24 h and 48 h at 37 °C in a 5% CO_2 atmosphere. It should be pointed out that within 10 min, K562 cells adhere to the coverslips and that these cells continue to grow in an anchorage-dependent-like manner on the coverslips throughout the entire time period of exposure. For flow cytometric analyses, K562 cells were seeded direcly in Petri dishes covered with sterile polylysine as described above.

2.3. Immunofluorescence labeling

For detection of the cytoskeletal elements actin and tubulin, control K562 cell suspensions were fixed with 3.7% paraformaldehyde for 15 min at room temperature. After washing with phosphate-buffered saline (PBS), the cells were adhered to polylysine-coated cover slips for 10 min and then permeabilized with 0.5% Triton X-100 (Sigma) for 5 min at room temperature. Cells exposed to polylysine by direct growth on polylysine-coated coverslips for 30 min, 1 h, 2 h, 24 h and 48 h were also fixed directly on the coverslips with 3.7% paraformaldehyde for 15 min at room temperature, washed with PBS and permeabilized with 0.5% Triton X-100 for 5 min at room temperature. For actin detection, all samples were stained with fluorescein-phalloidin (Sigma) for 30 min at 37 °C. For labeling of tubulin, incubation with monoclonal antibody (a mixture of monoclonal antibodies against α- and β-tubulin 1 : 1) (Sigma) was conducted at 37 °C for 30 min. After washing, cells were incubated with antimouse immunoglobulin G (IgG) fluorescein-linked whole antibodies for 30 min at 37 °C (Sigma). Samples were then mounted with glycerol/PBS (1 : 1) and analyzed with a Nikon Microphot fluorescence microscope. The percentage of cells with a rearranged cytoskeletal network in the polylysine-exposed K562 cells was determined by counting, at high magnification (500 ×), 10 microscopic fields chosen randomly. At least 500 cells were counted for each sample.

2.4. Flow cytometric analyses

For flow cytometric determination of actin and α- and β-tubulin, control K562 cells and polylysine-exposed cells were collected from the Petri dishes, fixed with 2% paraformaldehyde for 10 min at 4 °C and a 0.05% Triton X-100 solution was added directly for 5 min at 4 °C. The cells were then washed twice with PBS. For actin detection, samples were stained with fluorescein-phalloidin (Sigma) for 30 min at 4 °C. Unstained cells were considered as negative controls. For labeling of tubulin, cells were incubated with monoclonal antibody (a mixture of monoclonal antibodies against α- and β-tubulin 1 : 1) (Sigma) at 4 °C for 30 min, washed with PBS and incubated with antimouse IgG fluorescein-linked whole antibodies for 30 min at 4 °C (Sigma). For negative controls, cells were incubated with the second antibody only. After washing with PBS, all samples were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a 15 mW, 488 nm, air-cooled argon ion laser. Fluorescence emission was collected through a 530 nm band-pass filter and acquired in log mode. The analyses were performed on a Hewlett-Packard model 310 computer interfaced with the FACScan.

2.5. Scanning electron microscopy (SEM)

Control K562 cells were collected from the Petri dishes, centrifuged (1000 ***g*** for 5 min) and fixed in suspension

with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 20 min. After washing twice with the same buffer, the cells were seeded on glass coverslips coated with polylysine for 10 min at room temperature. Treated cells grown directly on glass coverslips for 30 min, 1 h, 2 h, 24 h and 48 h were also fixed with 2.5% for 20 min at room temperature and washed twice with the 0.1 M cacodylate buffer. All samples were post-fixed in 1% osmium tetroxide for 30 min at room temperature, dehydrated through graded ethanols, critical point dried in CO_2 and gold coated by sputtering. The samples were examined with a Cambridge 360 scanning electron microscope.

2.6. Statistical analyses

The statistical significance of the values obtained from the cytofluorimetric analyses was calculated by using the Kolmogorov–Smirnov (K/S) test included in Lysys II software (Becton Dickinson).

3. Results

The possible effects of polylysine on the cell cytoskeleton were studied at different times of growth (30 min, 1 h, 2 h, 24 h and 48 h). As can be seen in Fig. 1, actin filaments were reorganized in cells grown for 30 min on polylysine (Fig. 1c and d) with respect to controls (Fig. 1a and b). In fact, in control cells, the actin filaments have a ring appearance which is typical of cells which grow in suspension (Fig. 1a and b) while in the majority of treated cells this ring structure is lost (Fig. 1c and d). In addition, in treated cells, protrusions are present which are labeled by actin and which give the cells a flattened contour. The same results were also obtained with cells grown for 1 and 2 h on polylysine (data not shown). Cells grown on polylysine for 48 h are shown in Fig. 2. It appears from Fig. 2a and b that the cells are no longer flattened, but rather actin filaments resemble the organization observed in 30-min controls which are identical to 48 h controls. However, in the focal plane where the cells come into direct contact with the polymer surface (Fig. 2c), cell protrusions in which actin is strongly present can still be seen. This rearrangement at the cell base was not noted in controls (Fig. 1b). Similar results were obtained at 24 h of growth onto polylysine (data not shown).

In order to better understand the role of the cytoskeleton, the microtubular network of K562 cells was also investigated. In Fig. 3a are shown control cells in which a ring structure can also be observed while in cells grown for 30 min on polylysine (Fig. 3b) this ring appearance is lost in about 30% of the cells. In cells grown for 1 and 2 h (Fig. 4a and Fig. 4b, respectively),

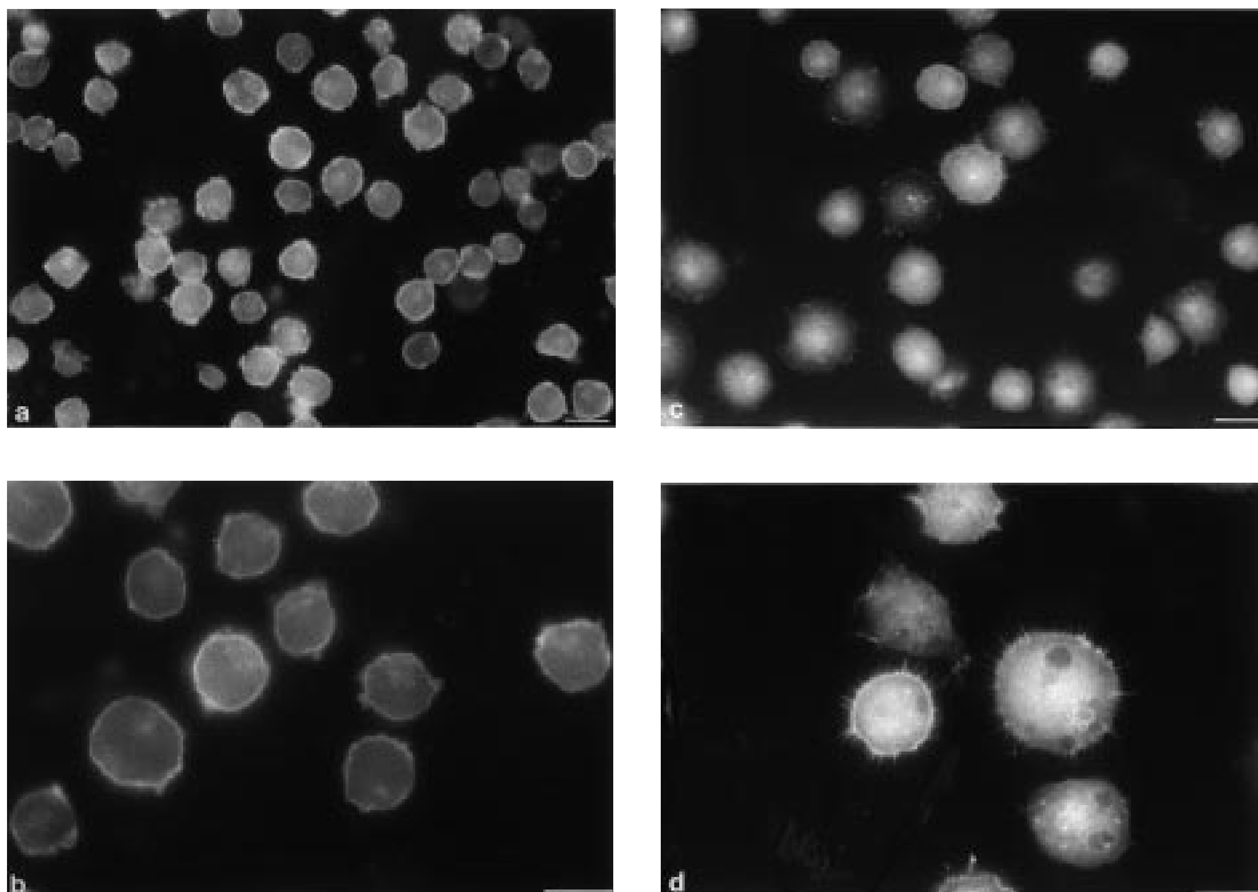
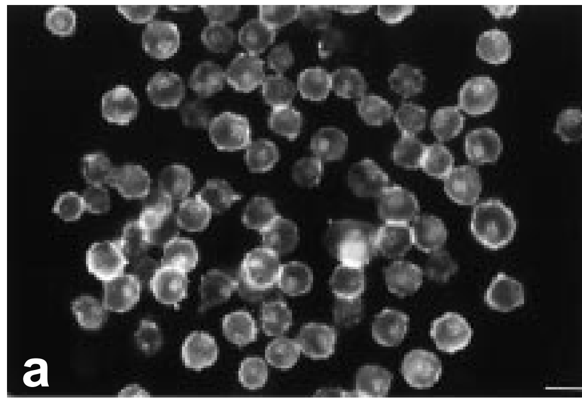
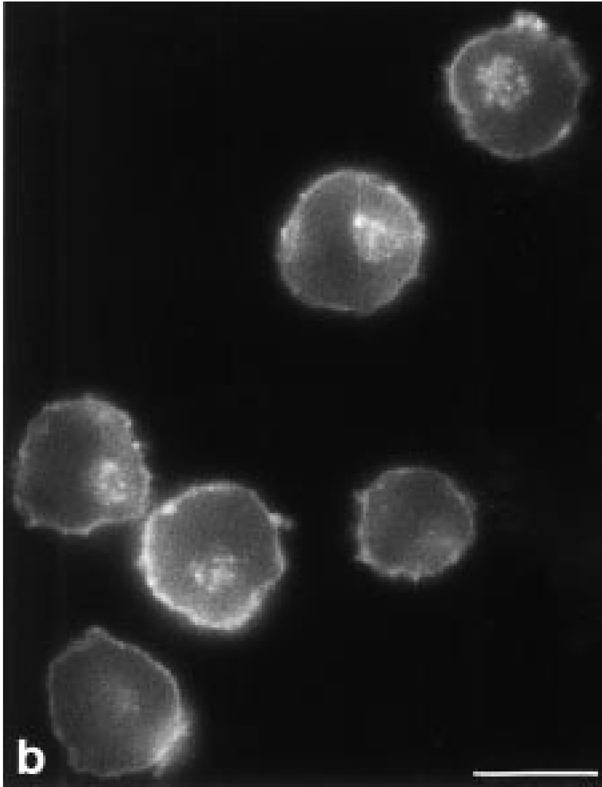


Figure 1 Fluorescence microscopy of control K562 cells (a and b) and of these cells observed after 30 min of growth onto polylysine (c and d). Cells were labeled with phalloidin-FITC to reveal actin microfilament organization. As can be seen, in cells grown onto polylysine for 30 min (c and d) actin filaments appear completely rearranged with respect to controls (a and b). This reorganization is particularly evident at higher magnification (d versus b). The bars represent 10 μm.





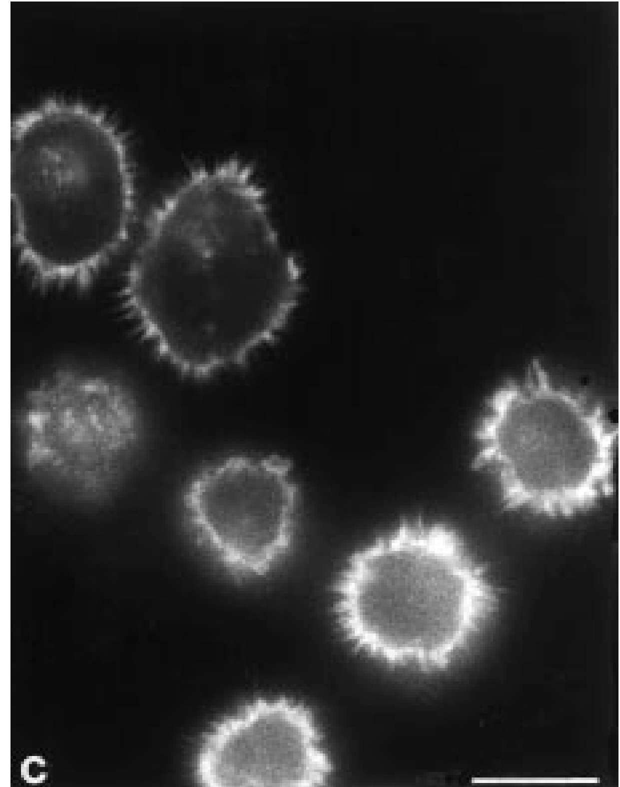
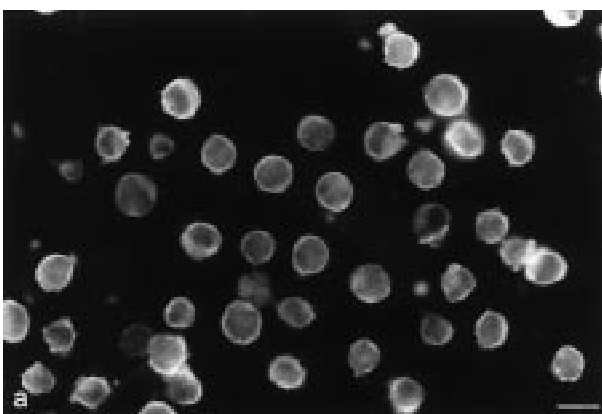


Figure 2 Fluorescence microscopy of K562 cells observed after 48 h of growth on to polylysine labeled with phalloidin-FITC to reveal actin microfilaments. Actin filaments appear very similar to 30-min controls which are identical to those of 48 h (Fig. 1 a and b), but in the focal plane in which the cells come into direct contact with polylysine, protrusions are evident (c). The bars represent 10 μm.



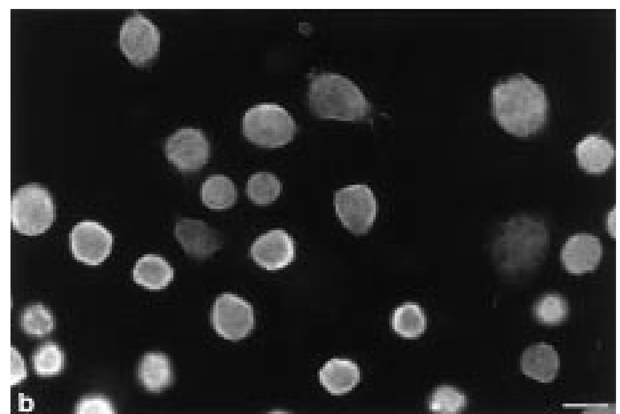


Figure 3 Immunofluorescence microscopy of control K562 cells (a) and of these cells grown onto polylysine for 30 min (b) labeled with antibodies directed against α- and β-tubulin. In the majority of exposed cells (b), the microtubular network is completely modified with respect to controls (a). The bars represent 10 μm.

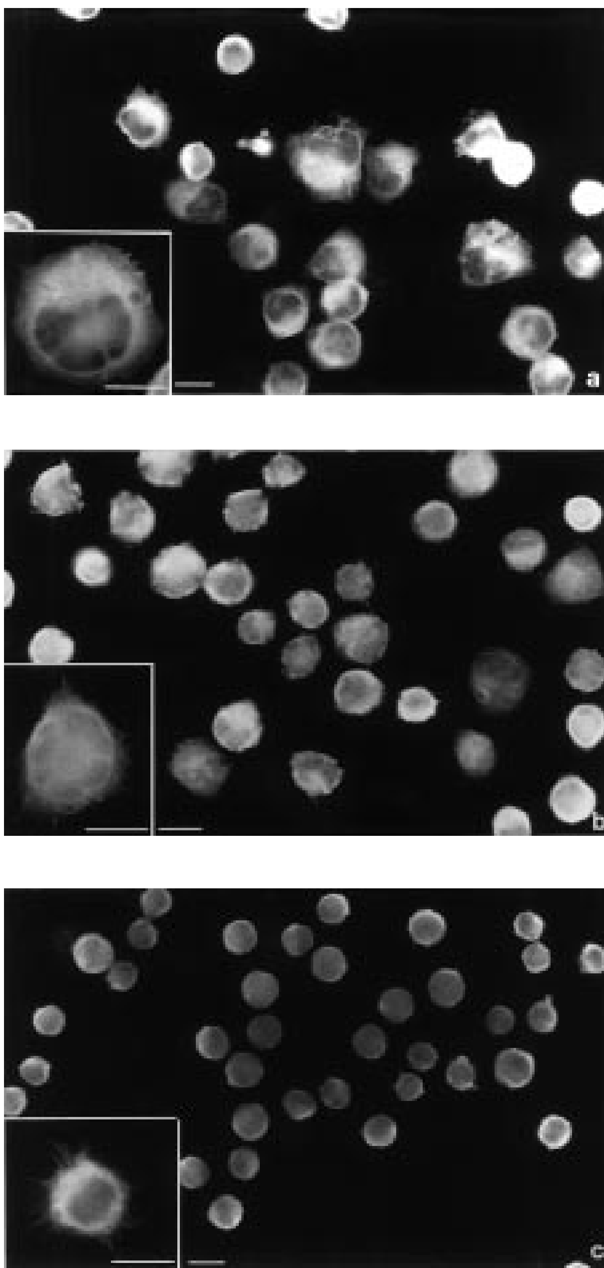


Figure 4 Immunofluorescence microscopy of K562 cells grown onto polylysine for 1 h (a), 2 h (b) and 24 h (c) labeled with antibodies directed against α- and β-tubulin. An evident flattening of the cells can be observed after 1 h of growth onto polylysine (a). In particular, in cells which appear the most flattened, there is a polar patching of tubulin (a, insert). After 2 h of growth (b), tubulin patching is diminished and labeling of tubulin is present at the cell protrusions in the focal plane where cells are attached to the substratum (b, insert). After 24 h of growth (c), tubulin distribution is very similar to that observed in 30 min controls which are identical to those of 24 h (Fig. 3a) even if labeling of tubulin at the cell protrusions in the focal plane of cell/substratum attachment still remains evident (Fig. 4c, insert). The bars represent 10 μm.

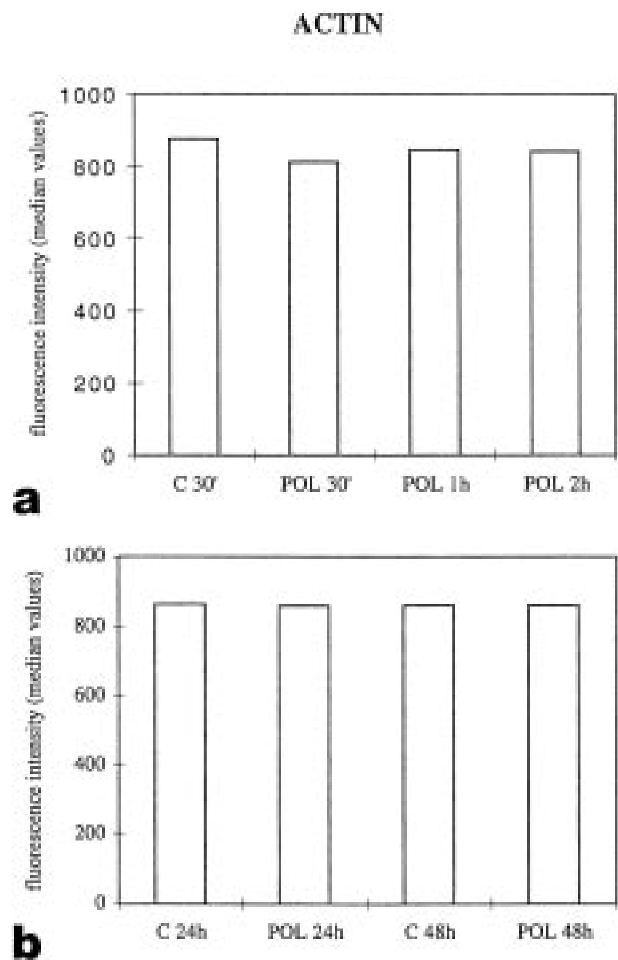


Figure 5 Quantitative analysis of actin microfilament expression performed by flow cytometry on the 30-min control and K562 cells exposed to polylysine for 30 min, 1 h and 2 h (a), and the 24-h and 48-h controls and K562 cells exposed to polylysine for the same exposure time (b). Cells were labeled with phalloidin-FITC to reveal actin microfilaments. Calculation of fluorescence intensity was carried out after conversion of logarithmically amplified signals into values of a linear scale and expressed as median values. No significant differences were revealed between control and polylysine exposed K562 cells ($P < 0.005$). One representative experiment of three is shown.

tubulin appears completely rearranged in about 90% of the cells. In fact, the cells appear very flat with respect to controls (Fig. 3a) after these times of treatment. In addition, after 1 h of exposure, in many of the cells which appear particularly flat, patching of tubulin at a cell pole is present (Fig. 4a, insert). However, after 2 h of growth (Fig. 4b), tubulin patching seems to have diminished and labeling of tubulin is present at the cell protrusions in the focal plane where cells are attached to the substratum (Fig. 4b, insert). After 24 h of growth (Fig. 4c), the situation appears to be completely reversed. In fact, tubulin distribution is very similar to that observed in 30-min controls (Fig. 3a), which are identical to 24-h controls, even if labeling of tubulin at the cell protrusions in the focal plane of cell/substratum attachment still remains evident (Fig. 4c, insert). Similar results were obtained at 48 h of growth of K562 cells onto polylysine (data not shown).

Reorganization of both the actin microfilament and microtubular networks observed by fluorescence microscopy is not accompanied by quantitative variations in their expression as revealed by flow cytometric analyses. Specifically, as can be seen in Fig. 5a, no significant differences in the fluorescence intensity of actin stained with fluoroscein isothiocyanate (FITC)-phalloidin was present between the 30 min control of K562 cells and the cells grown onto polylysine for 30 min, 1 h and 2 h (median values 877 versus 813, 847 and 842,

respectively). In addition, no significant differences were observed between 24-h and 48-h controls and their respective cells treated for the same exposure times (Fig. 5b; 862 versus 858 and 859 versus 860, respectively). Similarly, no significatant differences were detected in the amount of α- and β-tubulin between 30-min controls and cells treated for 30 min, 1 h and 2 h (Fig. 6a; 451 versus 463, 440 and 470, respectively) and between 24-h and 48-h controls and their respective cells treated for the same exposure times (Fig. 6b; 394 versus 414 and 417 versus 450, respectively). Since all the controls (30 min, 1 h and 2 h) are identical only the 30-min control was shown.

Because of the close association between the cytoskeleton and the cell membrane which determines cell shape, the possible changes induced on the membrane surface by different times of growth (30 min, 1 h, 2 h, 24 h and 48 h) of K562 cells on polylysine were investigated by SEM. As can be seen in Fig. 7, it is possible to observe that controls (Fig. 7a) and exposed cells (Fig. 7b, c and d) differ in their surface morphology. In fact, controls appear rounded and have many microvilli on their surface while 30 min of growth onto polylysine (Fig. 7b) induces an evident flattening of the cells with many zones of contact between the cells and the polylysine surface. This flattened appearance supports the same observation made with actin and tubulin described above for cells grown on polylysine for 30 min, 1 h and 2 h. The same SEM results were obtained for cells grown for 1 h and 2 h onto polylysine (data not shown). After 24 h and 48 h (Fig. 7c and Fig. 7d, respectively) of growth onto polylysine, the cells return to a spherical shape very similar to controls although some cells show few microvilli and a surface characterized by ''lamellar-like structures'', especially at 48 h. It should also be noted that many cells at 24 h have numerous small blebs on their surface which are no longer visible at 48 h. This reversal of the blebbing phenomenon seems to indicate a reorganization of membrane structure and not cell damage. The morphological characteristics of cells grown onto polylysine give them an appearance which is dramatically different from that of controls. In addition, these cells have ''pseudopodia-like protrusions'' at the points of cell contact with the surface (Fig. 7c and d, arrows) which are similar to the protrusions noted with actin and tubulin labeling (Fig. 2c and insert of Fig. 4c).

4. Discussion

Cell adhesion plays an important role in numerous biological functions. The cell membrane, the cytoskeleton and CAMs are closely related to the adhesive process and all three participate in transducing environmental stimuli of cell attachment (or lack of this attachment) into signals which allow the cell to survive. In fact, it is widely accepted that cell adhesion receptors function in signal transduction and that the cell cytoskeleton is intimately involved [3–5]. In addition, because of the close connection between the cytoskeleton and CAMs with the cell membrane, this membrane is also involved in adhesion and signal transduction. Forced adhesive growth of K562 cells onto the positively charged model polymer, polylysine, induces important alterations in the cytoskeletal organization as well as the surface morphology of these cells. Thus, because of the interrelationships which exist between the cytoskeleton, the cell membrane and CAMs, the data presented in this report are closely linked to the results obtained previously regarding CAM redistribution [1] and changes in membrane electrical properties [2] observed when these cells were forced to grow on polylysine. In fact, in a previous paper [1], it was hypothesized that the redistribution in CAM expression is an early event in the interaction between K562 cells and polylysine and that this event may be necessary for the continued growth of these cells in their new charge environment. Since

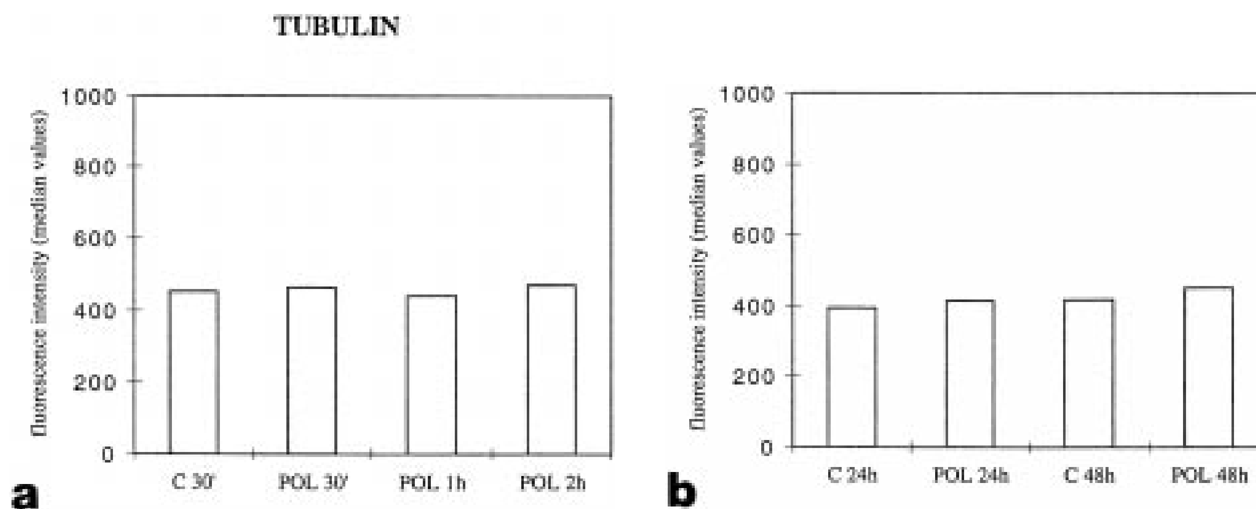


Figure 6 Quantitative analysis of α- and β-tubulin expression performed by flow cytometry on the 30-min control and K562 cells exposed to polylysine for 30 min, 1 h and 2 h (a), and the 24-h and 48-h controls and K562 cells exposed to polylysine for the same exposure time (b). Cells were labelled with antibodies directed against α- and β-tubulin. Calculation of fluorescence intensity was carried out after conversion of logarithmically amplified signals into values of a linear scale and expressed as median values. No significant differences were revealed between control and polylysine exposed K562 cells ($P < 0.005$). One representative experiment of three is shown.

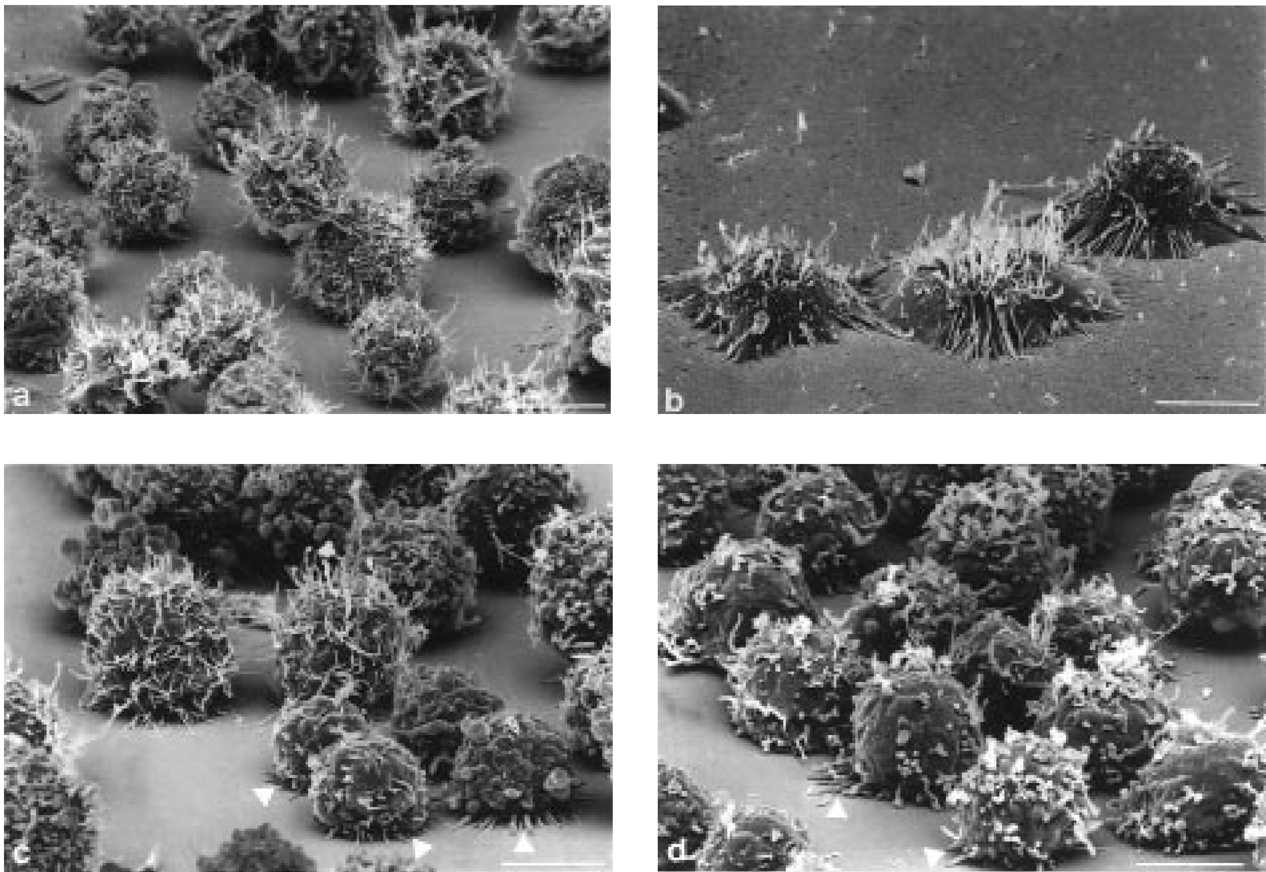


Figure 7 Scanning electron microscopy of control K562 cells (a) and of these cells grown onto polylysine for 30 min (b), 24 h (c) and 48 h (d). Controls appear rounded and have many microvilli on their surface while 30 min of growth onto polylysine (b) induces an evident flattening of the cells. After 24 h and 48 h (c and d, respectively) of growth onto polylysine, the cells return to a spherical shape very similar to controls although some cells show few microvilli and a surface characterized by ''lamellar-like structures''. Cells grown onto polylysine for 24 h and 48 h have ''pseudopodia-like protrusions'' at the points of cell contact with the surface (c and d, arrows). The bars represent 10 μm.

K562 cells do not die, but rather proliferate normally when growing on polylysine, it can be deduced that they adapt to adhesive growth on this model polymer. However, this adaptation induces (or is the result of) long-term variations in cell structure, particularly at the cell membrane level as evidenced by the alterations in membrane conductivity and membrane permittivity noted at long exposure times [2]. The alterations in membrane structure are also closely related to the variations in cell surface morphology observed in the present study at both short and long exposure times. In fact, the surface characteristics of K562 cells after 48 h of growth onto polylysine indicate that an important structural reorganization necessary for long-term adaptation has taken place. On the other hand, the much more evident but transient perturbations in surface morphology observed at shorter exposure times (the cells are completely attached to the polylysine surface and flat) seem to demonstrate that these changes are signalling cues for more long-lasting perturbations. This hypothesis is enforced by the fact that cytoskeletal rearrangement is observed only at short exposure times and that it is not accompanied by quantitative variations at both shorter and longer exposure treatment times. How do cell signalling events occur in K562 cells? In recent years, it has become increasingly evident that cell signalling through the CAMs and the cytoskeleton occurs through the phosphatidylinositol lipids [6] and that these lipids may directly regulate cytoskeletal events [7]. With this in mind, studies are underway in our laboratory in order to determine if changes in phosphatidylinositol lipids do, in fact, occur in K562 cells grown on polylysine. Preliminary evidence utilizing proton nuclear magnetic resonance (NMR) spectroscopy seems to suggest that such variations in these lipids do occur (paper in preparation).

In conclusion, if the evidence presented in this paper is considered together with that of our previous reports, it can be stated that charge interactions play an extremely important role in determining cell function. Together with other effects, of particular relevance may be the variations in mechanical tension generated in the cytoskeleton of cells by these charge interactions. In fact, changes in intracellular tension are of great importance in cell behavior, especially with regard to mechanotransduction through the cytoskeleton [8–10]. Finally, the data presented also seems to suggest that both the cytoskeleton and CAMs along with the cell membrane may be targets of charge interactions as is the case with other toxic agents [11]. Thus, the pivotal

role played by these cell components should be kept in mind in studies regarding cell/biomaterials interactions and in the design of more biocompatible biomaterials.

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