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Rho-dependent cell spreading activated by *E.coli* cytotoxic necrotizing factor 1 hinders apoptosis in epithelial cells

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Abstract

Cell-cell and cell-matrix interactions play a pivotal role in numerous cell functions including cell survival and death. In this work, we report evidence that the Rho-dependent cell spreading activated by a protein toxin from *E. coli*, the cytotoxic necrotizing factor 1 (CNF1), is capable of hindering apoptosis in HEp-2 cells. In addition to the promotion of cell spreading, CNF1 protects cells from the experimentally-induced rounding up and detachment and improves the ability of cells to adhere to each other and to the extracellular matrix by modulating the expression of proteins related to cell adhesion. In particular, the expression of integrins such as α_5, α_6 and α_v, as well as of some heterotypic and homotypic adhesion-related proteins such as the Focal Adhesion Kinase, E-cadherin, α and β catenins were significantly increased in cells exposed to CNF1. Our results suggest, however, that the promotion of Rho-dependent cell spreading is the key mechanism in protecting cells against apoptosis rather than cell adhesion *per se*. A toxin inducing cell spreading without activating Rho, such as Cytochalasin B, was in fact ineffective in favouring cell survival. These data are of relevance (i) for the understanding of the role of the actin-dependent and especially Rho-dependent cellular activities involved in apoptosis regulation and (ii) in providing some clues to understanding the mechanisms by which bacteria, by controlling cell fate, might exert their pathogenic activity.

Keywords: apoptosis; CNF1; actin cytoskeleton; cell adhesion; cell spreading; Rho

Abbreviations: CNF1, cytotoxic necrotizing factor 1; CB, cytochalasin B; FCS, fetal calf serum; PBS, phosphate buffered saline; BSA, bovine serum albumin; BrdU, bromodeoxyuridine; FAK, focal adhesion kinase; MAP, mitogen-activated protein

Introduction

Apoptosis is a physiological form of cell death which plays an important role in tissue development and homeostasis, maintaining a correct cell number in the body by balancing cell growth and death (Martins and Earnshow, 1997). It is a multi-phase process characterized by three distinct steps: in the first cells receive the death stimulus, in the second the cell is triggered to undergo apoptosis (effector phase) and, during the last step, typical irreversible morphological and molecular markers of apoptosis can be detected (Kroemer, 1997). Without apoptosis, detached cells from endothelia or epithelia could possibly reattach to inappropriately sited matrices, including the matrix that they would eventually synthesize themselves, and resume growth (Ruoslahti and Reed, 1994). Apoptosis occurring in detached cells would abrogate this escape mechanism. In addition, it is now established that cells forced to extend themselves over a large surface (spreading cells) survive better and proliferate faster than cells with a more rounded shape (Ruoslahti, 1997). Consequently, an increase in cell adhesion and spreading can protect cells against apoptosis.

Cell adhesion and cell spreading are different and well-defined phenomena mainly dependent on the integrity and function of the actin cytoskeleton in terms of focal adhesion plaque assembly or cell contractility (Burridge *et al*, 1997; Nobes and Hall, 1995). The actin cytoskeleton organization is controlled by proteins belonging to the Ras-like p21 Rho family (Hall, 1998) which are also involved in a number of cellular functions influencing cell fate. Among factors able to interfere with pathways leading to cell survival or death, we have very recently reported that a bacterial toxin from *E. coli* is capable of protecting epithelial cells against apoptosis (Fiorentini *et al*, 1997b; 1998b). The toxin, named Cytotoxic Necrotizing Factor 1 (CNF1), belongs to that protein toxin family which controls the actin cytoskeleton via modulation of regulatory G-proteins (Aktories, 1997). CNF1 permanently activates the p21 Rho protein by deamidation of glutamine 63 (Flatau *et al*, 1997; Schmidt *et al*, 1997). By activating Rho, the toxin stimulates a number of kinases in cells (Fiorentini *et al*, 1997a; Lacerda *et al*, 1997) which in turn promote actin assembly (Falzano *et al*, 1993; Fiorentini *et al*, 1995) and cell contractility (Fiorentini *et al*, 1997a).

In the present work, we explored the role of some Rho-dependent cell activities in hindering apoptosis. Our data clearly indicate that, rather than the augmented adhesiveness to the cell matrix, the Rho-dependent cell spreading and contractility (associated with the increase in stress fiber organization) are the factors favouring cell survival.

Results

CNF1 protects HEp-2 cells from UVB-induced apoptosis

We have reported that UV radiation induces cytoskeleton-dependent surface blebbing and cell death in epithelial cells (Malorni *et al*, 1994; Straface *et al*, 1995) and that these effects are partially counteracted by drugs capable of improving cell adhesion (Malorni *et al*, 1995). We have thus investigated whether the promotion of actin assembly and cell spreading due to CNF1 could play a key role in the toxin-induced reduction of apoptosis that we have very recently observed in epithelial cells (Fiorentini *et al*, 1997b; 1998b). All data herein reported refer to cells which maintained contacts with the substrate after the various treatments. We did not include the observations on detached cells floating in the culture medium because all of them, irrespective of the treatment, showed nuclear changes typical of apoptosis. Figure 1 shows the results obtained by phase contrast microscopy (Figure 1a, c, e, g) and fluorescence microscopy (Figure 1b, d, f, h). The former gives a general picture of the monolayer after each treatment, and the latter shows chromatin structure and clumping typical of apoptosis by using Hoechst 33258, a fluorescent probe which specifically binds DNA (Bursh *et al*, 1992). HEp-2 cells growing in monolayer are polygonal-shaped (Figure 1a) with a single rounded nucleus per cell (Figure 1b). Upon exposure to CNF1, cells became large, flattened and with prominent membrane ruffles (Figure 1c). Treated cells were also multinucleated but such a modification did not interfere with chromatin organization, all the nuclei being regularly-shaped (Figure 1d).

After exposure to UVB radiation, cells maintained for 24 h in fresh medium underwent cell retraction, rounding, and displayed a typical sign of cell injury named surface blebbing (Figure 1e). Most cells still adhering to the substrate (about 49%, as shown in Figure 2), displayed nuclei with condensed or fragmented chromatin (Figure 1f). These cells, however, easily underwent detachment since the cell-substrate adhesion was much weaker. Pretreatment with CNF1 for 48 h allowed cells to resist the UVB-induced effects, most cells still adhering to the substrate (Figure 1g) with normal-shaped nuclei (Figure 1h). When a quantitative and statistical analysis of these phenomena was performed on six different experiments, it became evident that the protection offered by CNF1 was highly significant ($P<0.01$, Figure 2). Noticeably, CNF1 also counteracted apoptosis when induced by stimuli other than UVB. In fact, apoptosis caused by the antineoplastic drug Etoposide (58% of apoptosis after 48 h) or by the withdrawal of growth factors (serum starvation, 28% after 48 h) was significantly prevented when cells were pre-exposed to CNF1 ($\triangle$=48% and 88%, respectively; $P<0.01$). In this paper, however, only data obtained by using UVB as apoptotic inducer are reported.

To rule out the possibility that CNF1-promoted multinucleation could induce a more quiescent state in the cell population thus influencing susceptibility to apoptosis, we have performed a cytofluorimetric analysis of DNA. Our results clearly indicated that multinucleation did not influence the capability of toxin-treated cells to duplicate their DNA. In fact, the percentage of cells in S phase after 24 and 48 h of treatment (29.6% and 23.5%, respectively)

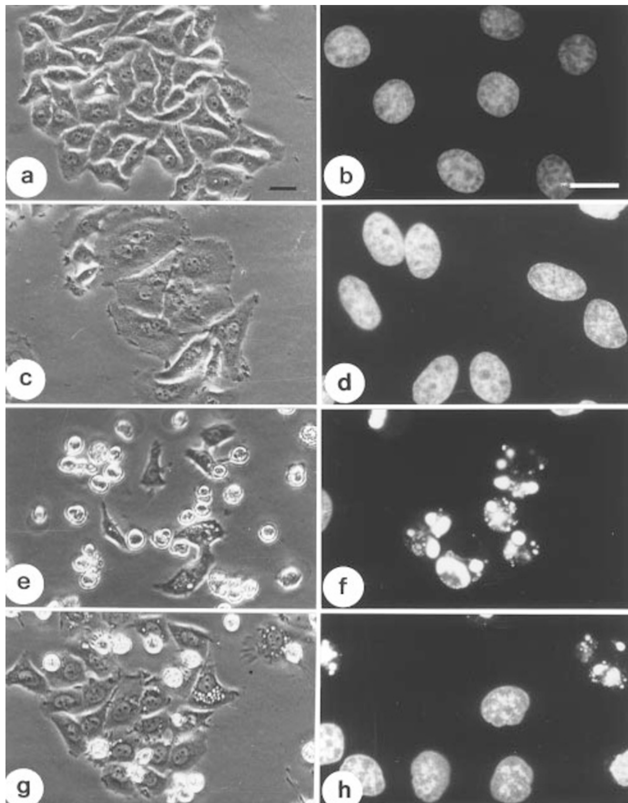

Figure 1 CNF1 treatment prevents the UVB-induced rounding up and apoptosis in HEp-2 cells. Left panel: phase contrast and right panel: fluorescence microscopy of cells stained with Hoechst 33258. (**a,b**) Control cells; (**c,d**) cells treated for 48 h with CNF1; (**e,f**) UVB-treated cells; (**g,h**) cells treated for 48 h with CNF1 and then with UVB

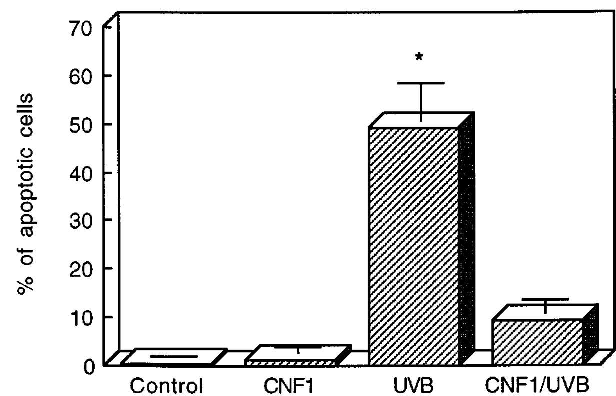

Figure 2 CNF1 significantly protects cells from UVB-induced apoptosis. Percentages of apoptotic cells after different treatments. Data refer only to cells which remained in the monolayer. It is noteworthy that CNF1 is capable of strongly decreasing the percentage of apoptosis with respect to UVB (*, $P<0.01$). The values are the means$\pm$S.D. from six separate experiments. Statistical significance was determined by Student's *t*-test

was comparable to the percentages in control cells at the same time (23.1% and 25.2% at 24 and 48 h, respectively).

Cell spreading promoted by CNF1 protected HEp-2 cells from the radiation-induced actin cytoskeleton changes

Scanning electron microscopy analyses showed that, in control cells (Figure 3a), UVB exposure induced cell retraction, alterations of surface microvillous structures and the formation of small bulb-like protrusions in cells adhering to the substrate (Figure 3c). In contrast, CNF1 pre-treated cells appeared to be firmly attached to the substrate (Figure 3d) and showed a CNF1-like (Figure 3b) surface ultrastructure even after exposure to UVB. In fact, the cells maintained stable cell-to-cell and cell-substrate interactions without undergoing rounding or detachment from the substrate (Figure 3d). When cells were labeled with rhodamin-phalloidin for F-actin detection, the disappearance of actin organization induced by UVB (Figure 3g) was evident. On the contrary, an increase in actin stress fibers was observed in HEp-2 cells treated with CNF1 (Figure 3f) with respect to control cells (Figure 3e) and the actin disorganization induced by radiation was significantly prevented by pretreatment with CNF1 (Figure 3h).

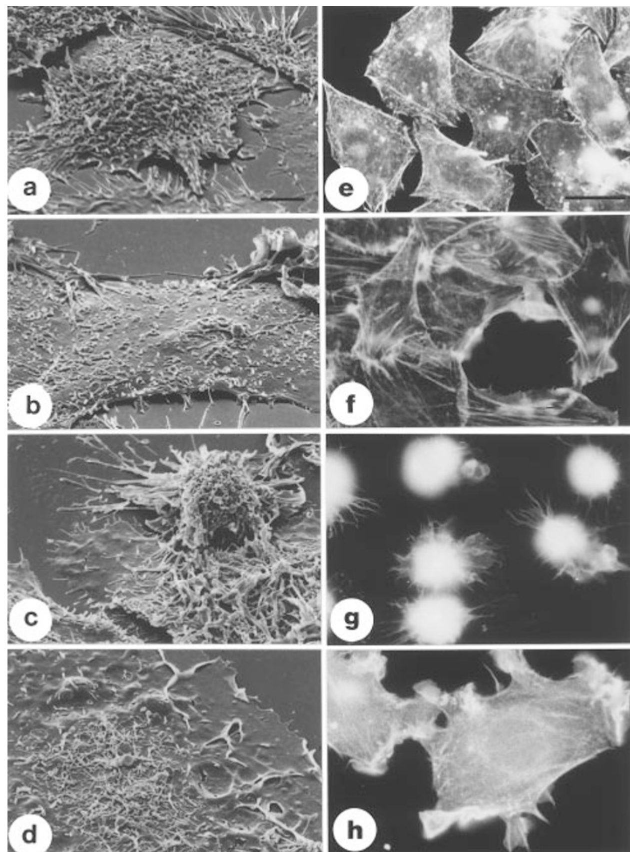

Figure 3 CNF1 treatment protects from the UVB-induced cell damage. Left panel: scanning electron micrographs and right panel: fluorescence micrographs of cells stained for F-actin detection of (**a**,**e**) control cells, (**b**,**f**) cells treated for 48 h with CNF1, (**c**,**g**) UVB-treated cells, (**d**,**h**) cells treated for 48 h with CNF1 and then with UVB

The actin filament-disrupting agent CB, although inducing multinucleation and cell spreading, does not protect cells against apoptosis

We next investigated the possibility that the protective effect exerted by CNF1 on apoptosis could be related to the ability of this toxin to induce multinucleation, cell spreading and to increase the cell volume. For this purpose, we used CB, which is able, in a certain range of concentrations, to cause multinucleation, to promote cell spreading and to increase cell volume by directly interacting with the actin filaments without involving the Rho proteins (Cooper, 1987). The CB dose used in this study was 0.5 μg/ml, i.e. the minimal toxin dose causing the formation of flattened, multinucleated cells

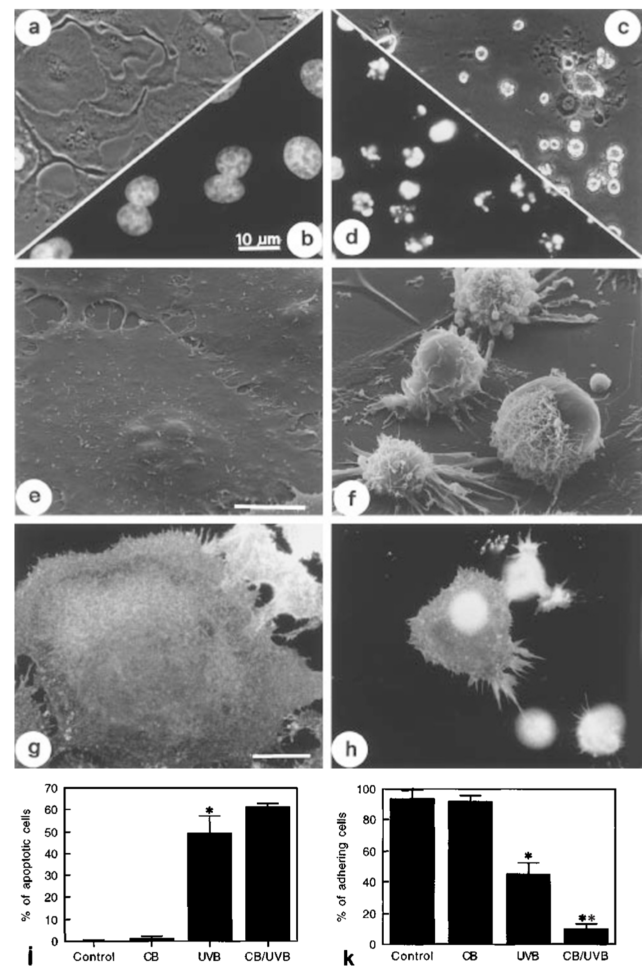

Figure 4 CB treatment does not prevent cell retraction and apoptosis induced by UVB in HEp-2 cells. (**a**,**c**) Phase contrast, (**b**,**d**) fluorescence microscopy of cells stained with Hoechst 33258, (**g**,**h**) fluorescence microscopy of cells stained for F-actin detection, and (**e**,**f**) scanning electron microscopy. Cells treated for 48 h with CB are shown in (**a**,**b**,**e**,**g**); cells treated 48 h with CB and then with UVB are shown in (**c**,**d**,**f**,**h**). (**j**) Percentages of apoptotic cells after different treatments. (**k**) Percentages of cells still adhering to the substrate after different treatments. Note that the percentage of apoptosis shown in (**j**) is inversely proportional to the percentage of adhering cells shown in (**k**). The values are the means$\pm$S.D. from four separate experiments. Statistical analysis was performed by Student's *t*-test. (*) in (**j**) and (**k**) indicates $P<0.01$ *versus* control and CB-treated cells. (**) in (**k**) indicate $P<0.01$ *versus* UV-radiated cells

within 48 h. Figure 4 shows the results obtained by phase contrast (Figure 4a and c), fluorescence (Hoechst: Figure 4b and d; FITC-phalloidin: Figure 4g and h) and scanning electron microscopy (Figure 4e and f). After exposure to CB, HEp-2 cells became large, flattened and multinucleated (Figure 4a), with two or more nuclei regularly-shaped (Figure 4b) and devoid of stress fibers (Figure 4g). UVB radiation of CB-treated cells induced a remarkable loss of adhesion to the substrate in a number of cells which finally detached (Figure 4c). The nuclei of most adherent cells showed apoptotic features (Figure 4d), and the actin organization was completely compromised (Figure 4h). In accordance with these morphological observations, the percentage of apoptotic cells after treatment with CB/UVB was significantly increased (61.4%) with respect to UVB-irradiated cells (49.2%) as viewed in Figure 4j. Interestingly, the percentage of cells undergoing apoptosis after each treatment (Figure 4j) was inversely related ($P<0.01$) to the percentage of cells which, in the same conditions, still remained adherent to the substrate (Figure 4k). In fact, the percentage of these cells was significantly decreased by pre-exposure (48 h) to CB (Figure 4k). Thus, it seems that multinucleation, cell spreading and the increase in cell volume are not *per se* sufficient to counteract apoptosis.

CNF1 protects HEp-2 cells from the UVB-induced detachment by improving cell adhesion

We have recently reported that the activation of Rho by CNF1 is an event clearly detectable within the first hour of treatment (Flatau *et al*, 1997). We have also observed, however, that most of the properties of CNF1, such as multinucleation and phagocytic behaviour, are late events which occur after more than 12–24 h (Falzano *et al*, 1993). Interestingly, the ability of toxin-exposed cells to ingest particles increases with time and is inversely related to the property of such cells to counteract apoptosis (Fiorentini *et al*, 1997b). In fact, as shown in Figure 5a (▲), the protection against apoptosis was more efficient by prolonging the time of exposure to CNF1. This finding was in agreement with the time-dependent ability of CNF1-treated cells to: (i) impair the cell detachment which followed exposure to UVB (Figure 5a, □) and (ii) increase the adhesion capabilities as measured on plastic Petri dishes, the substrate on which all the experiments herein reported were performed (Figure 5b). Thus, the improvement in cell adhesion which accompanies CNF1 activity seems to be a requisite for counteracting apoptosis. Statistical analyses of the relationships between these

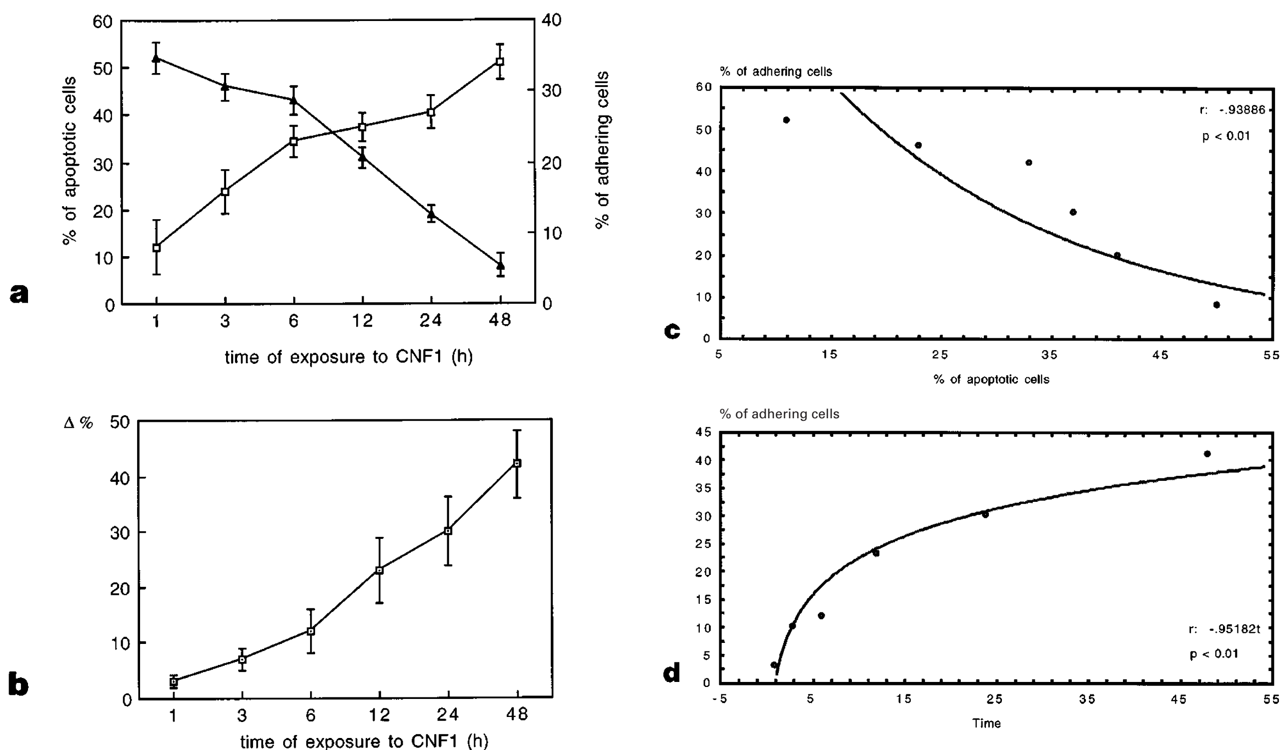

Figure 5 CNF1 protection of HEp-2 cells from UVB-induced detachment and apoptosis is a time-dependent phenomenon. (**a**) Percentage of apoptosis induced by irradiation (▲) and percentage of adhering cells after UVB exposure (□) as a function of different times of CNF1 pre-treatment. (**b**) Adhesion test of HEp-2 cells after different times of exposure to CNF1 represented in △% of adherent cells with respect to controls. The values are the means ± S.D. from six separate experiments. Statistical significance determined by Student's *t*-test indicates that CNF1 significantly increases cell adhesion capability with respect to untreated cells ($P<0.01$). Statistical analyses of these results indicated a significant inverse correlation between the percentage of apoptotic cells and that of cells adhering to the substrate (**c**). In addition, a significant correlation between CNF1 exposure and cell adhesion was also found (**d**)

events indicated in fact a significant correlation between cell adhesion and apoptosis following increased time of exposure to CNF1 (Figure 5c, d).

CNF1 modulates, in HEp-2 cells, the expression of antigens involved in cell adhesion

We then quantitatively evaluated whether CNF1 administration could modulate the expression of some surface and cytosolic antigens related to cell adhesion. By flow cytometry we analyzed (as reported in Table 1): (i) proteins controlling cell-substrate relationships, such as the immunoglobulin-like molecule CD44 hyaluronic acid receptor), integrins such as α1 (collagen receptor), α2 (collagen receptor), α3 (laminin, collagen and fibronectin receptor), α4 (fibronectin receptor), α5 (fibronectin receptor), α6 (laminin receptor), αv (vitronectin, fibronectin and collagen I receptor) and the adhesion plaque-related molecule FAK; and (ii) proteins involved in cell-cell contacts, such as ICAM-1, E-cadherin, α-catenin and β-catenin. No significant quantitative alterations of CD44, α1, α2, α3 and α4 were detectable by FACS analyses. However, 48 h of exposure to CNF1 caused a significant ($P<0.01$) decrease (20%) of ICAM-1 and a parallel increase in α5 (36%), α6 (20%), αv (35%), FAK (48%), E-cadherin (52%), α-catenin (33%) and β-catenin expression (24%) (Table 1 and Figure 6). Thus, both cell-cell and cell-substrate interactions can be modulated by CNF1 and these changes are probably involved in the toxin-induced mechanisms preventing apoptosis.

Table 1 Quantitative evaluation of the expression, detected by flow cytometry, of surface and cytosolic antigens related to cell adhesion

Antigens related to cell adhesion	Control	CNF1	Δ %
ICAM-1 (s)*	513	415	−20
E-cadherin (c)**	186	283	+52
α-catenin (c)*	258	345	+33
β-catenin (c)*	309	385	+24
FAK (c)**	172	255	+48
CD44 (s)	693	684	−2
α1 (s)	461	449	−3
α2 (s)	451	437	−3
α3 (s)	617	625	+1
α4 (s)	145	151	+4
α5 (s)*	527	682	+36
α6 (s)*	520	624	+20
αv (s)*	332	449	+35

Calculation of fluorescence was carried out after conversion of logarithmically amplified signals into values on a linear scale and expressed as median values. (s) and (c) indicate cell surface and cytosolic labeling, respectively. *indicates a $P<0.003$; **indicates a $P<0.001$ (calculated by using K/S test)

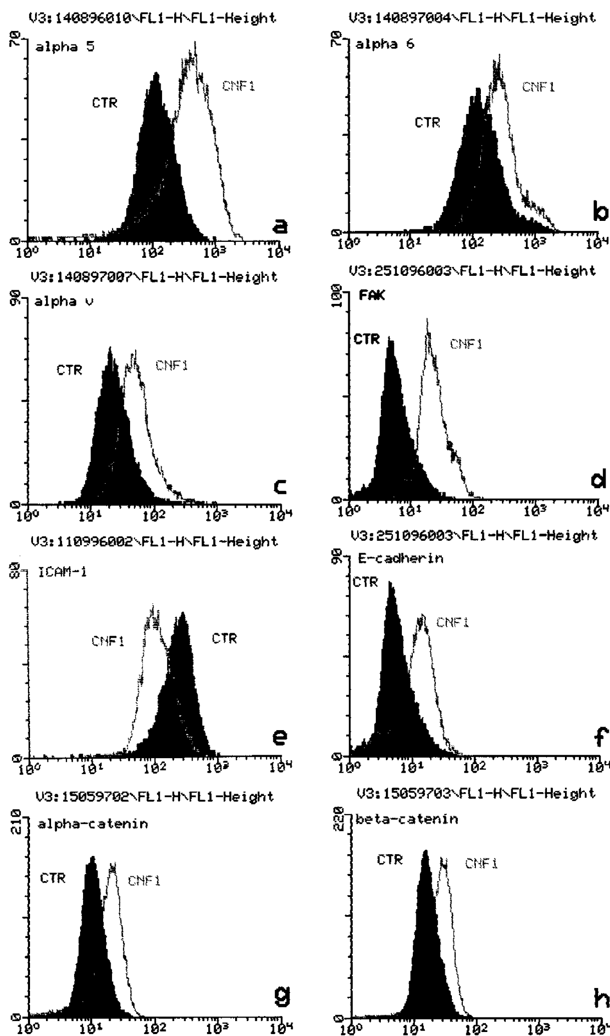

Figure 6 CNF1 provokes a change in the expression of some surface and cytosolic molecules related to cell adhesion. Histograms of flow cytometric analysis of the cell-surface or cytoplasmic molecules α5 (**a**), α6 (**b**), αv (**c**), FAK (**d**), ICAM-1 (**e**), E-cadherin (**f**), α- (**g**) and β- (**h**) catenin. A shift of curves indicates a decrease of ICAM-1 and a parallel increase in α5, α6, αv, FAK, E-cadherin, α-catenin and β-catenin expression after exposure to CNF1 for 48 h. On the abscissa, FL1 indicates green fluorescence (log. scale), the ordinate indicates the relative cell number. One experiment representative of six is shown

CB does not influence the expression of antigens involved in cell adhesion in HEp-2 cells

We analyzed the quantitative expression of some surface and cytosolic antigens related to cell-cell and cell-substrate relationships after 48 h of CB treatment. In particular, we performed this analysis comparing the median values of the histograms relative to control and CB-treated cells. No significant quantitative alterations of α5 (control: 427; CB: 431), α6 (control: 537; CB: 520), αv (control: 345; CB: 347), FAK (control: 320; CB: 340); ICAM-1 (control: 499; CB: 501); E-cadherin (control: 210; CB: 209); α-catenin (control: 261; CB: 259); β-catenin (control: 304; CB: 309) were detectable by FACS.

Discussion

The importance of cell adhesion processes in the induction of apoptosis has recently been underlined by several authors (Meredith and Schwartz, 1997). For instance, those cell types which require attachment to a substrate in order to grow undergo apoptosis when detached from the substrate (or when attachment is prevented) (Guadagno *et al*, 1993, Meredith *et al*, 1993). This type of apoptosis due to a

'homeless' condition has been called 'anoikia' (from greek $\alpha\nu\alpha$ (without) $\omega\lambda\kappa\lambda\alpha$ (home); Frisch and Francis, 1994; Frisch, 1997). Our work may add new insights in this field dealing with the promotion of cell spreading by a toxin-driven Rho-dependent mechanism which significantly protects epithelial cells against apoptosis.

In order to adhere to a substrate or to other cells in a monolayer, a cell needs the active involvement of several proteins which form organized complexes, i.e. adhesion plaques and intercellular junctions. Such protein complexes are in turn linked to the cytoskeleton network, in particular to the actin microfilament system (Yamada and Geiger, 1997). For instance, establishment of cadherin-dependent cell-cell contacts requires the activity of the small GTPases Rho and Rac (Braga *et al*, 1997). The augmented expression, in cells exposed to CNF1, of E-cadherin as well as of α-catenin and β-catenin, all proteins controlling in different ways the interactions between cells (Huber *et al*, 1996), suggests a role played by these molecules in hindering apoptosis. This is consistent with the observation that the Rho-independent effects of CB in cells did not provoke the overexpression of such molecules, CB-treated cells undergoing apoptosis at a significantly higher percentage than control cells. The importance of cell-cell adhesion in favouring cell survival has been previously reported, for example, in intestinal cells forced to detach by a bacterial toxin known to perturb the actin cytoskeleton (Mahida *et al*, 1996). Such cells, however, although in suspension, remained alive while linked to each other.

To date, however, the majority of studies on apoptosis and cell adhesion have been focused on those mechanisms which control the interaction between a cell and the extracellular matrix (ECM). Cell-substrate relationships could be considered as composed by at least two different processes: a 'static' one (cell attachment) and a 'dynamic' one (cell spreading). The cooperation between them leads to the cell 'homing' process, which was suggested by Frisch and Francis (1994) to be a complex regulatory mechanism of cell survival and death associated with the modulation of cell-substrate interactions. In fact, integrin-mediated attachment to the extracellular matrix promotes cell survival (Boudreau *et al*, 1995; Brooks *et al*, 1994; Frish and Francis, 1994; Meredith *et al*, 1993; Montgomery *et al*, 1994). On the other hand, lack of adhesion leads to anoikia and the acquisition of anoikia resistance could facilitate anchorage-independent growth and, perhaps, transformation (Frisch *et al*, 1996). Zhang *et al*. (1995) first showed that integrin $\alpha5\beta1$ binding to fibronectin was especially efficient at preventing the death of cells in serum-free medium. The crucial role was played by the $\alpha5$ cytoplasmic domain possibly by regulating the expression of the cell death suppressor Bcl-2 (Zhang *et al*, 1995). Ligation of integrin $\alpha5\beta1$ in $\alpha5$-transfected tumor cells which exhibit reduced proliferation as compared with untransfected cells, also prevented apoptosis by inducing Bcl-2 expression (Stromblad *et al*, 1996). Ligation of integrin $\alpha v\beta3$ in endothelial cells suppresses p53 activity and increases the Bcl-2:Bax ratio, promoting cell survival (Clarke *et al*, 1995; Stromblad *et al*, 1996). In contrast, blocking integrin $\alpha v\beta3$ ligation with integrin antagonists induced p53 activation and blocked Bcl-2 expression (Stromblad *et al*, 1996). Thus, in these cell types, ligands to integrins have been shown partially to suppress the apoptotic cell death program. As concerns CNF1, we have recently reported that the toxin can increase the expression of Bcl-2 and Bcl-X_L in epithelial cells (Fiorentini *et al*, 1998b), findings which are consistent with the augmented expression of $\alpha5$ and αv (and possibly $\alpha6$) that we have measured by flow cytometry in CNF1-treated cells. Moreover, since the growth matrix did not influence the destiny of cells exposed to the toxin (data not shown), we speculate that, accordingly to other reports (Zhang *et al*, 1995; Levkau *et al*, 1998), it is probably the activity of integrins as signal transducers in the cytosol more than the link to ECM which drives cells towards survival or death. The direct activation of Rho in cells by CNF1, which in turn increases the expression of anti-apoptotic proteins, may perhaps allow cells to overcome the need for a specific substrate for growth.

Interactions of integrins with extracellular matrix proteins can activate integrin signal transducers such as the focal adhesion kinase (FAK), which participates in the control of anchorage dependence. FAK can regulate apoptosis in normal epithelial and endothelial cells and the conferral of resistance to anoikia may suffice to transform certain epithelial cells (Frish *et al*, 1996, Levkau *et al*, 1998). In this study, we have observed that CNF1 increases the expression of FAK. Interestingly, CNF1 has previously been shown to induce a Rho-dependent tyrosine phosphorylation of FAK (Lacerda *et al*, 1997). How can FAK mediate the integrin-dependent survival signals? One possibility is that FAK, upon activation by CNF1, in turn activates the mitogen-activated protein (MAP) kinase through the Ras-Raf pathway (Schlaepfer *et al*, 1994). Interestingly, MAP kinase activation has been reported to suppress apoptosis (Xia *et al*, 1995).

Beside the phosphorylation of FAK (Lacerda *et al*, 1997), CNF1-promoted Rho activation induced contractility and cell spreading (Fiorentini *et al*, 1997a) as well as the polymerization of actin into prominent stress fibers, probably via activation of a cytoskeleton-associated phosphatidylinositol 4-phosphate 5-kinase (Fiorentini *et al*, 1997a; Burridge *et al*, 1997). Cell spreading is a phenomenon which is driven by forces deriving from actin polymerization (Theriot and Mitchison, 1991) and by actin-myosin-based forces (Cramer and Mitchison, 1995). Cell adhesion to extracellular matrix proteins is responsible for cell spreading through integrin receptors which promotes integrin clustering and cytoskeletal organization and forces cells to spread (Hynes, 1992). It is current opinion that cell spreading induced by integrin ligation, and the subsequent intracellular signaling, are needed to avoid apoptosis (Re *et al*, 1994, Ruoslahti, 1997) and that attachment *per se* does not prevent cell death (Ruoslahti and Reed, 1994). This is because the ability of anchorage-dependent cells to proliferate is mainly linked to the cell spreading and not to the attachment (Ruoslahti, 1997). We have to underline that, at least in our system, cell spreading *per se* was not enough to circumvent the apoptotic response. In fact, the flattening (spreading) provoked by certain doses of CB in epithelial cells, was inefficient in triggering cell survival.

Thus, in order to thrive, cells need additive factors which, in case of the CNF1-induced cell response, may be represented by the actin assembly into prominent stress fibers and, overall, by the Rho activity as controller.

The hypothesis of Rho as 'supervisor' of cell survival or death is now supported by several lines of evidence. Rho plays a selective role in early thymic development as a critical determinant for proliferation and cell survival signals, its inactivation by *C. botulinum* C3 leading to apoptosis (Henning *et al*, 1997). Inactivation of Rho proteins has also been reported to induce apoptosis in murine T lymphoma cells (Moorman *et al*, 1996) and GDI, an abundant cell GDP-dissociation inhibitor for the Ras-related Rho family GTPases, is a substrate of the apoptosis protease CPP32/ Caspase 3 (Na *et al*, 1996). Moreover, bacterial protein toxins inhibiting the activity of proteins of the Rho subfamily may induce apoptosis in intestinal cells (Fiorentini *et al*, 1993; 1998a). Consistent with these findings which indicate that inactivation of Rho is a negative signal for survival, is our observation that CNF1, which activates Rho, protects epithelial cells from apoptosis (Fiorentini *et al*, 1997b). Very recently, a direct link between the functional state of Rho and Bcl-2 expression in controlling the apoptotic pathway has also been suggested (Fiorentini *et al*, 1998b; Gomez *et al*, 1997).

Taking our results together with data previously reported (for a review see Meredith and Schwartz, 1997), promotion of Rho-dependent cell spreading and actin bundling results in protection against apoptosis. Thus, we propose CNF1 as a new and extremely useful tool for studying the relationships between apoptosis and the cytoskeleton. Not all data herein reported, however, can be directly referred to cytoskeletal modulation and several questions remain unanswered. For instance, it remains to be clarified why CNF1 decreases the expression of ICAM-1 and how this can be involved in apoptosis protection. Speculatively, since ICAM-1 is a molecule which binds leukocyte-specific surface antigens (Malik and Lo, 1996), the lack of interaction between CNF1-altered cells and immune cells might protect the former from being removed. This might allow the survival of damaged cells and their entry into the cell cycle, thus avoiding apoptosis. On the other hand, we have also to remember that CNF1 is a toxin produced by intestinal bacteria and that apoptosis is critical for maintaining the integrity of intestinal epithelium. As intestinal stem cells ascend upward from basement membrane along the crypt-villous axis toward the intestinal lumen, cellular differentiation and proliferative arrest do occur. Having reached the villi surface, cells become fully differentiated, undergo apoptosis and are shed in the lumen. Factors capable of prolonging cell survival could influence the integrity and function of the intestinal mucosa. Prolonged cell survival, together with increased adhesion to matrix components might have significant biological consequences and affect the tumorigenic potential of epithelial cells as previously reported (Tsujii and DuBois, 1995). Thus, considering the role of CNF1 in human pathology (Caprioli *et al*, 1987; Cherifi *et al*, 1990; De Rycke *et al*, 1990) apoptosis may turn out as being a new virulence mechanism for CNF1-producing bacteria.

Materials and Methods

Cell cultures

HEp-2 cells were grown at 37°C in DMEM medium, supplemented with 10% fetal calf serum (FCS) (Flow Laboratories, Irvine, UK), 1% non-essential aminoacids, 5 mM L-glutamine, penicillin (100 U ml) and streptomycin (100 μg ml)) in a 37°C incubator containing an atmosphere of 95% air and 5% CO_2. The subcultures were serially propagated after harvesting with 10 mM EDTA and 0.25% trypsin in phosphate buffer solution (PBS, pH 7.4).

Treatments

10^{-10} M CNF1 (purified as previously described (Falzano *et al*, 1993) from the *E coli* BM2-1 strain) or 0.5 μg ml cytochalasin B (CB, Sigma Chemical Co, St Louis, MO) were directly added to the culture medium at the same time of seeding. After different times (i.e. 1, 3, 6, 12, 24 and 48 h) of toxin treatment, HEp-2 cells were exposed to different apoptotic inducers (UVB, Etoposide or serum starvation). Samples were then prepared for fluorescence and scanning electron microscopy or for flow cytometry. All the experiments were performed at least four times with triplicate samples for each point.

Induction of apoptosis

Three different apoptotic inducers have been used: (i) a physical agent; (ii) a chemical agent and (iii) the withdrawal of growth factors.

UVB exposure Control and CNF1-treated HEp-2 cells were exposed to UVB irradiation in phosphate-buffered saline (PBS) using a Philips TL 20 W/12 lamp localized in a sterile hood. The plastic Petri dishes containing the cells were placed without covers at the vertical distance of 10 cm from the centre of the tube to UVB. In order to eliminate UVC radiation, a Kodak filter (Kodacell TL 401) with an optical density of less than 0.4 for wavelengths below 285 nm was employed and was placed on the Petri dishes during exposure. In these conditions, the UVB radiant flux density to the cells was 2.2 Wm^{-2}, as verified by an Osram Centra UV meter. The filter used in our experiments has an optical density of 2.5 at 285 nm, 3 at 280 nm and above 4 for wavelengths below 270 nm. Therefore we can estimate that the contamination by UVC to cells does not exceed 0.003% of the total UVB. Twenty-four hours after the end of the UVB irradiation, control and treated cells were prepared for fluorescence and scanning electron microscopy or for flow cytometry.

Etoposide treatment Etoposide is an anticancer drug capable of inducing apoptosis in epithelial cells (Oberhammer *et al*, 1993). Forty-eight hours after cell seeding or CNF1 exposure, 200 μM Etoposide (Sigma; stock solution 0.01 M in ethanol) was added directly to the culture medium for 48 h. Cells treated with an equal volume of ethanol were considered as control.

Serum starvation Forty-eight hours after seeding or CNF1 exposure, HEp-2 cells were completely deprived of the FCS contained in the growth medium and then further cultured for 48 h.

To evaluate the percentage of apoptosis in adherent control and treated cells the chromatin dye Hoechst 33258 (Molecular Probes Inc, Or) was used. Quantitative evaluation of apoptotic cell death was performed by counting at least 500 cells at high magnification (500 $\times$) in triplicate samples for each point as previously described (Malorni *et al*, 1994). The numbers reported were the mean values $\pm$ standard deviation (S.D.) from the experiments repeated six times.

Adhesion assay

Stock preparations of collagen I, vitronectin, fibronectin, laminin, hyaluronic acid or bovine serum albumin (BSA) were distributed in 60 mm diameter Petri dishes (Costar, Cambridge, MA, USA) at the final concentrations of 10 mg cm^3, 0.1 mg cm^3, 5 mg cm^3, 2 mg cm^3, 5 mg cm^3 and 3%, respectively. After 2 h at room temperature, the coated dishes were washed three times with PBS to remove non-immobilized proteins. To saturate the remaining protein binding sites 2 ml of PBS supplemented with 1% heat-denatured BSA were added and the plates were incubated at room temperature for 1 h. The attachment assay was adapted from that described by Malorni *et al*, (1995).

Control and CNF1-treated cells (5×10^4) were suspended in 4 ml DMEM containing 10% fetal calf serum, seeded into coated Petri dishes and incubated for 30 min at 37°C. After incubation, the 'unattached' cells were removed and the 'attached' cells were harvested from the assay dishes using 10 mM EDTA and 0.25% trypsin. Unattached and attached cells were counted by ZBI Coulter Counter.

Percentages of attachment were calculated as follows:

$$\frac{\text{Counts in matrix fraction}}{\text{Counts in matrix fraction counts in supernatant}} \times 100$$

The data obtained represent the average of measurements made on three dishes. All the experiments were repeated four times.

Fluorescence microscopy

HEp-2 cells were grown on 13 mm-diameter glass coverslips in separate wells (5×10^4 cells/well) in a 37°C incubator containing an atmosphere of 95% air and 5% CO_2. Following toxin and UVB treatments, both control and treated cells were fixed with 3.7% formaldehyde in PBS with 2% bovine serum albumin (BSA), for 10 min at room temperature. After washing in the same buffer, cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. To analyze the nuclei, cells were stained with Hoechst 33258 (Sigma, working dilution 1 : 1000) at 37°C for 30 min. For F-actin detection, cells were stained with fluorescein-phalloidin (Sigma, working dilution 1 : 500) at 37°C for 30 min. Finally, after washings, coverslips were mounted with glycerol-PBS (2 : 1) and analyzed with a Nikon Microphot fluorescence microscope.

Scanning electron microscopy

Control and treated cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 20 min. Following post-fixation in 1% OsO_4 for 30 min, cells were 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.

Flow cytometry

Detection of intracellular antigens Control and treated HEp-2 cells were pelleted, fixed in 70% ice-cold methanol, and washed twice with cold PBS. For the detection of focal adhesion kinase (FAK), E-cadherin, α-catenin or β-catenin cells were stained with specific monoclonal antibodies (mAb) (Chemicon International, Inc., Temecule, CA) for 30 min at 4°C. After washing, cells were incubated for 30 min at 37°C with FITC-labeled anti-mouse mAb (Sigma), washed and analyzed.

Determination of cell-surface molecules The membrane expression of ICAM-1 (CD54), CD44, α1 (CD49a), α2 (CD49b), α3 (CD49c), α4 (CD49d), α5 (CD49e), α6 (CD49f) and αv (CD51) was studied by incubating cells for 30 min at 4°C with specific monoclonal antibodies (Chemicon). After washings with ice-cold PBS containing 10 mM NaN_3, 1% BSA (Sigma) and 0.002% EDTA, cells were incubated for 30 min at 4°C with FITC-labeled anti-mouse mAb (Sigma), washed and immediately analyzed by a Hewlett Packard computer using the Lysys II software (Becton Dickinson, San José).

Cell cycle analysis Biparametric DNA/BrdU analysis was performed by using propidium iodide (PI; 40 μg ml), Bromodeoxyuridine (BrdU; 20 μM) and mAb against BrdU (Chemicon) as previously reported by Vindelov and Christensen, 1990. The samples were then analyzed on a FACScan flow cytometer (Becton Dickinson) performing excitation at 488 nm and collecting FL1=green fluorescence (FITC-BrdU) at 515–540 nm and FL2=red fluorescence (PI-DNA) above 620 nm. The percentage of cells in different phases of the cell cycle was determined by a Hewlett Packard computer using the Lysys II software (Becton Dickinson).

Statistical analyses

Values reported in Figures 2, 4 and 5 are given as the mean $\pm$ S.D. Student *t*-test ('Statistics' program for Macintosh) for correlated samples was used. A *P* value of less than 0.01 was considered significant. Concerning flow cytometry, the statistical significance of the values obtained, reported in Table 1, was calculated by using the Kolmogorov-Smirnov (K/S) test included in Lysys II software (Becton Dickinson). Asterisks in Table 1 indicate antigens which undergo significant quantitative variation after CNF1 treatment.

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