The inhibition of cell spreading on a cellulose substrate (cuprophan) induces an apoptotic process via a mitochondria-dependent pathway

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Abstract Cell shape was found to be a strong indicator of whether individual cells grow or die, and may play an important role in controlling apoptosis as well as cell growth. We compared here the behaviour of rounded Swiss 3T3 cells aggregated on a cellulose cuprophan membrane to those cultured on dish polystyrene. We demonstrated that cells aggregated on cellulose substrates for up to 48 h underwent programmed cell death that was associated with phosphatidylserine flipping and caspase 9 and caspase 3 activation, suggesting a mitochondria-dependent apoptotic process. In addition, we found that this phenomenon cannot be entirely explained by disengagement of α5β1 integrin ligation.

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Key words: Apoptosis; Caspase; Cell aggregation; Biomaterial; Fibroblast

1. Introduction

Cell shape influences whether individual cells grow or die, and may also be important in controlling apoptosis [1–3]. The growth of endothelial cells on micropatterned substrates has shown that altering the geometry of cell spreading can determine whether the cells enter the three major genetic programmes that govern angiogenesis – growth, apoptosis and differentiation [3,4]. The inhibition of anchorage-dependent endothelial cell spreading [5] also triggers rapid apoptosis, as does the loss of matrix adhesion in fibroblasts [6] or the unligated state of T24E carcinoma cell integrins [7].

Studies with Swiss 3T3 fibroblasts have indicated that cell spreading is mediated by both cell surface integrin receptors and the small GTP binding protein RhoA [8] – a Rho GTPase member of the Ras superfamily that regulates many cell functions [9]. Recent data suggesting that RhoA is downstream of integrin engagement [10] have confirmed that the former is implicated in cell shape modification [11,12]. RhoA is also involved in the regulation of integrin activation by promoting avidity modulation, a process known as 'inside-out signalling' [13–15].

Our previous studies have focused on early cellular events induced by attachment to substrates which differ in their ability to adsorb serum adhesive proteins. We showed that Swiss 3T3 cells remained rounded when grown on cuprophan (CU, a cellulose substrate to which serum adhesive proteins are poorly adsorbed) and that they then underwent cAMP-dependent aggregation. In contrast, control cells grown on polystyrene (PS, which favours vitronectin (VN) and fibronectin (FN) adsorption) spread well and contained little cAMP [16]. CU does not promote the formation of either focal contacts or actin stress fibres, and the plasma membranes of cells on CU contain less RhoA than those of control cells on PS [17]. Other authors have reported that contact with CU causes neutrophils and monocytes to undergo apoptosis [18,19]. Here, we compared the behaviour of cells cultured on CU or PS for 24 and 48 h; we also investigated the apoptotic changes that occur in rounded Swiss 3T3 cells aggregated on CU by examining both mitochondria-dependent and death receptor (Fas) apoptosis pathways. The viability and proliferation rates of cells aggregated on CU were first compared to those for cells spreading on PS. The initiation and progress of apoptosis were then assessed by monitoring phosphatidylserine flipping [20–22] and caspase activity [23–25]. Our results indicate the occurrence of a mitochondria-dependent apoptotic process in cells aggregated on cellulose substrates. This phenomenon cannot be fully explained by integrin ligation disengagement.

2. Materials and methods

2.1. Materials

All studies were performed with sterile CU membranes (Lundia Alpha 500, Gambro, Sweden) cut into 35 or 60 mm diameter discs and placed in Petri dishes. Controls were Nunclon® PS culture plates.

2.2. Cell culture

Swiss mouse 3T3 cells (European Collection of Cell Cultures, ECACC number 85022608, UK) were grown in PS culture plates with Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Eragny, France) supplemented with 10% foetal bovine serum (batch number 40F5493K, South American origin, Gibco BRL), penicillin (100 U/ml Eurobio, Les Ulis, France) and streptomycin (100 μg/ml, Eurobio). After having been passaged 2 or 3 days prior to use, cells were seeded onto CU or PS at a density of 1×10^4 cells/cm^2 and were cultured for 6, 24 or 48 h before being detached by trypsinisation.

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2.3. Proliferation assays

Cell growth was assessed using the PKH26 (Sigma Aldrich Chimie, St Quentin Fallavier, France) dye dilution method [26]. Briefly, flow cytometry (FC) was used to measure the fluorescence intensity of PKH26-stained cells immediately after labelling and following culture for various lengths of time. The percentage decrease in fluorescence intensity is proportionate to cell growth. Cell proliferation was also assessed by counting unlabelled cells in a Malassez haemocytometer.

2.4. Assessment of cell death

Cell membrane integrity was analysed using the propidium iodide (PI) fluorescent label in combination with FC. The trypan blue exclusion assay was also performed using a Malassez haemocytometer.

2.5. Annexin V binding assays

Annexin V labelling reveals the early stage translocation of phosphatidylserine from the inner leaflet to the outer leaflet of the bilayer plasma membrane. The cells were incubated with fluorescein isothiocyanate-labelled annexin V (Boehringer Mannheim, Meylan, France) and PI, and the percentage of apoptotic cells at time zero and after each culture time point was analysed by FC.

2.6. Measurement of mitochondrial transmembrane potential by $DiOC_6(3)$ labelling

Disruption of the mitochondrial transmembrane potential ($\Delta\Psi_m$) was evaluated with the $DiOC_6(3)$ cationic, cell-permeable, voltage-sensitive, lipophilic, fluorescent carbocyanine dye. A shift in the fluorescence profile of labelled cells indicates a change in the mitochondrial membrane potential, i.e. $DiOC_6(3)$-positive cells become $DiOC_6(3)$-negative when the potential is lost. Cells were washed in phosphate-buffered saline–5 mM EDTA and incubated for 20 min with 40 nM $DiOC_6(3)$ (Calbiochem, VWR, Fontenay-sous-Bois, France) and 5 μg/ml of PI at room temperature. Samples were immediately analysed by FC.

2.7. Caspase assays

2.7.1. Fluorometric measurement of caspase activities. Caspase activities were measured as previously described [27]. Briefly, cells were collected, lysed in buffer A (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride, supplemented with 10 μg/ml pepstatin, aprotinin and leupeptin), sonicated and centrifuged at $14\,000\times g$ for 5 min at 4°C. The supernatants were kept and were considered to be representative cytosol extracts. Aminomethylcoumarin (AMC)- or aminotrifluoromethylcoumarin (AFC)-conjugated peptide substrates corresponding to each caspase (to a final concentration of 2×10^{-5} M) were added to 20 μg (for caspase 3 and 8) or 30 μg (for caspase 9) of total cytosol protein. The coumarin dyes were measured spectrofluorometrically, with excitation at 360 nm and emission at 460 nm for AMC and excitation at 400 nm and emission at 505 nm for AFC. The specific substrates used were as follows: acetyl (Ac)-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) for caspase 3 (Bachem, King of Prussia, PA, USA), Ac-Ile-Glu-Thr-Asp-AMC (Ac-IETD-AMC) for caspase-8 (Peptide Institute, Osaka, Japan) and Ac-leu-Glu-his-Asp-AFC (Ac-LEDH-AFC) for caspase 9 (Enzyme Systems Products, Livermore, CA, USA). Caspase 3 activity was checked in the presence or absence of the Boc-D-FMK broad-spectrum caspase 3 inhibitor (Calbiochem).

2.7.2. Colorimetric assay of caspase 8 activation. Caspase 8 activity was measured colorimetrically at 405 nm using the specific substrate IETD-*p*NA (Calbiochem) in the presence of 100 μg of total cytosol protein. Cleavage of the C-terminal peptide bond releases *p*-nitroaniline. The caspase 8 activity in total cell lysates was evaluated in the presence or absence of the specific inhibitor Ac-IETD-CHO (Calbiochem). The activation capacity of caspase 8 was assessed by cross-linking the Fas antigen with a monoclonal antibody (mAb) (RK8 mAb, Immunotech, Marseille, France).

2.8. Flow cytometry

Flow cytometry was carried out in an EPICS Elite Cytometer (Beckman Coulter, Villepinte, France). For each sample, 20 000 stained cells were analysed.

2.9. Assessment of adhesion-dependent survival

As there was no available activating mAb to mouse α5β1 integrins, we used soluble FN, their natural ligand, to cross-link these adhesion molecules. Cells were first incubated for 1 h in 500 μl serum-free DMEM supplemented or not with 30 μg/ml of FN (Roche Diagnostics, Mannheim, Germany), then cultured for 6 h on CU in medium containing FN. Experiments were performed in quadruplicate and the percentage of apoptotic cells was measured by annexin V binding assays, as described in Section 2.5.

2.10. Statistical analysis

Data are presented as the means of triplicate measurements and were analysed using the Kruskal–Wallis non-parametric test. All statistical computations were performed with Graphpad Instat® 2.00 software.

3. Results

3.1. Growth of Swiss 3T3 fibroblasts decreases on CU

PKH26 dye dilution assays showed that cells grown on CU proliferated significantly more slowly than those grown on PS. The change in fluorescence intensity of cells cultured on CU for 24 and 48 h was significantly less than that of cells grown on PS (Fig. 1). These data were confirmed by daily counts of unlabelled cells. Cells on CU had proliferation rates of 1.03 and 1.6 at 24 h and 48 h respectively, whereas the comparable figures for cells on PS were 2.05 and 5.25. The growth rate differences at each time point were statistically significant ($P<0.001$).

3.2. Death of Swiss 3T3 fibroblasts increases on CU

The death rate for cells cultured on CU for 24 and 48 h was significantly higher than for cells spread on PS, as evaluated by PI labelling and FC (Fig. 1). These data were confirmed by daily trypan blue exclusion assays: 13.6% and 20.1% of dead cells on CU at 24 and 48 h respectively, versus 2.3% and 1.6% on PS. The death rate differences at each time point were statistically significant ($P<0.01$ at 24 h and $P<0.001$ at 48 h).

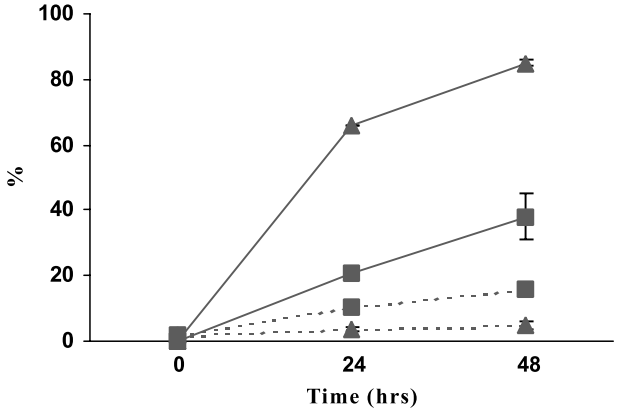

Fig. 1. Growth of Swiss 3T3 fibroblast cells cultured on CU (–■–) and on PS (–▲–) (control), as measured by the PKH26 dye dilution method and flow cytometry (decrease in fluorescence intensity %). The decrease in the fluorescence intensity of cells cultured on CU for 24 and 48 h was significantly smaller than that of cells cultured on PS, showing that cells aggregated on CU proliferate more slowly (Kruskal–Wallis test: $P<0.01$). Viability of Swiss 3T3 fibroblasts cultured on CU (- -■- -) and on PS (- - ▲ - -) (control), as assessed by PI (% cell death PI labelling) and flow cytometry. Cells cultured on CU for 24 or 48 h showed significantly higher death rates than cells on PS (Kruskal–Wallis test: $P<0.01$).

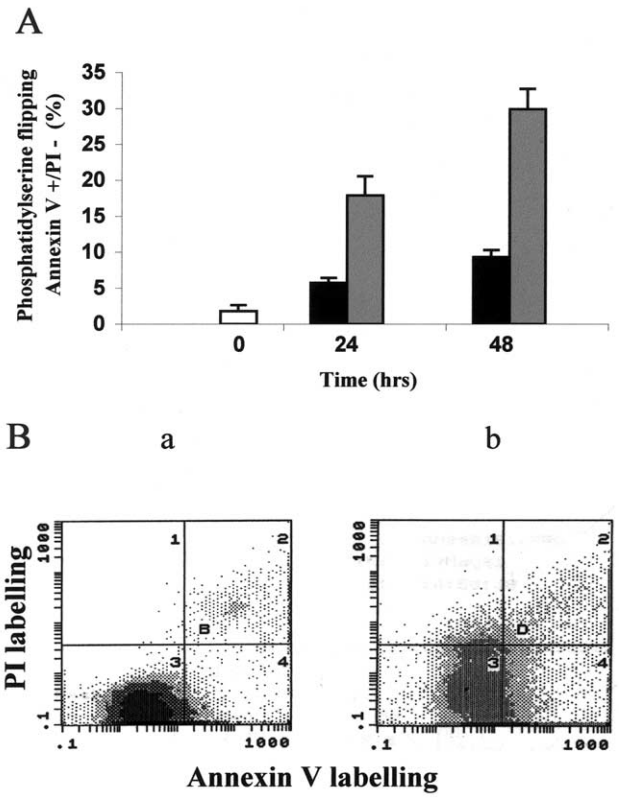

Fig. 2. Phosphatidylserine flipping of Swiss 3T3 fibroblasts cultured on CU (grey bars) and on PS (black bars) (control), assessed by annexin V binding and flow cytometry. Cells before culture (white bar). A: Fraction of annexin V-positive and PI-negative cells (i.e. live cells engaged in apoptosis). Phosphatidylserine flipping was significantly higher in cells cultured for 24 and 48 h on CU than in cells cultured on PS (Kruskal–Wallis test: $P < 0.001$). B: Shift in the spectra of cells cultured for 48 h on PS (a) and CU (b). Cell populations in the fourth quartile reveal the proportions of annexin V-positive and PI-negative cells.

3.3. *Apoptosis in Swiss 3T3 fibroblasts on CU is revealed by early stage phosphatidylserine flipping and annexin V labelling*

The percentage of cells engaged in early apoptosis (i.e. phosphatidylserine flipping) was assessed by annexin V labelling. Phosphatidylserine flipping was significantly higher in cells cultured for 24 and 48 h on CU than for cells on PS (Fig. 2A). This significant difference was also illustrated by the difference in the spectra of cells cultured for 48 h on CU (Fig. 2Bb) and on PS (Fig. 2Ba).

In light of these results, we then sought to determine whether the mitochondria-dependent pathway initiated by caspase 9 (upstream of the effector caspase 3) was involved in the apoptotic process seen on CU. We also checked for involvement of the Fas-dependent pathway by measuring caspase 8 activity.

3.4. *CU-induced apoptosis involves a mitochondria-dependent pathway, as evidenced by increased caspase 9 activity and disrupted mitochondrial transmembrane potentials in cells cultured on CU for 6 h*

We explored the state of the mitochondria-dependent pathway of the apoptotic cascade (i.e. upstream of the effector caspase 3) in Swiss 3T3 fibroblasts cultured on CU and on PS. Initiator caspase 9 activity was first measured fluorometrically. Disruption of the mitochondrial transmembrane potential was then assessed using $DiOC_6(3)$ labelling. Since the mitochondria-dependent pathway is an early stage in the apoptotic cascade, we measured these parameters in cells cultured for just 6 h, as well as for our usual 24 and 48 h time points.

3.4.1. Increased caspase 9 activity in cells cultured for 6 h on CU. After 6 h of culture, initiator caspase 9 activity (measured fluorometrically) was significantly greater in cells cultured on CU than in cells on PS, although this was not the case after 24 or 48 h (Fig. 3A).

3.4.2. Loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) in cells cultured for 6 h on CU. $DiOC_6(3)$ labelling showed that significantly more cells cultured on CU for 6 h had disrupted mitochondrial function (as indicated by a loss of $\Delta\Psi_m$) than cells cultured on PS for the same length of time, although again there was no difference between cells cultured on PS or CU for 24 and 48 h (Fig. 3B).

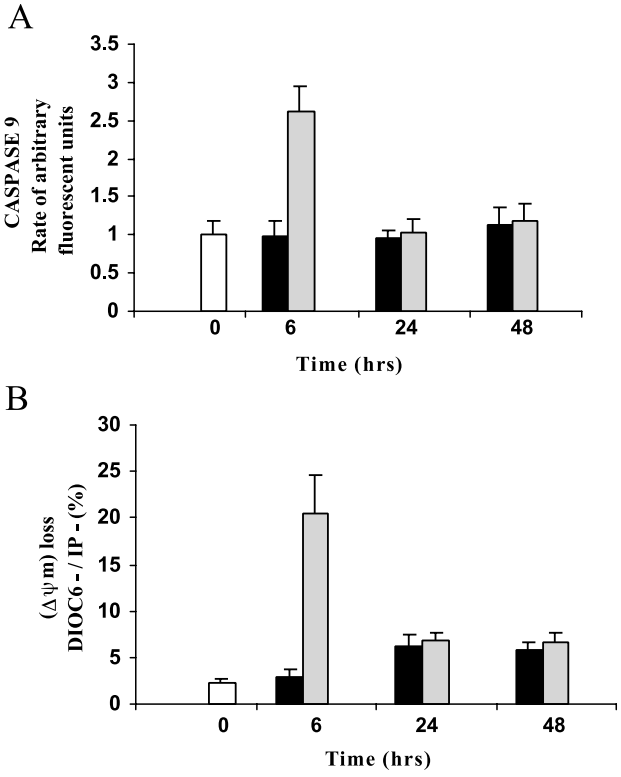

Fig. 3. Implication of the mitochondria-dependent apoptosis pathway in Swiss 3T3 fibroblasts cultured on CU (grey bars) and on PS (black bars) (control). Cells before culture (white bars). A: Fluorometric measurements of caspase 9: the activity in cells cultured on CU for 6 h was significantly greater than in cells on PS (Kruskal–Wallis test: $P < 0.001$), although there were no significant CU v. PS differences for cells cultured for 24 and 48 h. B: Loss of mitochondrial membrane potential ($\Delta\Psi_m$), as measured by $DiOC_6(3)$ labelling and flow cytometry. A shift in the fluorescence profile of labelled cells revealed a change in mitochondrial membrane potential, i.e. $DiOC_6(3)$-positive cells became $DiOC_6(3)$-negative. The graph shows the fraction of $DiOC_6(3)$-negative and PI-negative cells, i.e. live cells with $\Delta\Psi_m$ disruption. Significantly more cells cultured for 6 h on CU had lost their membrane potential than had cells on PS (Kruskal–Wallis test: $P < 0.001$), although there were no differences for cells cultured for 24 and 48 h.

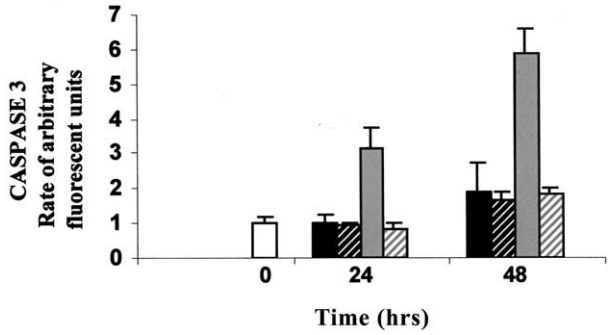

Fig. 4. Caspase 3 activity in Swiss 3T3 fibroblasts cultured on CU (grey bars), (CU+Boc-D-FMK) (light hatched bars) and on PS (black bars) (control) (PS+Boc-D-FMK) (dark hatched bars), cells before culture (white bar), as measured by fluorometry in the presence and absence of the Boc-D-FMK broad-spectrum caspase 3 inhibitor. Cells cultured on CU for 24 and 48 h had significantly greater caspase 3 activity than cells cultured on PS (Kruskal–Wallis test: $P < 0.001$). On CU, caspase 3 activity was significantly reduced in the presence of Boc-D-FMK (Kruskal–Wallis test: $P < 0.01$). The activity in cells on PS was not significantly different from the basal level measured immediately upon cell seeding (time 0), and the presence of Boc-D-FMK had no effect.

3.5. *Apoptosis in Swiss 3T3 fibroblasts on CU is associated with caspase 3 activation*

Effector caspase 3 activity in cells cultured on CU for 24 and 48 h was significantly higher than in cells cultured on PS (Fig. 4). This activity was significantly decreased by the broad-spectrum caspase 3 inhibitor Boc-D-FMK. Throughout the 48-h experiment, the activity in cells cultured on PS did not significantly differ from the basal level measured immediately upon cell seeding (time 0). The presence or absence of Boc-D-FMK had no effect on the measured activity. Thus, compared to PS, the CU substrate activated caspase 3 in Swiss 3T3 fibroblasts. We then checked whether the apoptosis detected on CU involved caspase 8 activation.

3.6. *Caspase 8 activity is not involved in the apoptosis of Swiss 3T3 fibroblasts on CU*

We checked initiator caspase 8 activity in order to explore the state of the Fas-dependent pathway (i.e. upstream of the effector caspase 3) in 3T3 cells cultured on CU and PS. Fluorometric and colorimetric assays showed that for each time point (time 0, then 6, 24 and 48 h of culture), caspase 8 activities were not significantly different for cells on CU compared to those on PS (data not shown).

Furthermore, there were no significant differences in caspase 8 activity in cells cultured on CU or PS in the presence and absence of the specific inhibitor Ac-IETD-CHO.

We also checked whether the apically signalling caspase 8 could be activated in Swiss 3T3 fibroblasts cultured on PS by inducing apoptosis with a specific anti-Fas monoclonal antibody. Apoptosis was significantly activated in cells cultured on PS for 6, 24 and 48 h ($P < 0.01$), and at each time point this activation was significantly inhibited (data not shown) by Ac-IETD-CHO ($P < 0.001$).

The results thus indicate that caspase 8 is not implicated in the apoptotic cascade triggered by CU.

3.7. *The ligation of β1 integrins does not fully protect the cells cultured on CU from apoptosis*

In order to explore whether apoptosis induced on CU might be associated with an integrin disengagement, cells were incubated for 1 h with FN (30 μg/ml), the ligand of α5β1 integrins, before seeding on CU in medium containing FN. Annexin V assays were performed at 6 h of culture, using untreated cells as control. In this system, FN induced significant changes in cell morphology that was associated with the presence of a larger number of isolated cells and smaller aggregates. FN ligation also induced a slight decrease in the percentage of apoptotic Swiss 3T3 cells on CU: 11.60% ±1.84 (treated) versus 15.93% ±3.02 (untreated). Nevertheless the difference was not statistically significant ($P > 0.05$).

4. Discussion

In the present study we demonstrated that both cell growth and viability were markedly decreased when the Swiss 3T3 cells were aggregated on CU as compared to cells spread on PS. The apoptotic process was shown to involve the mitochondria-dependent pathway but not caspase 8 activation. Recent studies have delineated two primary pathways which lead to apoptosis. The first involves the interaction between cytokine signals (e.g. tumour necrosis factor) and their cell membrane death receptors, leading to the activation of initiator caspases such as caspase 8 [28]. The second is a stress response pathway that involves the mitochondria [24] and leads to the formation of an apoptotic complex in which caspase 9 is activated [29]. The caspase 8 pathway does not appear to be activated under our experimental conditions, although we cannot rule out the possibility that caspase 10 might be involved [30]. In contrast, the caspase 9 pathway is undoubtedly implicated, since the mitochondrial membrane potential ($\Delta\Psi_m$) was altered in cells cultured on CU. It has been demonstrated that an extracellular apoptotic signal can be transmitted via two pathways, depending on the cell type [31]. In type I cells, apoptosis can activate downstream caspases independently of mitochondria, whereas the mitochondria-dependent pathway is activated in type II cells [31]. Our results suggest that in our model the Swiss 3T3 fibroblasts act as type II cells when they are cultured on CU. Further studies would be required to assess the status of the Bid, Bax and Bak proteins, whose translocation to the outer mitochondrial membrane induces mitochondrial transmembrane potential changes and apoptosome formation [24,31,32]. There is increasing evidence to suggest that proteins involved in apoptosis also control cellular activation and proliferation pathways [30]. Integrins that regulate cell viability through their interaction with the extracellular matrix convert information from the matrix to chemical signals modulating intracellular signal transduction. Under our experimental conditions, the surface integrins of cells on PS bind VN and/or FN favouring the attachment, spreading and proliferation of cells.

Cells on PS have well-defined focal adhesion complexes and stress fibres [33]. This reflects the effective 'outside–inside' transmembrane signalling produced by attachment of integrins to substrate-adsorbed proteins. In contrast, CU, which adsorbs adhesive proteins poorly, does not support cell spreading, the formation of focal contacts and stress fibres.

Recent studies have shown that the ligation of β1 integrin with anti-β1 integrin antibody can protect fibroblasts from apoptosis [34]. The results we obtained with Swiss 3T3 fibroblasts using FN as the ligand of α5β1 integrins suggest that the apoptotic process induced on CU cannot be fully ex-

plained by a disengagement of integrins. However, we cannot rule out the possibility that cross-linking of α5β1 integrins was not optimal using soluble FN. Anyway, we have been able to demonstrate coexistent cell survival with FN secretion and FAK Tyr 397 phosphorylation, from other cells aggregated on a cellulose substrate for 48 h (unpublished results). In the present study, the mechanisms that regulate apoptosis and cell survival on CU need further investigation to become clarified. Our observation that cell growth is not completely suppressed on CU after 48 h (Fig. 1) suggests, indeed, that aggregated cells either are the target of conflicting signals or differ in their capability to react to stimuli.

In conclusion, our results suggest that CU causes the apoptosis of aggregated Swiss 3T3 fibroblasts via activation of the mitochondria-dependent cell death pathway. An integrin disengagement does not seem to be entirely responsible for this phenomenon. CU thus appears to be an interesting model of triggered cell death, which may be useful for studying cell signalling mechanisms involved in this process.

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