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Decrease in Survival Threshold of Quiescent Colon Carcinoma Cells in the Presence of a Small Molecule Integrin Antagonist

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ABSTRACT

The role of adhesion molecules, such as α_v integrins, in the control of the survival of quiescent tumor cells is unclear. We used S 34961, a novel small molecule α_v integrin antagonist, to investigate the role of integrin-signaling in the survival of populations of quiescent human HT-29 and HCT 116 colon carcinoma cells. S 34961 at 1 μM induced detachment, but cells retained viability, existing as clusters. Nonligated β-integrins may recruit and activate caspase-8 [*J Cell Biol* **155:**459–470, 2001]. However, congruent with the absence of apoptosis, no activation of caspase-8 in these cells was detected after incubation with S 34961. A rapid (2 h) change in conformation of the N terminus of proapoptotic Bak was observed before detachment, together with a decrease in phosphorylation of focal adhesion kinase (2 h) and subsequent (8 h) decreases in phosphorylation of extracellular signal-regulated kinase-1/2 and Akt. Together, these results suggested that although treatment with S 34961 has no effect on survival per se, it may reduce the survival threshold of the tumor cells, with Bak in an activated state. Indeed, concomitant incubation of S 34961 with 10 μM U-0126 (a mitogen-activated protein kinase kinase inhibitor) was found to lead to apoptosis (at 24 h), whereas U-0126 alone had no effect. Together, these observations could guide the use of combination therapy with integrin antagonists in the clinic.

One of the major obstacles to successful cancer treatment is the existence of populations of quiescent malignant cells that are relatively insensitive to chemotherapy and thus have a high survival potential. Tumor growth is a balance between cell proliferation, cell survival, and cell death, and factors affecting this balance have profound effects on tumor growth. Novel treatments to reduce the survival threshold of the quiescent tumor cells should be of significant benefit in the clinic.

Integrins are heterodimeric cell surface receptors that link structural and functional components within the cell to the extracellular matrix (ECM), and have been shown to play a major role in the regulation of cell survival. Detachment of normal cells from the ECM rapidly induces apoptosis [anoikis (i.e., detachment-induced cell death or suspension-induced apoptosis)] and is a physiological process necessary for the control of normal tissue architecture (Grossmann, 2002). Recent reports have shown that unligated integrins, apart from turning off survival signals, may also activate signals for apoptosis. For example, caspase-8 activation has been proposed as an initiating event in anoikis (Rytömaa et al., 1999), and a recent study has shown that nonligated β-integrins may recruit and activate caspase-8 (Stupack et al., 2001).

Unlike nontransformed cells, many tumor cells seem not to depend on signals from adhesion molecules such as the α_v integrins for survival (Schwartz, 1997). However, integrins are also implicated in cell migration, proliferation, and invasion; tumor cells often express high levels of the vitronectin-binding integrins such as $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Clezardin, 1998; Hood and Cheresh, 2002). Although resistance of some tumor cells to anoikis is well documented, the nature of intracellular events after loss of integrin ligation in tumors remains poorly defined. We have sought to determine whether the absence of integrin ligation to a natural substrate, after treatment with S 34961, a novel and potent small molecule antagonist of α_v integrins synthesized in our laboratory (Perron-Sierra et al., 2002), leads to a reduction in the survival threshold of quiescent anoikis-resistant colon carcinoma cells.

One set of intracellular proteins that has been shown to play a pivotal role in setting the cellular survival threshold is the Bcl-2 family. Some members of this family, such as Bcl-X_L and Bcl-2, promote cell survival, whereas others, such

ABBREVIATIONS: ECM, extracellular matrix; S 34961, (7-{[4-(pyridin-2-ylamino)-butyrylamino]-methyl}-6,9-dihydro-5H-benzocyclohepten-5-yl)-acetic acid hydrochloride; PI3K, phosphatidylinositol-3 kinase; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; BrdU, bromodeoxyuridine; PARP, poly ADP-ribose polymerase; U-0126, bis[amino[(2-aminophenyl)thio]methylene]butanedinitrile; FCS, fetal calf serum; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBST, phosphate-buffered saline/0.1% Tween 20; BSA, bovine serum albumin; FAK, focal adhesion kinase; MEK, mitogen-activated protein kinase kinase kinase; MEKK, mitogen-activated protein kinase kinase kinase.

as Bax, Bak, and the "BH3-only" members Bid and Bmf, promote apoptosis (Antonsson, 2001). The proapoptotic functions of these proteins are regulated at several levels, including transcription, proteolysis, phosphorylation, intracellular translocation, protein-protein interactions, and conformational changes. In the present study, S 34961 was found to have a marked effect on the N-terminal exposure of Bak, suggesting a change in survival threshold of nonproliferating tumor cells. In addition, S 34961 was seen to lead to reduced intracellular signaling via both the PI3K/Akt and the Raf/mitogen-activated protein kinase kinase/ERK1/2 pathways, both of which have been implicated in cell survival. This decrease in survival threshold was demonstrated by the increased sensitivity of the S 34961-treated quiescent colon carcinoma cells to the mitogen-activated protein kinase kinase-1/2 inhibitor U-0126.

Materials and Methods

Antibodies. Primary antibodies used were: mouse monoclonal anti-integrin α_v (BD Biosciences PharMingen, San Diego, CA), mouse monoclonal anti-integrin β_3 (Chemicon, Temecula, CA), goat polyclonal anti-integrin β_5 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-bromodeoxyuridine (BrdU)-FITC (BD Biosciences PharMingen), rabbit polyclonal anti-Bak, residues 14 to 36 (BD Biosciences PharMingen), rabbit polyclonal anti-Bak, residues 23 to 37 (Upstate Biotechnology, Lake Placid, NY), rabbit polyclonal anti-Bax, residues 11 to 30 (Santa Cruz Biotechnology), rabbit polyclonal anti-Bax, residues 1 to 171 (Santa Cruz Biotechnology). Rabbit polyclonal anti-focal adhesion kinase (FAK) and anti-phospho-FAK were from Upstate Biotechnology, and rabbit polyclonal anti-Akt, anti-phospho-Akt, anti-ERK1/2, anti-phospho-ERK1/2, anti-PARP, anti-Bid, and mouse monoclonal anti-caspase-8 were from Cell Signaling Technology (Beverly, MA). The anti-Fas monoclonal antibody (CH-11) used to induce caspase-8 activation in cell culture was from Upstate Biotechnology. The secondary antibodies used were peroxidase-conjugated anti-mouse IgG, anti-rabbit IgG (Cell Signaling Technology), anti anti-goat IgG (Santa Cruz Biotechnology) for Western blotting, Cy3-conjugated anti-mouse IgG, and anti-rabbit IgG (Jackson ImmunoResearch, West Grove, Pennsylvania) for immunofluorescence, and FITC-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) for flow cytometry.

Compounds. S 34961 was synthesized as described previously (Perron-Sierra et al., 2002) and solubilized at 10 mM in dimethyl sulfoxide. Staurosporine (Calbiochem, San Diego, CA), U-0126 (Calbiochem), and wortmannin (Calbiochem) were dissolved at 10 mM in water. All compounds were aliquoted and stored at −20°C, and diluted into the culture medium as required.

Models of Cell Quiescence. HT-29 and HCT 116 colon carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA), and were routinely passaged in RPMI 1640 medium supplemented with 10% decomplemented fetal calf serum (FCS), 2 mM *l*-glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin, and incubated at 37°C in 5% CO_2/95% air. Media and supplements were from Invitrogen (Cergy-Pontoise, France), except for FCS, which was from Sigma. For the models of tumor cell quiescence, cells were seeded at a density of 25,000 cells per 35-mm tissue culture dish or per well of six-well tissue culture plates (Techno Plastic Products, Trasadingen, Switzerland) in 4 ml of RPMI containing 10% FCS. Two days later, the culture medium was removed; cells were washed once in phosphate-buffered saline (PBS), and 4 ml of serum-free RPMI containing 0.5% BSA (cell-culture tested; Sigma) was added. The subconfluent cell cultures were further maintained for a maximum of 12 days with no medium change.

MTT Cell Viability Assay. For the assay of cell viability, quiescent cell cultures were incubated with a solution of 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) in PBS for 4 h at 37°C. A 20% solution of SDS (Sigma) in 20 mM HCl was added, and cultures were incubated overnight at 37°C to solubilize the formazan metabolite. Global cell viability was estimated by measurement of optical density at 540 nm.

BrdU Incorporation. The proportion of cells in the cell cycle over a given 24-h period was measured by flow cytometric analysis of BrdU incorporation. Briefly, cells were incubated 24 h at 37°C in the presence of 10 μM BrdU (Sigma), detached by trypsin-EDTA (Invitrogen), and fixed in 70% ethanol at 4°C for at least 4 h. Samples were washed in PBS and incubated in 2 M HCl (30 min, 20°C) to denature DNA, then washed twice with PBS containing 0.5% Tween 20 and incubated with 20 μl of anti-BrdU-FITC for 45 min at 20°C. After washing with PBS, cells were incubated for 30 min at 20°C with 100 μg/ml RNase (Sigma) and 10 μg/ml propidium iodide (Sigma), and analyzed by flow cytometry. FITC and propidium iodide fluorescences were collected through 520- and 630-nm bandpass filters, respectively.

Western Blot Analysis. Whole-cell extracts were prepared with radioimmunoprecipitation assay extraction buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.8, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholic acid) to which was added protease [1% (v/v); Sigma] and phosphatase [1% (v/v); Sigma] inhibitor cocktails. Protein concentration was determined by Bradford protein assay (Bio-Rad Laboratories, Marnes la Coquette, France), and extracts were diluted into Laemmli sample buffer (Bio-Rad) containing 5% (v/v) β-mercaptoethanol, heated for 3 min at 95°C, and resolved on Tris-glycine gels (Novex, San Diego, CA). Biotinylated molecular mass standards (Cell Signaling Technology) were included in all gels. Proteins were transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences, Freiburg, Germany), which were blocked in PBS/0.1% Tween 20 (PBST) containing 5% milk, and probed at 4°C overnight with primary antibodies. Antibody dilutions were: anti-integrin α_v, 1/250 (5% milk); anti-integrin β_3, 1/500 (5% milk); anti-integrin β_5, 1/100 (5% milk); anti-Bak residues 23 to 37, 1/1000 (5% milk); anti-Bax residues 11 to 30, 1/2000 (5% milk); anti-FAK and anti-phospho-FAK, 1/1000 (5% milk); anti-Akt, anti-phospho-Akt, anti-ERK1/2 and anti-phospho-ERK1/2, 1/1000 (5% BSA); anti-caspase-8, 1/1000 (5% milk); anti-Bid, 1/300 (5% milk); and anti-PARP, 1/1000 (5% BSA). Anti-mouse and anti-rabbit peroxidase conjugated secondary antibodies were used at 1/3000 dilutions in PBST containing 5% milk (1 h, 20°C). Chemiluminescence detection was performed using the ECL Plus Western blotting detection kit and was recorded on ECL Plus hyperfilm (both from Amersham Biosciences).

Immunofluorescence Microscopy and Flow Cytometry. For immunofluorescence microscopy, cells in 35-mm culture dishes were fixed 5 min in 1% formaldehyde in PBS, washed in PBS, and incubated in primary antibody solution (600 μl) diluted in 500 μg/ml digitonin in PBS for 1 h at 20°C. Antibody dilutions were: anti-Bak residues 14 to 36, 1/500; anti-Bax residues 11 to 30, 1/100; and anti-Bax residues 1 to 171, 1/100. Cells were washed, then incubated with the relevant Cy3 conjugated secondary antibody for 1 h in PBS at 20°C at 1/200 dilution. Cells were washed, mounted in Vector-Shield (Vector Laboratories, Burlingame, CA) under a glass coverslip, and images were captured using an Olympus upright fluorescent microscope. For flow cytometric analysis of Bak N-terminal exposure, cells were cultured and fixed as for microscopic analysis, collected as a single-cell suspension by incubation at 37°C in 20% (v/v) cell dissociation solution (Sigma) in trypsin-EDTA, washed in PBS, and incubated 1 h with 200 μl of anti-Bak (residues 14–36) diluted as above. Cells were washed, then incubated with FITC-conjugated secondary antibody for 1 h in PBS at 20°C at 1/10 dilution and analyzed by flow cytometry. FITC fluorescence was collected through a 520-nm bandpass filter.

Results

S 34961 (Fig. 1) is a novel and potent small molecule antagonist of α_v integrins with IC_{50} values in binding studies of 2 nM for $\alpha_v\beta_3$, 1 nM for $\alpha_v\beta_5$, and 4 μM for $\alpha_{IIb}\beta_3$ (compounds 1–2 in Perron-Sierra et al., 2002). Anoikis of human umbilical vein endothelial cells and cluster formation of anoikis-resistant tumor cells occur at submicromolar doses of S 34961 on tissue culture plastic in the presence or absence of adsorbed vitronectin (an α_v integrin ligand) but not in the presence of adsorbed collagens or laminin (unpublished observations).

The α_v Integrin Antagonist S 34961 Induced Detachment of Quiescent HT-29 and HCT 116 Cells from Tissue Culture Plastic, but Cells Retained Viability, Existing as Clusters. Models of tumor cell quiescence were developed by culturing HT-29 or HCT 116 colon carcinoma cells in serum-free medium. After an initial 5-day period during which viable cell number increased, there was a plateau phase of 7 days in which total cell number remained constant, as indicated by MTT cell viability assay (Fig. 2A). During this plateau phase, cells were subconfluent (typically 70% confluence), and were thus not contact-inhibited. Over a 24-h period, 77% of these HT-29 cells were BrdU-negative, as opposed to 1% of cells in exponentially growing culture (Fig. 2B). Similar data were obtained for HCT 116 (data not shown). These results thus indicate that although total viable cell numbers remained constant, a small proportion of the cell population remained in the cell cycle. At all time points during the plateau phase, approximately 5% of cells were apoptotic, as judged by light-microscopic observation of nuclear fragmentation. This model may be considered to be representative of solid tumors in which cells are often proliferating at a slow rate, and in which many cells are quiescent. All experiments described in this report used quiescent populations of HT-29 and HCT 116 cells during the plateau phase from 7 to 11 days. Each cell line was found to express all of the α_v, β_3, and β_5 integrin subunits in both the proliferating and quiescent states (Fig. 2C). When quiescent populations (day 7) of HT-29 or HCT 116 cells were incubated with 1 μM S 34961, the cells were seen to begin to detach from the tissue culture plastic after 2 (HT-29) or 4 h (HCT 116), and to form free-floating cell clusters within 24 h (Fig. 2, D and E). The number of viable cells in these cultures was unchanged even after 4 days of culture in continued presence of S 34961, as assessed by MTT assay (data not shown).

Detachment of Quiescent HT-29 and HCT 116 Cells after Incubation with S 34961 Was Preceded by Exposure of the N Terminus of Bak. Recent studies have shown modulation of the N termini of the proapoptotic Bcl-2 family members Bak and Bax after stress to various normal and tumor cell types (Griffiths et al., 2001; Makin et al., 2001; Mandic et al., 2001). The reversible activation of these proteins via exposure of their N termini represents a critical event in the cell's sensing of damage, and was reported to occur before the commitment to apoptosis. Antibodies to N-terminal epitopes of both Bak and Bax were used to determine whether S 34961 antagonism of the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin-substrate interactions induced changes in exposure of N-terminal epitopes in these Bcl-2 family proteins in the quiescent HT-29 and HCT 116 cells. All data shown in this section are for HT-29 cells; similar data were obtained for HCT 116 cells. A marked increase in punctate Bak N-terminal staining with an antibody against residues 14 to 36 was observed in a general manner in the whole cell population within 2 h (Fig. 3A). This result was confirmed by flow cytometric analysis of fixed cells, where a global shift in Bak N-terminal reactivity was seen after 2-h treatment with S 34961 (Fig. 3B). The exposure of the N-terminal epitope of Bak was thus apparent before cell detachment occurred and was also evident in the cell clusters after 24 h. The majority (approximately 95%) of cells showing increased Bak staining had normal nonfragmented nuclei. Staurosporine (1 μM, 4 h), used as a positive control to induce apoptosis (Griffiths et al., 2001), led to an increase in staining of only ~50% of cells. The more punctate expression pattern here can be explained by the fact that these staurosporine-treated cells are undergoing apoptosis (unlike the S 34961-treated cells) and have condensed mitochondria. These results were confirmed by two further antibodies against residues 2 to 14 and 23 to 37 of Bak. In contrast to these results for Bak, very few cells (< 1%) showed a change in the N-terminal exposure of Bax (residues 11–30) in the presence of 1 μM S 34961, although an increase in staining was observed (approximately 20% of cells) when incubated with 1 μM staurosporine, used as positive control (Fig. 3A). The use of a further antibody directed against residues 1 to 21 confirmed this result. Antibodies directed against the whole Bax protein (except C-terminal; residues 1–171; Fig. 3A) demonstrated no change in total protein staining, as did a BH3-proximal epitope of Bax (residues 43–61; data not shown). Importantly, no changes in protein levels of either Bak or Bax were detected by Western blotting during this 24-h period (Fig. 3C) or during the subsequent 48 h (data not shown). Similarly, no changes in the protein levels, by Western blotting, of the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-XL were seen during the same time course (data not shown).

A Decrease in Phosphorylation of FAK and Subsequent Decreases in Phosphorylation of ERK1/2 and Akt Were Observed after Incubation of Quiescent Cells with S 34961. The tyrosine phosphorylation of the integrin-linked FAK, and downstream Akt and ERK1/2, has an important role in adhesion-mediated signal transduction (Schwartz and Ginsberg, 2002). In the present study, phosphorylation of FAK was seen to be reduced in quiescent populations of HT-29 cells after 2-h incubation with 1 μM S 34961 and then to decrease progressively to 8 h (Fig. 4, top left). Only a slight change in total phosphorylation of FAK could be detected by Western blot in the quiescent HCT 116 cells incubated for 24 h with 1 μM S 34961 (Fig. 4, top right). A decrease in phosphorylation of Akt was observed after 8-h incubation with 1 μM S 34961 for both cell lines; phosphorylation of this protein became virtually undetectable by 24 h (Fig. 4, middle). A similar decrease in phosphorylation of

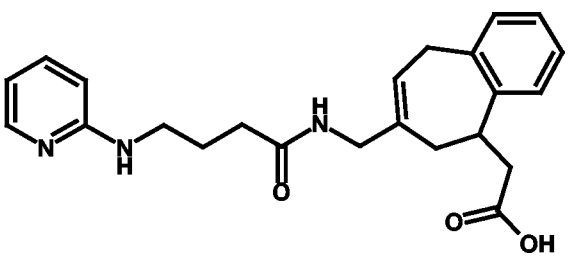

Fig. 1. The structure of S 34961.

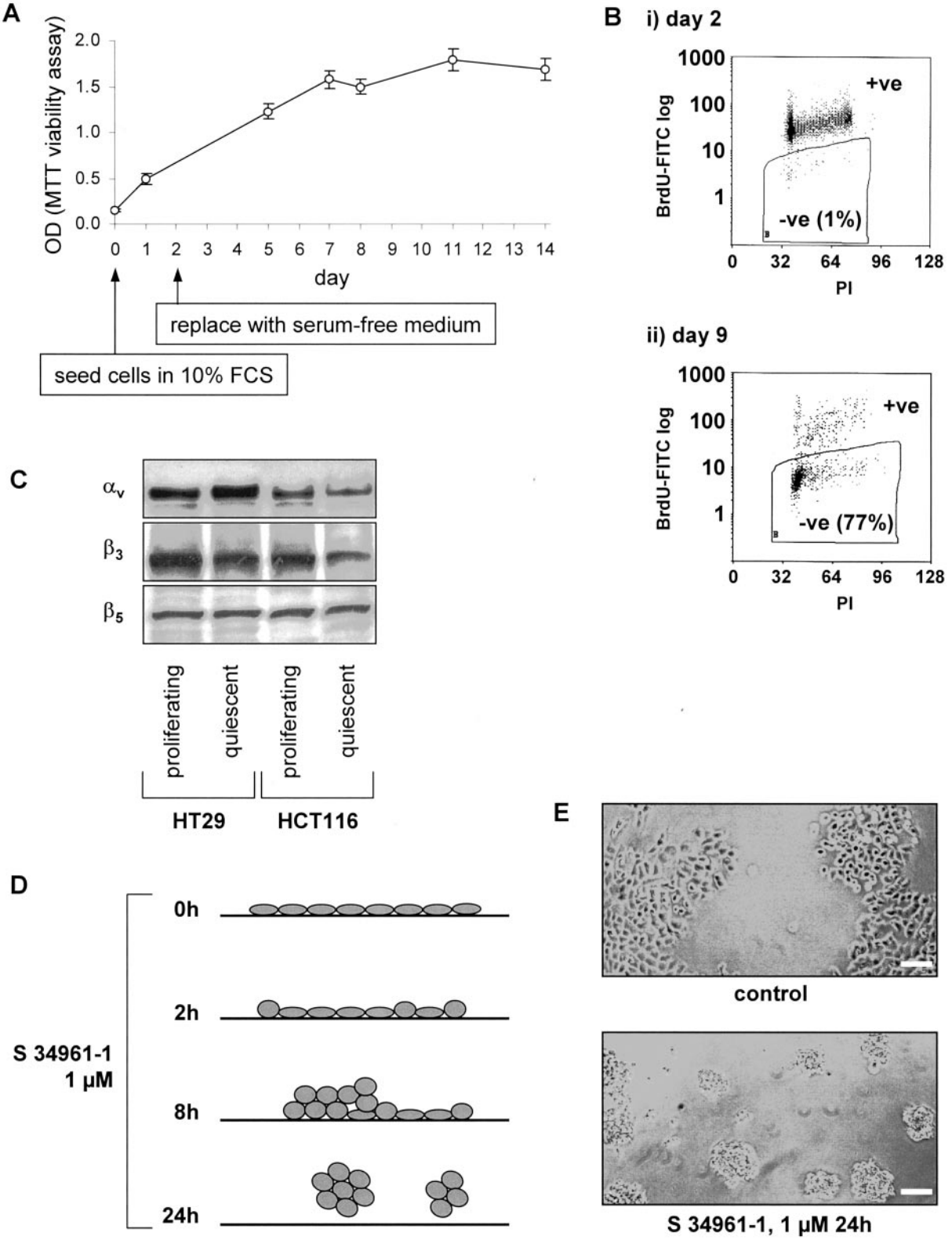

Fig. 2. A model of tumor cell quiescence. HT-29 colon carcinoma cells were cultured for 2 days in complete medium, then in serum-free medium for a further 12 days. A, MTT cell viability assay performed as described under *Materials and Methods* showing HT-29 growth kinetics. A subconfluence plateau phase from 7 to 14 days was observed during which total viable cell numbers remained constant. Error bars represent S.E.M. for six data points for a representative experiment. B, flow cytometric analysis of 24h BrdU incorporation showed that 1% HT-29 cells were noncycling on day 2 (i), as opposed to 77% on day 9 (ii). C, Western blots showing expression of all the α_v, β_3, and β_5 integrin subunits in the HT-29 and HCT 116 cell lines, both in the proliferating (day 2) and quiescent (day 9) states. D, a schematic representation of the effect of the α_v integrin antagonist S 34961 on the quiescent HT-29 cell populations, in which cells begin to detach after 2 h and to form free-floating cell clusters within 24 h. E, photomicrographs showing control populations of adherent quiescent HT-29 cells and the formation of cell clusters after 24 h incubation with 1 μM S 34961. Scale bars, 50 μm.

ERK1/2 was observed after 8-h incubation, although for these proteins, no further phosphorylation decrease was observed at 24 h (Fig. 4, bottom). Total protein levels of all these proteins were unchanged during the experiment, and phosphorylation levels of each in the control cultures were identical at both the start and end of treatment.

Incubation of Quiescent Cells with S 34961 Did Not Induce Caspase-8 Activation. A recent study using a number of cell lines has shown that nonligated β-integrins may recruit and activate caspase-8, hence contributing to detachment-initiated apoptosis (Stupack et al., 2001); these authors went on to suggest that integrin antagonists might also induce apoptotic signals via the same mechanism. We thus investigated the activation of this caspase after antagonism of integrin-substrate interactions by 1 μM S 34961. In quiescent HT-29 cells, no activated isoforms of caspase-8 and no cleavage of the caspase-8 substrate Bid were detected (Fig. 5, A and B). An anti-Fas monoclonal antibody and staurosporine were used as positive controls for caspase-8 activation. In agreement with the low percentage of cells with apoptotic morphology in the quiescent HT-29 cultures, the activity of downstream caspases (such as caspase-3) was very low, as evidenced by minimal PARP cleavage (Fig. 6A, top).

Simultaneous Incubation of Quiescent HT-29 Cells with 1 μM S 34961 and 10 μM U-0126 Led to Caspase Activation and Apoptosis, Whereas U-0126 Had No Effect Alone. Together, the above results show that incubation

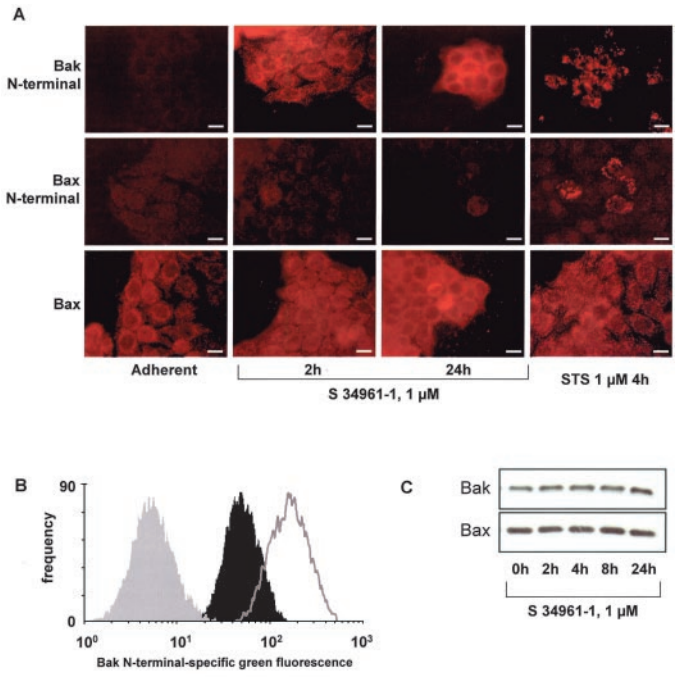

Fig. 3. S 34961-induced detachment of quiescent HT-29 cells was preceded by exposure of the N terminus of Bak. A, using fluorescence microscopy, a marked increase in punctate Bak N-terminal staining (residues 14–36) in the majority of HT-29 cells was seen after 2 h. This staining was also evident in the cell clusters after 24 h, at which time cells presented no signs of apoptosis. In contrast, very few cells (< 1%) showed a change in the N-terminal exposure of Bax (residues 11–30), even after 24 h. Antibodies directed against the whole Bax protein (except C-terminal; residues 1–171) demonstrated no change in total protein staining. Staurosporine (1 μM) was used as a positive control in each case. Scale bars, 10 μm. B, flow cytometry-generated frequency histograms of fluorescence associated with the antibody to residues 14 to 36 of Bak in untreated cells (filled histogram) and after 1 μM S 34961 treatment for 2 h (open histogram). The histogram for the secondary antibody alone is shown in gray. C, Western blot showing no change in total protein levels of either Bak or Bax during this 24-h period.

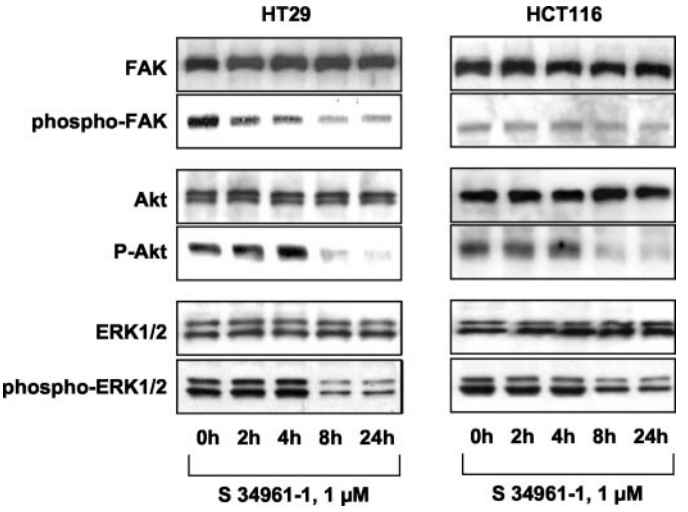

Fig. 4. Western blots showing levels of expression and activation (phosphorylation) of FAK, Akt, and ERK1/2 in quiescent HT-29 and HCT 116 cell populations after incubation with S 34961. Time 0 h corresponds to day 8 of cell culture at which cells were in the plateau phase of cell growth. All cell lysates were collected at "0 h", and incubations with S 34961 were begun 24, 8, 4, and 2 h before this time. For HT-29 cells, FAK phosphorylation was seen to decrease progressively to 8 h, whereas little change was seen for HCT 116. For both cell lines, phosphorylation levels of both Akt and ERK1/2 were unchanged at 2 and 4 h but were reduced at 8 and 24 h. This reduction was especially marked for Akt. Total protein levels of all these proteins were unchanged during the experiment. Cells at all time points were viable before protein extraction and showed no signs of apoptosis.

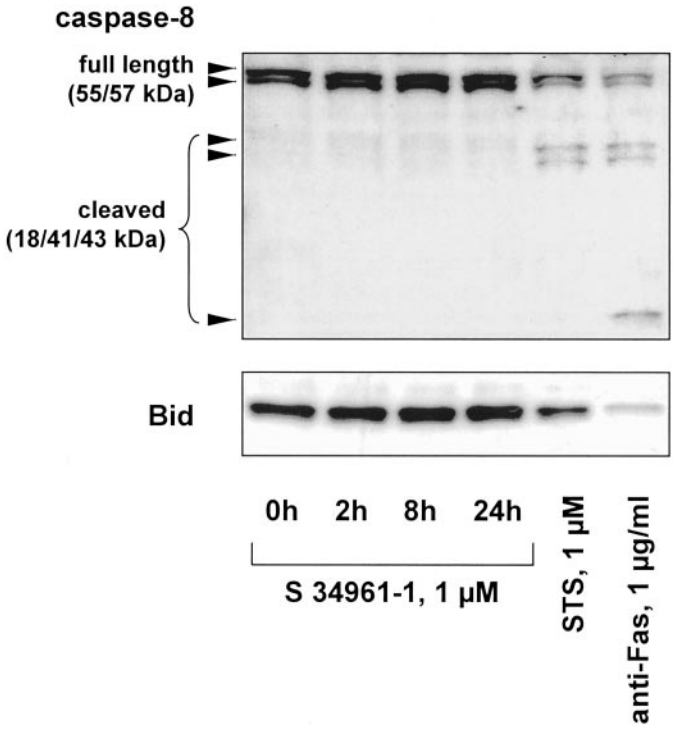

Fig. 5. Incubation with S 34961 did not induce caspase-8 activation. In quiescent HT-29 cells, no activated isoforms of caspase-8, and no cleavage of the caspase-8 substrate Bid (evidenced by lack of change in levels of the full-length protein), were detected by Western blots. An anti-Fas monoclonal antibody (CH-11, 1 μg/ml, 24 h) and staurosporine (1 μM, 24 h) were used as positive controls for caspase-8 activation.

of the quiescent HT-29 and HCT 116 cells with 1 μM S 34961 led to activation of Bak, near-total inhibition of PI3K-mediated phosphorylation of Akt, and a reduction in the level of MEK1/2 phosphorylation of ERK1/2. Despite this reduction in intracellular signals for survival, no caspase activity was detected, and cells did not undergo apoptosis. To test the hypothesis that the S 34961-treated cells had nevertheless undergone a reduction in survival threshold, quiescent HT-29 cells were incubated simultaneously with 1 μM S 34961 and 10 μM U-0126, a MEK1/2 inhibitor. Whereas neither compound alone had an effect on survival, their combination led to a marked increase in apoptosis, as shown by high levels of PARP cleavage (Fig. 6, top) and a 52 $\pm$ 4% reduction in the surviving population of cells after 48 h (Fig. 6, bottom). Concurrent incubation with S 34961 and 500 nM wortmannin (a PI3K inhibitor) did not lead to apoptosis above control levels. The levels of phosphorylation of ERK1/2 and Akt in the presence of S 34961, U-0126, and wortmannin are also shown (Fig. 6, middle). HCT 116 cells, like HT-29 cells, were seen to be sensitive only to the association of 1 μM S 34961 and 10 μM U-0126, with a reduction in surviving cell numbers of 48 $\pm$ 5% at 48 h. No effect on survival was observed for U-0126 or wortmannin alone or the association of wortmannin with S 34961.

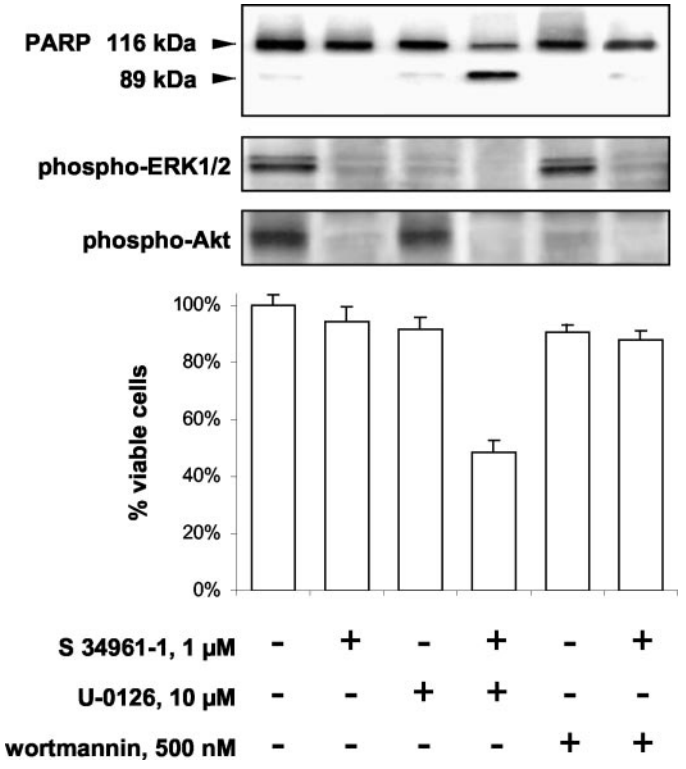

Fig. 6. Cells incubated with S 34961 were more sensitive to MEK1/2 inhibition. Quiescent HT-29 cells were incubated 48 h with either 10 μM U-0126 (MEK1/2 inhibitor) or 500 nM wortmannin (PI3K inhibitor) alone, or in association with 1 μM S 34961. Marked apoptosis, evidenced by high levels of PARP cleavage (top), was seen only for the association of U-0126 with S 34961. The levels of phosphorylation of ERK1/2 and Akt in the presence of S 34961, U-0126, and wortmannin are shown (middle). The lower part of the figure shows the effect of the different treatments on global cell survival as assessed using the MTT cell viability assay, performed as described under *Materials and Methods*. Error bars represent S.E.M. for three data points for a representative experiment.

Discussion

The α_v integrins are overexpressed in many tumor cell lines and have been shown to be important for tumor cell migration and invasion (Clezardin, 1998). Although normal cells require integrin-mediated attachment to a solid ECM for survival, many tumor cell lines have been described to survive prolonged periods in suspension (Grossmann, 2002). However, the intracellular events occurring in these anoikis-resistant tumor cells after abrogation of the survival-promoting integrin-ECM interactions are poorly understood. In the present study, quiescent cultures of colon carcinoma cells were used to assess the effect of a novel small molecule α_v integrin antagonist, S 34961, on tumor cell survival signaling. S 34961 (Fig. 1) has been shown to antagonize $\alpha_v\beta_3$ and $\alpha_v\beta_5$ binding to purified extracellular matrix ligands in the nanomolar range (Perron-Sierra et al., 2002). The colon carcinoma cell lines used in this study, HT-29 and HCT 116, were both found to express each of the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Fig. 2). When quiescent populations of these cells were cultured in the presence of 1 μM S 34961, cells rapidly became detached from the culture dishes and formed free-floating clusters (Fig. 2). No obvious effect on cell survival was seen, even after 4 days of culture.

The study of cell survival in the absence of substrate attachment typically involves the suspension culture of cells on nonadhesive substrates such as polyhydroxyethylmethacrylate (Gilmore et al., 2000). Recent studies have reported a positive correlation between the level of α_v integrin expression and apoptosis in suspension culture in both normal and tumor cell lines (Brassard et al., 1999; Kozlova et al., 2001; Stupack et al., 2001). Indeed, despite the widespread belief that tumor cells are innately resistant to anoikis, a number of tumor cell lines expressing high levels of α_v integrins have been shown to undergo apoptosis when detached from a solid substrate (Townsend et al., 2000; Kozlova et al., 2001; Stupack et al., 2001; Lewis et al., 2002). Thus, although the expression of α_v integrins confers a survival and proliferation advantage to both normal and tumor cells when adhering to a solid ECM, these same integrins may down-regulate survival signals, or possibly transmit death signals, when unligated. We were interested in how cells might respond to a small molecule inhibitor of α_v integrin binding.

The integrin antagonist S 34961 was seen not to affect survival of quiescent populations of HT-29 and HCT 116 cells in terms of viable cell numbers, and the low levels of cleaved PARP in cell extracts confirmed that activation of death-executing caspases, such as caspase-3, was unchanged in these cell populations compared with control cultures (Fig. 6). Caspase-8 activation has been proposed as a death receptor-independent initiating event in detachment-initiated apoptosis (Rytömaa et al., 1999), and a recent study by Stupack et al. (2001) has shown that nonligated β-integrins may recruit and activate caspase-8. It was suggested by Stupack et al. (2001) that integrin antagonists may induce similar events. We investigated the activation of this caspase after antagonism of integrin-substrate interactions by 1 μM S 34961. In quiescent HT-29 and HCT 116 cells, no activated isoforms of caspase-8 and no cleavage of the caspase-8 substrate Bid were detected (Fig. 5). These results suggest that S 34961 treatment may not be equivalent to the absence of an appropriate ligand. However, it is important to note that

Stupack et al. (2001) did not detect caspase-8 activation in anoikis-resistant HeLa cells, thus suggesting that such caspase activation is not a general phenomenon.

Although the integrin antagonist S 34961 was seen not to affect survival per se by initiating apoptosis, it was conceivable that this compound may affect the balance of integrin survival and apoptosis signals. A number of recent studies have pointed to the importance of changes in exposure of the N-terminal of the proteins Bak and Bax in the initiation of apoptosis (Griffiths et al., 2001; Makin et al., 2001; Mandic et al., 2001). These N-terminal conformational changes in Bak and Bax occurred before commitment to apoptosis and were reversible, representing an "activated state" after the cell had sensed perturbation. In the present study, a rapid exposure (2 h) of the N terminus of Bak was seen in virtually all cells after incubation with S 34961 (Fig. 3). Importantly, this occurred before the cells became detached from the culture dish. Detachment of both normal and tumor cells from substrate has been shown to lead to the exposure of the N-terminal of Bax (Gilmore et al., 2000; Makin at al., 2001). In our studies, a small number of S 34961-treated cells became positive for exposure of Bax N terminus, but only after a delay of 24 h and specifically in cells exhibiting apoptotic morphology. Thus Bax "activation" was secondary to exposure of the Bak N-terminal and seemed to be downstream to commitment to apoptosis. No changes in protein levels of the pro- or antiapoptotic Bcl-2 proteins were seen, even after 3 days of culture with S 34961. Although integrin ligation has been shown to affect transcription of Bcl-2 family proteins (Petitclerc et al., 1999; Matter and Ruoslahti, 2001), our data clearly corroborate that of other studies, showing that the role in damage sensing by these proteins, and in this case Bak, is much more rapid than could be explained by changes in protein levels (Griffiths et al., 2001, Makin et al., 2001).

Very little is known about how cellular stress signals are relayed to Bak or Bax, although a model has been suggested that involves activation by the Bcl-2 family BH3-only proteins, such as Bad, Bid, and Bmf (Cheng et al., 2001). Exactly how these proteins become activated when integrin ligation is disrupted is not clear. One upstream candidate in a signaling cascade from integrins is a cleavage fragment of MEKK1. This protein was shown to be cleaved when cells lose matrix contact (Cardone et al., 1997), and the proapoptotic conformation of Bak was induced in a MEKK1-dependent manner after DNA damage (Mandic et al., 2001). We detected no MEKK1 cleavage fragment after either 2- or 24-h S 34961 treatment of the HT-29 and HCT 116 cell lines (data not shown). Thus, the process whereby Bak becomes activated is not clear here, although its change in conformation suggests that the survival threshold of the cell may be compromised, a hypothesis we then went on to test.

A major upstream protein involved in adhesion-dependent cell survival signaling is FAK (Panetti, 2002). In the present study, phosphorylation of FAK was seen to be reduced after 2-h incubation of quiescent populations of HT-29 cells with 1 μM S 34961 and then to decrease progressively to 8 h (Fig. 4). Interestingly, total inhibition of phosphorylation of this protein was not observed, suggesting either alternative phosphorylation from other signaling molecules (e.g., activated Src; Biscardi et al., 1999) or that other FAK-linked integrins were present and activated in the detached cells. Only a slight change in total phosphorylation of FAK could be detected in HCT 116 cells incubated for 24 h with 1 μM S 34961, again possibly because of the presence of other integrin-linked signaling pathways.

Adhesion-mediated cell survival via FAK or other signaling proteins has been shown to involve primarily either ERK1/2 activation (Finlay et al., 2000; Le Gall et al., 2000; Howe et al., 2002) or PI3K signaling (Sonoda et al., 2000; Maeshima et al., 2002), apparently depending on the culture conditions and the cell lines used. In our studies, a decrease in the PI3K-mediated phosphorylation of Akt was observed after 8-h incubation with 1 μM S 34961 for both cell lines, phosphorylation of this protein becoming virtually undetectable by 24 h (Fig. 4). A recent study has shown similar data, whereby the integrin antagonist Tumstatin was shown to interact with $\alpha_v\beta_3$ integrin to inhibit activation of FAK, PI3K, and Akt in endothelial cells (Maeshima et al., 2002). A similar decrease in phosphorylation of ERK1/2 was observed after 8 h incubation with S 34961, although for these proteins no further phosphorylation decrease was observed after 24 h. Both the PI3K and ERK1/2 pathways have been shown to control pro- and antiapoptotic signals mediated by the Bcl-2 family proteins, including translocation and change in conformation of Bax (Boucher et al., 2000; Gilmore et al., 2000). However, in our experiments, the conformational change in Bak was seen well before changes in phosphorylation of ERK1/2 and Akt (2 h as opposed to 8 h; Figs. 3 and 4), suggesting that these signaling pathways were subsequent to this event.

Together, our results suggest that although treatment with S 34961 has no effect on caspase activation and survival per se, it may reduce the survival threshold of the tumor cells, with Bak in an activated state, a reduction in the level of ERK1/2 phosphorylation, and a near-total inhibition of PI3K-mediated phosphorylation of Akt. When quiescent HT-29 and HCT 116 cells were incubated concurrently with 1 μM S 34961 and the MEK1/2 inhibitor U-0126, marked apoptosis was seen, as evidenced by reductions in cell survival and high levels of PARP cleavage (Fig. 6). U-0126 had no effect on survival alone. This clearly demonstrates the markedly increased sensitivity of the S 34961-treated cells to inhibition of downstream survival signaling molecules. Concurrent incubation with S 34961 and 500 nM wortmannin (a PI3K inhibitor) did not lead to apoptosis. This agrees with the observation that 1 μM S 34961 alone caused a near-total inhibition of Akt phosphorylation in both cell lines.

In summary, S 34961 was seen to induce a two-step reduction in survival threshold in HT-29 and HCT 116 colon carcinoma cells. The immediate detection of cellular stress was signaled by the change in N-terminal exposure of Bak, followed by a decrease in PI3K and ERK1/2 survival signals. This reduction in survival threshold markedly increased the sensitivity of the HT-29 cells to a MEK1/2 inhibitor. This suggests that strategies based on blocking α_v integrin-mediated survival signals may represent a new therapeutic approach to improve the response to colon cancer chemotherapy in the clinic.

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