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TNF-α induces dyscohesion of epithelial cells. Association with disassembly of actin filaments

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TNF-α induced, in a time and dose-dependent fashion, cell-cell dissociation (dyscohesion) of endometrial epithelial cells. Within the time frame that dyscohesion was induced, TNF-α, in a dose-dependent fashion, reduced filamentous (F) actin and resulted in the loss of F-actin from the intercellular boundaries. Loss of F-actin mediated by TNF-α was not due to a reduction in the overall amount of actin or its β-isoform. Two proteins, Rho and Rho guanine nucleotide dissociation inhibitor (Rho-GDI), have been implicated in the regulation of organization of actin cytoskeleton. The reduced level of F-actin was not associated with altered expression of Rho protein, however, it was associated with an increase in the amount of Rho available for ribosylation *in vitro* by the C3 exoenzyme of Clostridium botulinum. The amount of Rho-GDI protein did not change after treatment with TNF-α suggesting that elevated expression of this protein is not responsible for the disassembly of actin filaments. These findings show that TNF-α induces dyscohesion. Dyscohesion induced by this cytokine is associated with perturbation of the actin cytoskeleton which may be due to the regulatory role of TNF-α on Rho.

Keywords: TNF-α; actin; epithelial cells; cell adhesion; endometrium

Introduction

The integrity of epithelial barriers is maintained by tight epithelial cell-cell binding. Under certain physiologic and pathologic conditions, the integrity of epithelia is disturbed. This occurs during menstruation when endometrial epithelial cells are shed (Ferenczy, 1979; Tabibzadeh, 1991b, 1994). The identity of the signals that participate in the loss of epithelial cell-cell binding is largely unknown. TNF-α is expressed in endometrium (Hunt *et al.*, 1992; Philippaeaux & Piguet, 1993; Tabibzadeh, 1991a, Tabibzadeh *et al.*, 1993a, 1995), and the expression of the TNF-α mRNA (Philippaeaux & Piguet, 1993) and protein (Tabibzadeh *et al.*, 1993a, 1995) peaks during the menstrual phase (Tabibzadeh *et al.*, 1993a, 1995). Based on this evidence and the fact that this cytokine may be utilized by leukocytes for their entry into epithelial domains, we tested whether TNF-α is potentially a signal involved in cell-cell dissociation (dyscohesion) induced by leukocytes. When epithelial cells were allowed to bind in three dimensions, they formed spheroidal structures. Within these structures, the epithelial cells were tightly bound. When spheroids were allowed to interact with leukocytes, lymphoid cells bound to and infiltrated the epithelial barriers. TNF-α enhanced the entry of lymphoid cells into epithelial domains and an antiserum against this cytokine inhibited this process (Tabibzadeh *et al.*, 1993b). These findings suggested that leukocytes utilize TNF-α in order to gain access to epithelial domains.

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The tight epithelial cell-cell cohesion is maintained by various junctions. Actin filaments are densely associated with the plasmalemmal undercoat, a feature most probably related to the tight association between adherens junctions and the force generating bundles of actin filaments (Mareel *et al.*, 1993). Agents that disrupt cortical actin filaments perturb cell-cell adhesion (Meza *et al.*, 1980; Hirano *et al.*, 1987; Jaffe *et al.*, 1990). The morphology of cortical actin filaments is likewise altered when cell-cell adhesion is compromised when level of extracellular Ca^{2+} is reduced (Volbert *et al.*, 1986). TNF-α impaired endothelial cell-cell binding and resulted in increased permeability of endothelial linings to macromolecules and low molecular weight solutes (Brett *et al.*, 1989). These effects were temporally associated with changes in the cytoskeleton and cell shape, development of intercellular gaps (Brett *et al.*, 1989; Goldblum *et al.*, 1993; Kohno *et al.*, 1993) and conversion of filamentous (F) actin to globular (G) actin (Goldblum *et al.*, 1993). Therefore, we have further examined the effect of TNF-α in its ability to induce dyscohesion in epithelial cells. In view of participation of actin filaments in epithelial cell-cell binding and in the formation and maintenance of adherens junctions, we also examined the impact of this cytokine on the expression of actin as well as its filamentous form in epithelial cells.

Results

Effect of TNF-α on cell binding; induction of dyscohesion

ECC1 cells grown as a monolayer were treated without and with TNF-α (1, 10, 100 ng/ml). Untreated cells grew as polygonal epithelial cells. Cohesive colonies of epithelial cells were tightly adherent at the cell borders (Figure 1A). In contast, TNF-α in a dose-dependent fashion induced morphologic changes in the epithelial cells. The effect of TNF-α on morphology was not apparent within the first few days. Typically, the initial impact of TNF-α on morphology was observed after 3 days of treatment. After this period, the epithelial cells gradually lost the polygonal shape and close cell-cell contacts, and over a period of days lost cell-cell adhesions and developed stariform or fusiform shapes and long cytoplasmic extensions (Figure 1B). These changes initially occurred at the borders of cell colonies rather than within their confluent centers. Continued treatment of epithelial cells with TNF-α resulted in rounding up of the cells and their separation from the substratum. The experiments reported here were performed during the time period that the majority of cells remained bound to the substratum. Within 7-10 days with 100 ng/ml of TNF-α, however, virtually all cells including those at the centers of the colonies underwent the morphologic change, became round and detached from the substratum. Incubation of the detached cells with the trypan blue at a given point of treatment demonstrated that some of the cells have lost their viability. We previously showed that this type of cell death induced by TNF-α is attributable to apoptosis (Tabibzadeh *et al.*, 1993a).

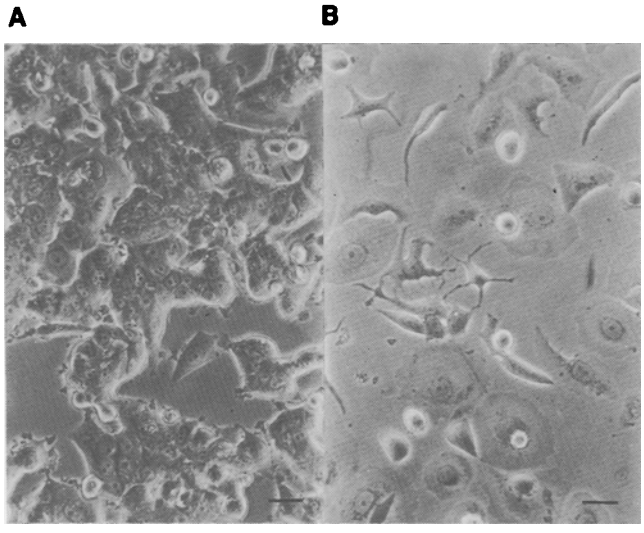


Figure 1 Effect of TNF-α on the morphologic configurations of monolayer cultures of epithelial cells. Cells were treated for 3 days with 0 (**A**) and 100 (**B**) ng/ml of TNF-α. (**A**) The untreated epithelial cells have formed tight epithelial clusters and show a polygonal morphology. Phase contrast microscopic photomicrograph (Bar: 20 μm). (**B**) TNF-α treated cells exhibit a stariform or fusiform morphology and have developed long cytoplasmic extensions. Some cells have become round. These cells may be mitotic or apoptotic. Phase contrast microscopic photomicrograph (Bar: 20 μm)

The separation of cells from each other within monolayer cultures could conceivably be due to several factors including dyscohesion, repulsion, spreading over the substratum or loss of some cells from the substrate. Therefore, we further examined the effect of TNF-α on cell-cell binding in three dimensional cultures. The ECC1 cells were stably transfected with the β-galactosidase gene to allow their histochemical detection. Monolayers of these cells were treated with various doses of TNF-α (0, 1, 10, 100 ng/ml). Control studies revealed that TNF-α does not modulate the amount of β-galactosidase in cells treated with various doses of cytokine. After a 3 day period of treatment, monolayer cultures were washed extensively in order to remove the round and non-viable cells. Cells which were still bound to the substratum were, after removal and as judged by trypan blue exclusion, more than 98% viable. 24 h prior to removal of these cells, ECC1 cells which were not transfected with the β-galactosidase gene and were grown as a monolayer in the absence of TNF-α were removed from the substratum and were allowed to form spheroids over a layer of agar. 24 h later, spheroids were allowed to settle for 5 min at 1 *g* in order to remove unbound cells. Spheroids were round and of rather uniform size. The single cell suspension of transfected cells treated without and with TNF-α were then incubated with the spheroids for 1 h. After binding to the spheroids, the unbound cells were removed. Spheroids were directly applied to glass slides and then histochemically stained to reveal the transfected cells. As shown in Figure 2A, the transfected ECC1 cells that were not treated with TNF-α bound to the spheroids. In addition, their binding together resulted in the bridging of various neighbouring spheroids. As shown in Figure 2B–D, the number of transfected cells bound to the spheroids progressively diminished in relation to the dose of TNF-α so that few transfected cells remained attached to the spheroids after treatment with 100 ng/ml of TNF-α. In order to quantitate the number of cells bound to the spheroids, other identical samples were trypsinized in order to separate spheroids into a single cell suspension. These cell suspensions were air dried over slides and histochemically stained and then the percentages of β-gal positive cells were determined by cell counting under a light microscope. The percentage of binding of β-gal positive cells to the non-transfected cells was diminished in relation to the dose of TNF-α (Figure 3). Similar samples were used to quantitate the number of β-gal positive cells by spectrophotometric measurement of β-galactosidase activity. Again, the detectable β-galactosidase activity was dependent on the dose of TNF-α (Figure 3).

We then examined the effect of TNF-α on the ability of the epithelial cells to attain tight cell-cell binding (compaction). A series of experiments indicated that ECC1 cells that bind together in 3-D cultures and form spheroids, attain compaction after a period of 24 h. After this period, a significant number of cells within spheroids could no longer be freed into the culture medium by vigorous pipetting. Then, monolayers of ECC1 cells were treated with TNF-α (0 and 100 ng/ml) for 3 days. The unbound and loosely bound cells were removed by extensive washing of the cultures. The cells bound to the substratum were removed and placed in equal numbers over a layer of agar in nutrient medium. Then, after 24 h, the epithelial spheroids were vigorously pipetted to allow release of loosely bound cells. The free cells were separated from the spheroids by allowing the spheroids to sediment. The number of viable cells within spheroids and those that were free in the culture medium were determined and % disaggregation (dyscohesion ratio) was calculated. As shown in Figure 4, at the starting point of assay, all cells were free and therefore the cell dyscohesion ratio was 1. However, after 24 h, about 40% of control cells were tightly associated and could not be freed from spheroids. TNF-α impaired the formation of tight epithelial cell-cell binding (compaction).

Effect of TNF-α on actin

Actin filaments are essential for the epithelial cell-cell binding and participate in the epithelial compaction (Hirano *et al.*, 1987; Mareel *et al.*, 1993). F actin is in equilibrium with G actin. Since the major impact of TNF-α was on the impairment of the ability of the epithelial cells to attain compaction, we decided to examine the effect of TNF-α on the overall amounts of actin, and its β-isoform, as well as the amounts and distribution of its F and G forms.

In order to examine whether the overall amount of actin and F-actin has changed in the cells treated with TNF-α, the following experiments were performed. Cells were treated with TNF-α (0, 1, 10, 100 ng/ml) and cytosolic and Triton-X100 soluble and insoluble membrane protein fractions were prepared. The extracted proteins were subjected to *in vitro* ribosylation by Clostridium perfringens iota toxin which ribosylates actin *in vitro* (Schering *et al.*, 1988). The amount of a 42 kD protein corresponding to actin and available for ribosylation by this toxin did not differ in proteins of various cell fractions after treatment of cells with TNF-α (Figure 4). In addition, Western blot analysis also failed to demonstrate modulation of expression of the total amount of the β-isoform of actin in the TNF-α treated cells (data not shown). Furthermore, monolayers of epithelial cells were treated with TNF-α (0, 1, 10, 100 ng/ml) for 72 h and then the cells bound to the substratum were removed. One group of cells were stained with the monoclonal antibody to β-actin while another group was stained with phalloidin-FITC and samples were then analysed by flow cytometry. As can be seen in lower panel of Figure 5, the total amount of β-actin in the cells treated with various concentrations of TNF-α remained essentially the same. However, as is seen in upper panel of Figure 5, the amount of F-actin was reduced in relation to the dose of TNF-α.

In order to examine the F and G-actin, monolayers of ECC1 cells treated with TNF-α (0, 1, 10 and 100 ng/ml) grown for four days were simultaneously stained with FITC-labeled phalloidin that binds to F-actin (Huang *et al.*, 1992) and Texas red-labeled DNAse I that binds to G-actin (Kabsch *et al.*, 1990). In the control untreated cells, F-actin was confined primarily to the intercellular boundaries (Figure 6A). Some aggregates of F-actin were within the cells and G-actin was diffusely present in these cells (Figure 6A). TNF-

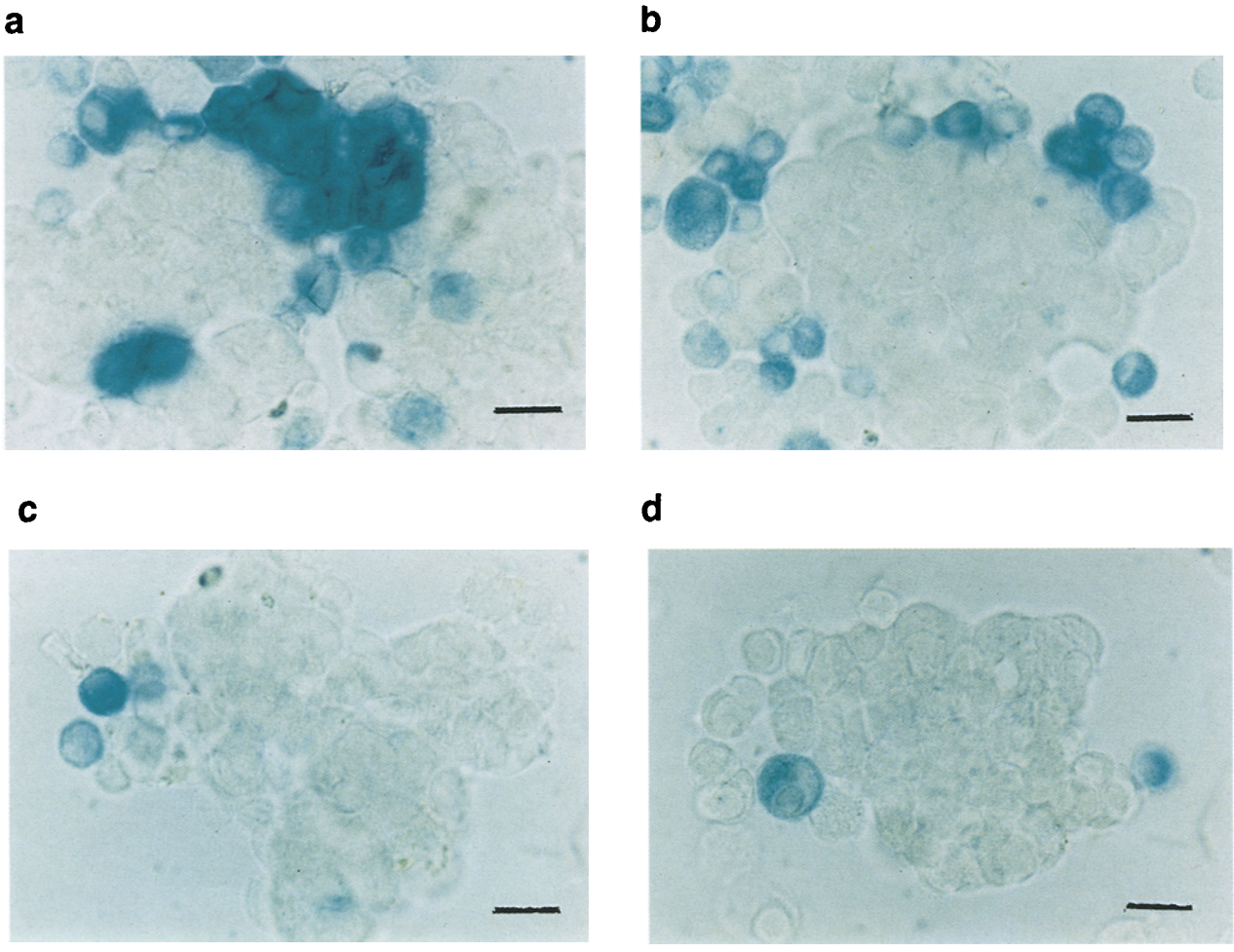


Figure 2 Demonstration of the effect of TNF-α on the cell-cell binding. Monolayer of β-gal positive ECC1 cells were treated with 0 (**a**), 1 (**b**), 10 (**c**) and 100 (**d**) ng/ml of TNF-α for 5 days. Following such treatment, cells bound to the substrate were removed and allowed to bind to ECC1 spheroids formed for a period of 24 h. After 1 h incubation at 37°C, and removal of unbound cells, the spheroids were air dried over slides and then stained with X-gal as described in the text. The untreated control β-gal positive darkly stained (blue) cells have bound to the neighboring spheroids causing formation of sheet-like structures. TNF-α has resulted, in a dose dependent manner, in reduced binding of β-gal positive cells to spheroids. As illustrated by the intensity of staining, TNF-α did not modulate the β-galactosidase activity (Bar: 20 μm)

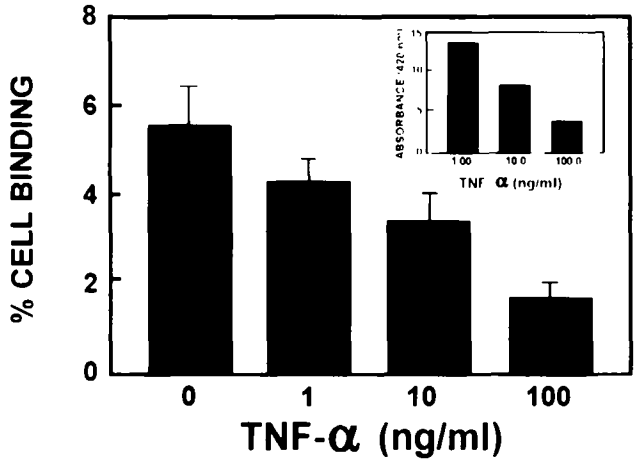


Figure 3 Quantitative determination of the effect of TNF-α in the binding of the β-gal positive cells to the ECC-1 spheroids. Monolayer of β-gal positive cells were treated without and with TNF-α and adherence assays were performed as described in Figure 2. The percentages of β-gal positive cells bound to the ECC1 spheroids were determined in air dried cell preparations. Histograms show the means and standard errors. TNF-α resulted, in a dose dependent manner, to the reduction of the number of cells bound to the spheroids. Inset demonstrates the TNF-α mediated, dose-dependent reduction, in the number of β-gal positive cells bound to the spheroids as reflected by the spectrophotometric measurement of the absorbence at 420 nm

α modulated the expression of F and G-actin initially at the borders and subsequently within the central regions of colonies. TNF-α in a dose-dependent fashion resulted in loss of cortical actin present at the intercellular borders (Figure 6B–6C). In cells that were stariform in shape, F-actin primarily accumulated to poles of these cells (Figure 6C). In the TNF-α treated cells, G-actin was predominantly distributed around the nuclei (Figure 6C). In some round cells, F-actin was still present whereas in other cells, it was completely replaced by G-actin (Figure 6C).

A group of 21 kD G-proteins of the Rho family of molecules regulate the assembly of actin cytoskeleton (Ridley & Hall, 1992). In order to examine whether TNF-α affects Rho proteins, cytosolic proteins of cells treated with various doses of TNF-α were subjected to SDS–PAGE and probed with an antiserum to RhoA. The total amount of RhoA remained the same in the cytosolic proteins of cells treated with TNF-α as compared with the untreated controls (Figure 4). However, when the same proteins were used for ribosylation by Clostridium botulinum C3 exoenzyme, the amount of ribosylated Rho was increased in relation to the amount of TNF-α (Figure 4). Examination of the membranes revealed identical results (data not shown). Time response studies showed that the amount of Rho that can be ribosylated *in vitro* with C3 exoenzyme significantly increased in hours after treatment with TNF-α and reached to a plateau within 24 h after treatment (Figure 7).

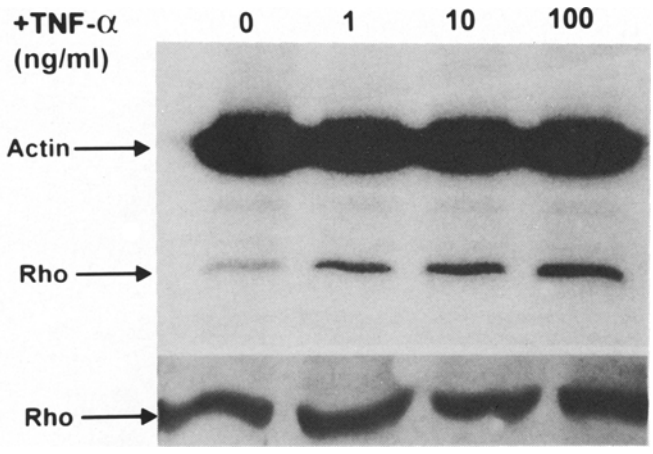


Figure 4 Dose-dependent effect of TNF-α on total amount of actin and Rho and amount of Rho accessible for in vitro ribosylation. Monolayers of cells were treated with TNF-α (0, 1, 10, 100 ng/ml) for 4 days. Upper panel: Cytosolic proteins were subjected to *in vitro* ribosylation by Iota toxin and C3 exoenzyme, and SDS PAGE followed by autoradiography. The total amount of actin as demonstrated after ribosylation by Clostridium perfringens toxin does not show any change after treatment with TNF-α. However, the amount of ribosylated Rho shows an increase in response to the amount of TNF-α. Lower Panel: Cytosolic proteins of cells treated with TNF-α (0, 1, 10, 100 ng/ml) were subjected to SDS-PAGE and Western blotting using the antiserum to RhoA. As compared to the untreated cells, the total amount of RhoA does not change after treatment of cells with various doses of TNF-α

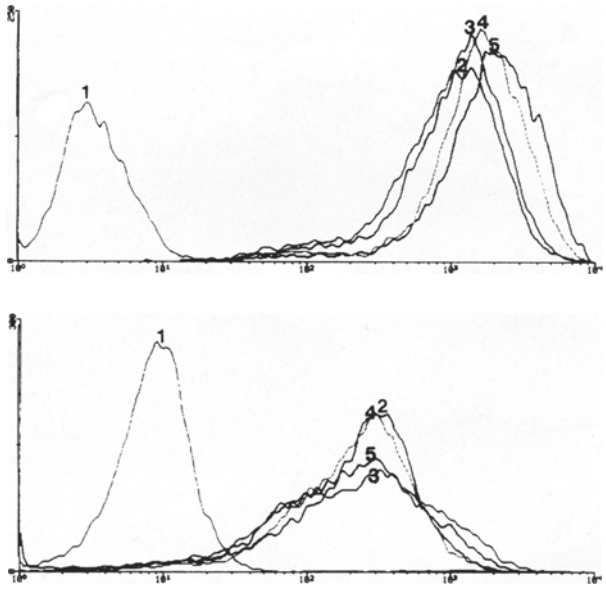


Figure 5 Effect of TNF-α on total amount F-actin and β-actin in cells. Monolayer of cells were treated with TNF-α at 0 (histogram #5), 1 (histogram #4), 20 (histogram #3) and 100 (histogram #2) ng/ml. After a period of 24 h, cells were removed and stained to reveal the F-actin and total amount of β-actin. Upper panel: Cells were stained without (#1) or with phalloidin-FITC (histograms #2–5). Lower panel: Cells were stained with antibody to β-actin (histograms #2–5) or with isotype specific antibody (histogram #1). After staining, cells were analysed by flow cytometry. X axis: fluorescence intensity, log scale. Y axis: number of events. As seen in the upper panel, cells do not show staining when incubated in the absence of phalloidin-FITC. Cells treated without TNF-α show the highest amount of F-actin. As demonstrated by shift to the left of the histograms, this amount is reduced in response to TNF-α in a dose-dependent fashion. As seen in the lower panel, cells stained with isotype specific antibody do not show staining. However, the histograms of cells stained with antibody to β-actin overlap regardless of the treatment with TNF-α

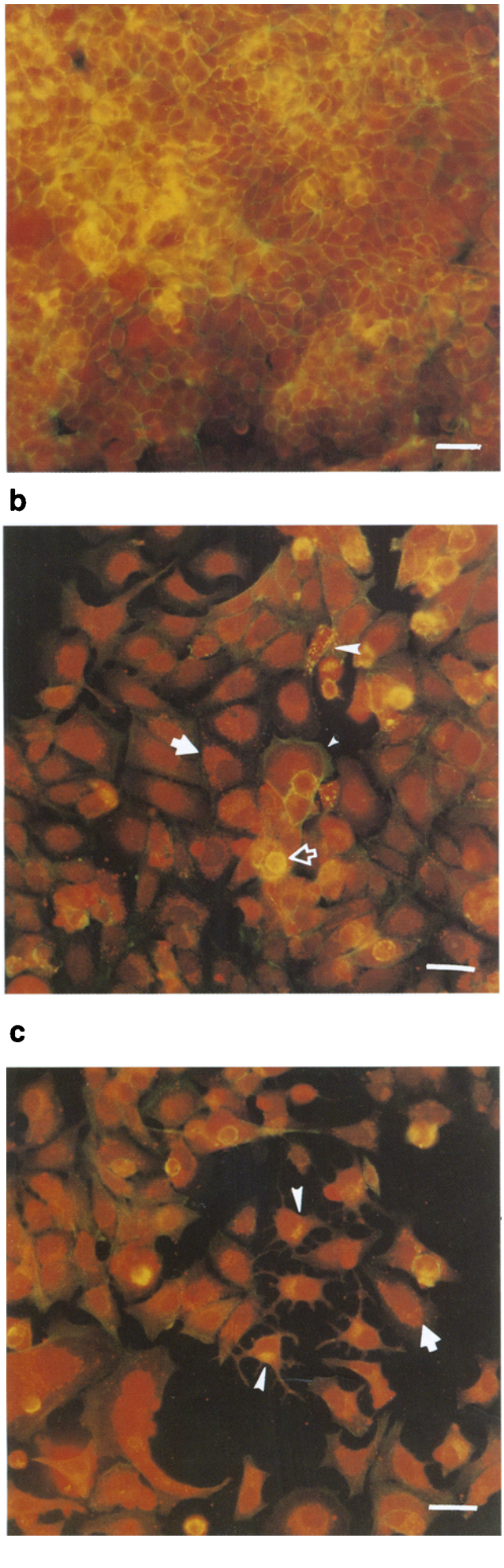
and active GTP-bound form (Madaule & Axel, 1985; Garrett *et al.*, 1989; Hori *et al.*, 1991) and are believed to be kept in the GDP-bound inactive form when associated with Rho-GDI (Kikiuchi *et al.*, 1992). Since overexpression of Rho-GDI protein also resulted in a round morphology and loss of stress fibers (Leffers *et al.*, 1993; Miura *et al.*, 1993), we tested whether the effects of TNF-α on morphology and on actin is potentially related to the regulation of expression of Rho-GDI protein. For this purpose, the cells were treated without and with TNF-α (0, 1, 10, 100 ng/ml) for 4 days and labeled for 14 h with [^{35}S]-methionine. Cell proteins were then subjected to 2D gel electrophoresis. These studies showed that treatment of cells with TNF-α did not induce changes in the amount of Rho-GDI proteins (Figure 8). Further analysis at different time points of 24, 72, 120 and 144 hours also failed to reveal any difference in the amount of Rho-GDI proteins (data not shown). However, at the same time points, TNF-α induced increased production of several known proteins as well as several proteins whose identities have not been determined as yet (Figure 8).

Discussion

The tight adhesion of epithelial cells was lost in the monolayer cultures after treatment with TNF-α. When the binding of epithelial cells treated with TNF-α in three dimensional cultures was compared with that of untreated cells, it became apparent that TNF-α impairs cell-cell binding and prevents this binding to become tight.

We tested the possibility that the impact of TNF-α on cell-cell binding may be exerted on the actin cytoskeleton. TNF-α did not modulate the overall amount of actin or the amount of its β-isoform in the epithelial cells within the time frame that it induced dyscohesion. However, TNF-α resulted in a dose-dependent fashion, in the loss of F-actin, particularly those at the cell-cell binding sites. Several lines of evidence suggest that actin filaments participate in cell-cell binding. For example, agents that disrupt cortical filaments perturb the cell-cell adhesion (Hirano *et al.*, 1987; Jaffe *et al.*, 1990; Meza *et al.*, 1980). Therefore, the impaired cell-cell binding associated with TNF-α treatment may be at least partially attributable to the effect of this cytokine on F-actin.

Loss of F-actin in the TNF-α treated cells was associated with morphologic configurations typified by stariform or fusiform shapes and long cytoplasmic extensions. Subsequently, the cells lost the extensions and developed a round morphology. These changes were associated with a progressive increase in the amount of Rho available for *in vitro* ribosylation by the C3 exoenzyme. This further availability of Rho was not due to its increased production of Rho as determined by Western blot analysis of proteins of TNF-α treated cells. The effect of TNF-α on the shape of the cells and the changes in the cytoskeleton may be mediated through regulation of Rho. Rho has been implicated in regulation of cell shape and actin cytoskeleton (Paterson *et al.*, 1990, Ridley & Hall, 1992). Microinjection of Val4Rho protein which is constitutively active resulted in rapid development of morphologic changes characterized by a contracted cell body and finger-like processes (Paterson *et al.*, 1990; Ridley & Hall, 1992). Microinjection of normal Rho resulted in the same morphologic changes induced by Val4Rho (Paterson *et al.*, 1990). Preincubation of Rho with the nonhydrolyzable GTP analogue, GTPγτS, before injection enhanced the activity of Rho fourfold (Paterson *et al.*, 1990). Microinjection of ADP-ribosylated Rho proteins or ADP-ribosylation of Rho proteins by C3 exoenzyme led to the rounding up of cells and loss of stress fibers (Paterson *et al.*, 1990). The increased amount of ribosylatable Rho after treatment with TNF-α may be due to increased free GDP-bound form of Rho. This free Rho may be due to separation of Rho from Rho-GDI since when bound to Rho-GDI, Rho



is not a good substrate for ADP-ribosylation (Kikuchi *et al.*, 1992). Rounding of cells and their death by apoptosis after treatment with TNF-α (Tabibzadeh *et al.*, 1993a) may also be attributable to the impact of Rho on actin cytoskeleton since cells microinjected with Val4Rho protein also detached from the substratum and died. However, whether this type of cell death was apoptotic in nature was not investigated (Paterson *et al.*, 1990). Overexpression of Rho-GDI protein in cells also induced rounding of cells and resulted in loss of stress fibers (Leffers *et al.*, 1993). However, the effects of TNF-α on actin cytoskeleton could not be attributed to regulation of amount of Rho-GDI protein in the treated epithelial cells.

Disruption of cell-cell binding among epithelial cells, stromal cells and endothelial cells leads to the characteristic tissue shedding and bleeding manifested during menstruation (Ferenczy, 1979; Tabibzadeh, 1991b; Tabibzadeh *et al.*, 1994). We showed that significant quantities of TNF-α is released by menstrual endometria (Tabibzadeh *et al.*, 1993a). Conversion of F to G actin occurs in endometrial glands shed during menstruation when amount of endometrial TNF-α is maintained at a high level (Tabibzadeh *et al.*, 1995). In view of the *in vitro* nature of the studies reported here and the cell line utilized, the findings reported here, suggest yet do not prove that TNF-α may contribute to the conversion of F to G actin in endometrial epithelial cells during the menstrual shedding.

In summary, we demonstrated that TNF-α impairs the ability of cells to bind together. This dyscohesion is associated with a range of abnormalities from overall loss of F-actin, loss of F-actin at the intercellular binding sites to abnormal distribution of F-actin within cells. This effect of TNF-α may be regulated by Rho since TNF-α modulates the amount of Rho available for ribosylation by the C3 exoenzyme of Clostridium botulinum. This effect, however, is independent of the modulation of expression of Rho-GDI proteins.

Materials and methods

Materials

E Coli recombinant human TNF-α (Specific activity 4.03×10^7 units/mg, Endotoxin <0.06 EU/mg by the limulus amoebocyte lysate assay) was obtained from Genentech (San Francisco, CA). FITC-labeled Phalloidin was obtained from Sigma Chemical Co (St Louis, MO). DNAse I-Texas red was obtained from Molecular probes (Eugene, OR). Monoclonal antibody to β-actin was obtained from Sigma Chemical Co. The rabbit antiserum against RhoA which was raised against recombinant RhoA recognizes predominantly the C terminus of this protein, so it is unlikely to react with RhoB or C (Ridley *et al.*, 1993). 5-bromo-4-chloro-3indyl-β-D-galactopy-

Figure 6 Effect of TNF-α on F and G forms of actin. Cells were treated for 4 days with 0 (**a**), 20 (**b**) and 100 (**c**) ng/ml of TNF-α. Cells were stained with phalloidin-FITC and DNAse I-Texas red as described in the text. The true fluorescence emitted from FITC is green (shown here as yellow) and from the Texas red is red. (**a**) In the control cells, cortical F-actin is seen at the intercellular boundaries. G-actin is diffusely present in the cytoplasm (Bar: 20 μm). (**b**) The cells treated with 20 ng/ml of TNF-α, have spread over the substratum and spaces have appeared among cells. Cortical F-actin is lost in majority of cells. A diffuse (small arrowhead) or aggregated (large arrowhead) cytoplasmic staining of F-actin is seen in some cells. G-actin is primarily localized around the nuclei (solid arrow). Cells that are round have a bright F-actin staining due to condensation of actin (bland arrow) (Bar: 20 μm). (**c**) Some of the cells that have been treated with 100 ng/ml of TNF-α have developed fusiform or stariform morphology (arrowheads). In these cells, the cortical F-actin has shifted to one pole of some cells. In other cells, there is a perinuclear F-actin. Perinuclear staining of G-actin is seen (arrow) (Bar: 20 μm)

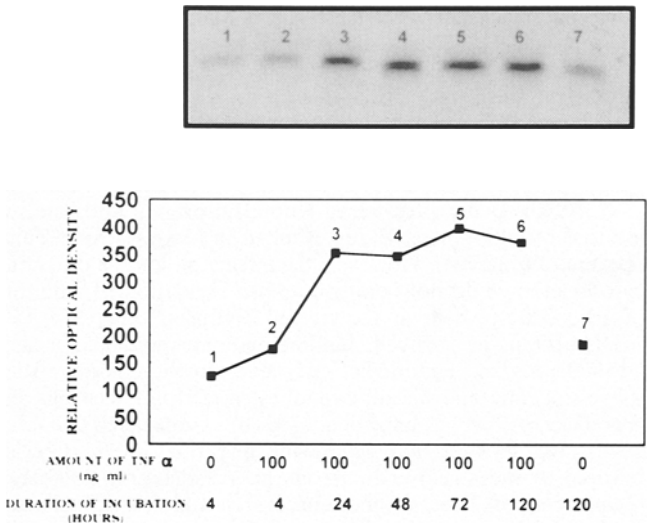


Figure 7 Time-dependent effect of TNF-α on ribosylation of Rho. Cells were treated with TNF-α (0 and 100 ng/ml) for the durations shown. Cytosolic proteins were subjected to *in vitro* ribosylation by C3 exoenzyme, SDS–PAGE followed by autoradiography. The amount of ribosylated Rho shows an increase in response to TNF-α. Within 4 h of treatment, the amount of ribosylated Rho is already above the control and continues to increase to reach to a plateau within 24 h and remains at this level as long as the treatment continues. Upper panel shows the *in vitro* ribosylated bands and the lower panel demonstrates the relative optical densities of the bands. The presented data are representative from one of three experiments

ranoside (X-gal), and o-nitrophenol-β-D-galactopyranoside (ONPG). The pRSV-LacZ II plasmid containing the *E. coli* β-galactosidase and neomycin resistant genes was kindly provided by Dr LA Culp (Case Western Reserve University, School of Medicine, Cleveland, Ohio) (Wen-Change & Culp, 1991). Fluorescein-di-galactoside (FDG) was from Molecular probes.

Monolayer and spheroid cultures of an epithelial cell line (ECC1)

ECC1 cells were cloned from EnCa101AE cell line, derived from a well differentiated adenocarcinoma of human endometrium transplanted in nude mice (Satyaswaroop *et al.*, 1983). The estradiol-17 sensitive cells of this cell line similar to normal and neoplastic epithelium form glandular structures in nude mice (Satyaswaroop *et al.*, 1988; Tabibzadeh *et al.*, 1990). These cells also form glandular structures when grown in Matrigel and exhibit tight cell-cell binding as well as various junctions both *in vitro* and *in vivo* (Satyaswaroop & Tabibzadeh, 1991; Tabibzadeh *et al.*, 1993c). ECC1 cells interact with each other and form spheroids when their attachment to the substratum is inhibited by culturing the cells over a layer of agar (Tabibzadeh *et al.*, 1993c). In order to make spheroids, ECC1 cells were removed from monolayer cultures by treatment with trypsin-EDTA. Spheroids were formed when 5×10^6 ECC1 cells were placed in 10 ml of medium over a 1 mm thick layer of 1.5% agar in a 100 mm Petri dish. Nutrient medium for all cultures consisted of DMEM-HAM's F-12 containing 10% heat inactivated and mycoplasma- and virus-tested fetal bovine serum, antibiotic-antimycotic mixture, human insulin (200 U/L), transferrin (25 mg/L) and glucose (400 mg/L). The spheroids were examined daily by phase contrast microscopy and culture media were changed every 3 days. Cell viability was determined by trypan blue exclusion. Contamination of cultures by bacterial and Mycoplasma was also repeatedly ruled out by PCR. The universal primers directed to conserved parts of the prokaryotic 16S rRNA and a set of internal primers specific to Mycoplasma species were used in the PCR reactions (Uphoff *et al.*, 1992).

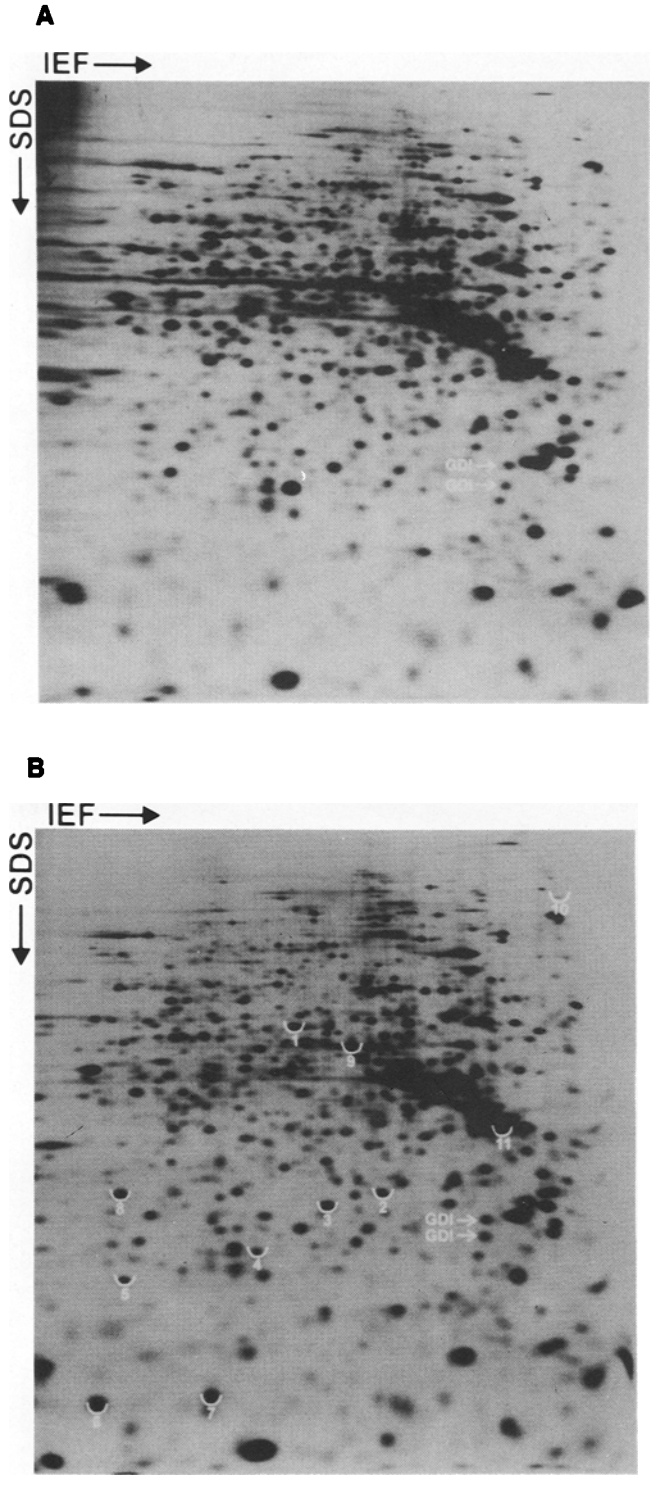


Figure 8 Effect on TNF-α on the amount of Rho-GDI proteins. Two dimensional gels (IEF followed by SDS–PAGE of [^{35}S]-methionine-labeled proteins from cells treated for 4 days with 0 (**A**) and 100 (**B**) ng/ml of TNF-α. The GDIs are shown by the arrows. TNF-α does not change the expression of the RHo-GDIs. Other spots shown by numbers are those proteins whose expression is enhanced by TNF-α. They include Spot #1: most likely tryptophanyl tRNA synthetase. Spot #2–8, 11: unknown, Spot #9: keratin 8, Spot 10: most likely cadherin.

Cell aggregation assay

ECC1 cells (1×10^6 cell/ml) were allowed to interact over a layer of agar. At the indicated time points, aggregated and non-aggregated cells were removed and the suspension was pipetted in and out. Our initial experiments showed that after a few times of pipetting, additional pipetting did not increase the number of free cells in the suspension of spheroids.

However, in order to completely assure that all the loosely bound cells could be freed, spheroids were pipetted 20 times. The large aggregates in the suspension that remained intact after multiple pipetting were allowed to settle for 5 min at 1 *g*. The cells within aggregates were dissociated by trypsinization. Then, the number of cells in the aggregates and the floating non-aggregated cells were determined. The extent of disaggregation (dyscohesion ratio) was determined as the ratio of free cells to the total number of cells.

Immunofluorescence staining of the F and G forms of actin

Cells were fixed for 5 min in 10% buffered formalin and then incubated in 0.5% Triton X-100 for 10 min at 25°C. Following a wash in PBS, samples were incubated at room temperature for 30 min with the appropriate dilution of FITC-labeled phalloidin and then with DNAse I-Texas red. The proper concentration for optimal staining of F and G actin was obtained by serial dilutions of FITC-labeled phalloidin and DNAse I-Texas red that respectively bind to F (Huang *et al.*, 1992) and G-actin (Kabsch *et al.*, 1990). After washing in PBS, tissue sections were examined and photographed by a fluorescent microscope.

Flow cytometry

Cells were fixed for 5 min in 10% buffered formalin and then permeated for 5 min with 0.5% Triton-X100. Cells were washed and then incubated with saturating amounts of fluorescent-labeled specific antibody to β-actin and the FITC-labeled phalloidin. The relative number of β-actin and F-actin positive cells in suspension were determined by analysing the cells by a flow cytometer-cell sorter (Becton Dickinson). In each assay, a minimum of 10 000 cells was analysed.

[^{32}P] ADP-ribosylation assay

Clostridium perfringens iota toxin (Stiles & Wilkens, 1986) and Clostridium botulinum C3 ADP-ribosyltransferases (Aktories *et al.*, 1986, 1987, 1988) were purified as described. Ribosylation by these ADP-ribosyltransferases was performed essentially as described previously (Aktories *et al.*, 1988; Schering *et al.*, 1988). Briefly, about 15 μg protein from the cytosolic and membrane fractions of TNF-α treated cells were incubated in the presence of 50 mM triethanolamine-HCl (pH 7.5), 1 mM EDTA, 1 mM PMSF, 2 mM $MgCl_2$, 1 mM DTT and 10 mM thymidine, 0.5 M [^{32}P]NAD (0.3-0.5 μCi) and 0.5 μg/ml iota toxin or 1 μg/ml C3 exoenzyme for 30 min at 30°C. The reaction was stopped by addition of sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Radiolabeled proteins were analysed by SDS-PAGE essentially as described by Laemmli (1970) and subsequently analysed with a phosphoimager or by autoradiography followed by scanning densitometry.

Labelling of cells with [^{35}S]-methionine, Two dimensional (2D) gel electrophoresis

Monolayers of cells were treated without and with TNF-α as indicated. Prior to removal, cells were incubated in presence of 200 μCi of [^{35}S]-methionine in a nutrient medium lacking methionine and containing 10% dialyzed fetal bovine serum. After an incubation period of 16 h, cells removed from culture vessels were resuspended in 300 μl lysis buffer solution (O'Farrell, 1975). 2D gel electrophoresis with isoelectric focusing (IEF) in the first dimension (O'Farrell, 1975) was carried out as described by Bravo *et al.* (1982) and Celis *et al.* (1990). Briefly, the first dimension of the gel electrophoresis was performed for 18 h at 400V in 130 × 1.2 mm, in 4% (W/V) polyacrylamide gels containing 2% carrier ampholytes (1.6% pH 5-7, Serva; 0.4% pH 3.5-10, LKB). First dimensional gels were equilibrated in 3 ml (3 min at room temperature) of equilibration solution (0.06 M Tris-HCl, pH 6.8; 2% SDS; 100 mM DTT and 10% glycerol) (O'Farrell, 1975). Gels were then stored at −20°C until use. First dimensional gels were applied to the second dimension with the aid of agarose solution (0.06 M Tris-HCl, pH 6.8; 2% SDS; 100 mM DTT; 10% Glycerol; 1% agarose and 0.002% bromophenol blue) (O'Farrell, 1975). SDS-PAGE (15% running gel and 5% stacking gel) was then carried out. The dried gels were exposed to X-ray films for various periods of time.

Western blotting

Cells were disrupted using a Dounce homogenizer. Nuclei were separated from cytosol and membrane fractions at low speed (200 *g*). Membranes were further separated from the cytosol by centrifugation at 100 000 *g* for 1 h. The proteins of plasma membranes were extracted in 0.5% Triton-X100 containing protease inhibitors (25 mM Tris pH 7.6, 25 mM sucrose, 0.1 mM EDTA, 5 mM $MgCl_2$, 5 mM DTT, 1 mM phenylethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). The insoluble fraction containing cytoskeletal and bound proteins were separated from the soluble fractions. SDS-solubilized cell proteins normalized for total protein were boiled in 1 × Laemmli buffer for 5 min. After centrifugation at 16 000 *g* for 5 min, supernatants were subjected to SDS-PAGE and separated proteins were transferred to nitrocellulose membranes. Blots were sequentially incubated for 2 h with TBST containing 2% milk powder and primary antibody, followed by 1 h incubation with secondary antibody and then 1 h incubation with avidin-biotin-complex. Each incubation was followed by two washes in TBST. Blots were developed in a mixture of 3, 3' diaminobenzidine tetrahydrochloride-H_2O_2.

Transfection

ECC1 cells were transfected with the pRSV-LacZ II plasmid by using lipofectin (Gibco/BRL, Grand Island, NY) (Sambrook *et al.*, 1989). Transfected cells were selectively grown in presence of the appropriate antibiotic (G418 sulphate). In order to select the cells with the high level of expression of β-galactosidase, the transfected cells were sorted by a flow cytometer/cell sorter (Becton Dickinson). This was achieved by a brief hypotonic shock followed by labeling with FDG. Fluorescence of β-galactosidase (β-gal) positive cells after staining with FDG allowed sorting of these cells with acquisition of virtually 100% stably transfected cells.

Staining of β-galactosidase positive cells

Staining was performed as described (Wen Chang & Culp, 1991). Briefly, air dried cells were fixed for 5 min at 4°C with 2% (v/v) formaldehyde-0.2% (v/v) glutaraldehyde in phosphate buffered saline (PBS; 0.1 M, pH 7.4). Cells were rinsed in PBS and incubated overnight at 37°C in a staining solution containing X-gal (1 mg/ml), potassium ferricyanide (20 mM), potassium ferrocyanide (20 mM) and $MgCl_2$ (2 mM) in PBS.

Spectrophotometric measurement of β-galactosidase activity

The amount of β-galactosidase was measured as previously described (Wen-Chang & Culp, 1991). Cell extracts were prepared by repeated freezing and thawing. Then, the cell extracts were incubated with the substrate ONPG (0.2 mg/ml) in presence of $mgCl_2$ (1 mM), β-mecaptomethanol (45 mM) and sodium phosphate (0.1 M; pH 7.5) for 12 h at 37°C. The reaction was stopped by adding sodium carbonate to the final concentration of 0.5 M. Absorbence of the reaction mixture was read at 420 nm.

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