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Resveratrol induces cell death in colorectal cancer cells by a novel pathway involving lysosomal cathepsin D

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In human colorectal cancer cells, the polyphenol resveratrol (RV) activated the caspase-dependent intrinsic pathway of apoptosis. This effect was not mediated via estrogen receptors. Pepstatin A, an inhibitor of lysosomal cathepsin D (CD), not (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester, an inhibitor of cathepsins B and L, prevented RV cytotoxicity. Similar protection was attained by small interference RNA-mediated knockdown of CD protein expression. RV promoted the accumulation of mature CD, induced lysosome leakage and increased cytosolic immunoreactivity of CD. Inhibition of CD or its post-transcriptional down-regulation precluded Bax oligomerization, permeabilization of mitochondrial membrane, cytosolic translocation of cytochrome c, caspase 3 activation and terminal deoxinucleotidyl transferase-mediated dUTP-biotin nick end labeling positivity occurring in RV-treated cells. The present study identifies the lysosome as a novel target of RV activity and demonstrates a hierarchy of the proteolytic pathways involved in its cytotoxic mechanism in which the lysosomal CD acts upstream of the cytosolic caspase activation. Our data indicate that metabolic, pharmacologic or genetic conditions affecting CD expression and/or activity could reflect on the sensitivity of cancer cells to RV.

Introduction

Resveratrol [3,4′,5-trihydroxy-*trans*-stilbene (RV)], a polyphenol enriched in red wine, grapes, peanuts and other food products, has recently attracted the interest of researchers owing to its protective effects in carcinogenesis (1). RV was shown to suppress cancer initiation and promotion (2), to reduce ascites tumor growth (3), to prevent chemical carcinogen-induced epithelial cell transformation (4,5) and to inhibit neoangiogenesis (6,7). In *in vitro* models, RV inhibited the growth of tumor cell lines derived from various human cancers (8–11). This effect has been associated with the ability of RV to arrest cell cycle progression (12,13), to promote cell differentiation (14) and to induce programmed cell death by caspase-independent or caspase-dependent apoptosis (15–17) or by autophagocytosis (11). These properties make RV an attractive chemotherapy and chemopreventive drug for cancer treatment (18). In this respect, it is interesting to note that RV exhibits estrogenic and antiestrogenic activities (19,20), a property that might influence the dynamics of estrogen receptor (ER)-positive cancers, such as breast (21,22) and colorectal (23–25) cancers. To fully exploit its potential as an anticarcinogenic drug, it is mandatory to elucidate the cytotoxic pathways activated by and the molecules and organelles targeted by RV in cancer cells.

Two principal pathways of apoptosis have been described: in the 'intrinsic' pathway, the cytotoxic stress affects primarily the mitochondrion inducing the release of molecules that promote the activation of caspases, whereas in the 'extrinsic' pathway, the activation of caspases follows the stimulation of the so-called death receptors on the plasma membrane (26). It is now clear that programmed cell death pathways independent of caspases also exist (27). In various circumstances, the initial trigger of the death machinery was shown to be a protease normally resident within the endosomal–lysosomal compartment. Lysosomal cathepsins B and D (CB and CD, respectively) have been shown to mediate apoptotic cell death induced by tumor necrosis factor α (28–31) and cytotoxic drugs (32–34). The type of lysosomal protease recruited in the death pathway seems to vary depending on the cell model and the trigger, and probably on the availability of specific substrates and other unknown metabolic factors. The aim of the present work was to examine the possible involvement of lysosomal cathepsins in the cytotoxic mechanism of RV in human colorectal cancer cells. Our data demonstrate that RV activates a lysosome-dependent cytotoxic pathway ending in caspase-dependent cell death. We provide evidences that CD, not CB or cathepsin L (CL), mediates RV cytotoxicity. The present data indicate the lysosome as a novel and primary target organelle of the cytotoxic activity of RV.

Abbreviations: CB, cathepsin B; CD, cathepsin D; CL, cathepsin L; E64d, (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; ER, estrogen receptor; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; Pst, pepstatin A; RV, resveratrol; siRNA, small interference RNA; TUNEL, terminal deoxinucleotidyl transferase-mediated dUTP-biotin nick end labeling; ZVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp.

Materials and methods

Unless otherwise specified, all reagents were from Sigma–Aldrich Corp., St Louis, MO.

Cell cultures, treatments and evaluation of cytotoxicity

Human colorectal cancer cell lines (DLD1 and HT29) were cultivated in standard culture conditions (37°C, 95% air:5% CO_2) in Dulbecco's modified Minimal Essential Medium supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA), 2 mM L-glutamine and 1% penicillin–streptomycin solution. Cells were seeded and let to adhere on sterile plastic dishes for 24 h prior to the start of any treatment. Treatments included 1–100 μM RV, 100 μM pepstatin A (Pst), 10 μM (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E64d) (Bachem AG, Bubendorf, Switzerland) and 30 μM benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (ZVAD-fmk) (Alexis Laboratories, San Diego, CA). Inhibitors were added to the culture medium 12 (Pst) or 1 h (E64d and ZVAD-fmk) before the incubation with RV. In some experiments, the ER antagonist fulvestrant (ICI 182,780 from Tocris, Bristol, UK) also was used at 1 μM concentration. Culture medium was changed and substances were re-added daily. At designated time points, adherent and suspended cells were collected, diluted in a solution containing trypan blue and counted. Cell death was also assessed by cytofluorometer analysis. To this end, 10^5 cells were washed in phosphate-buffered saline (PBS) and incubated for 15 min at room temperature with 2 μl annexin V–fluorescein isothiocyanate (FITC) (Alexis Laboratories), 5 μl propidium iodide (stock solution 50 μg/ml) and 98 μl of buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Alternatively, 10^6 cells were washed twice with cold PBS and fixed in ice-cold 70% ethanol for 1 h at 4°C. Cells were then washed twice with PBS and incubated with ribonuclease A (0.4 mg/ml) for 30 min at 37°C and with propidium iodide (0.1 mg/ml) for 15 min in the dark, at room temperature. Cells with hypodiploid content of DNA (sub-G_1 peak) were assumed as apoptotic. Cells (at least 10 000 per sample) were analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a 488 nm argon laser. Data were interpreted with the winMDI software.

Small interference RNA transfection

Post-transcriptional silencing of CD expression was achieved by the small interference RNA (siRNA) technology. Duplexes of 21 nucleotide siRNA including two 3′-overhanging TT were synthesized by MWG Biotech AG (Washington, DC). The sense strand of siRNA was GAACAUCUUCUCCUUCUAC, corresponding to the positions 724–742 relative to the start codon of the CD messenger RNA (28). An inefficient CD9 oligonucleotide corresponding to the AGGUAGUGUAAUCGCCUUG sequence was used as a negative control of transfection (referred to as control duplex). Transfection was performed with Lipofectamine 2000 (Invitrogen Corp.). Afterward, the transfection mixture was removed and cells were incubated for a further 24 h in fresh medium prior to any treatment.

Protein expression analysis

Cells were homogenized in buffer containing detergents and protease inhibitors. A total of 30 μg of cell protein was denatured with Laemmli sample buffer, separated by electrophoresis on a 12.5% polyacrylamide gel and then electroblotted onto nitrocellulose membrane (Bio-Rad, Hercules, CA). The filter was probed in two rounds with specific monoclonal antibodies against CD (EMD Biosciences, Calbiochem, San Diego, CA) or actin following standard western blotting procedure (35). Immunocomplexes were revealed by incubation with peroxidase-conjugated goat anti-mouse antibody and subsequent peroxidase-induced chemiluminescence reaction (Bio-Rad). Intensity of the bands was estimated by densitometric analysis (Quantity one software).

Assessment of caspase activity

Caspase 3 activity was assayed with the fluorogenic substrate Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin following the manufacturer's protocol (MBL, Naka-Ku, Nagoya, Japan). Total caspase activity was measured with the Caspase Detection kit (Merck Biosciences Ltd, Nottingham, UK) using FITC–Val-Ala-Asp-fluoromethylketone as a substrate following the manufacturer's protocol. Stained cells (at least 10 000 per sample) were analyzed by flow cytometry and data were interpreted with the winMDI software.

Immunofluorescence studies

Cells grown and treated on coverslips were fixed with methanol for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 15 min. The following primary antibodies were used: a monoclonal (EMD Biosciences) or a rabbit polyclonal anti-human CD (36), a rabbit polyclonal anti-Bax (Cell Signaling Technology, Danver, MA), a mix of mouse monoclonal antibodies against α- and β-tubulin and a monoclonal anti-cytochrome c (Alexis Laboratories). FITC- or tetramethylrodhamine isothiocyanate-conjugated secondary antibodies against mouse or rabbit IgG were used as appropriate. As negative control, the primary antibody was omitted or substituted with pre-immune antiserum.

Terminal deoxinucleotidyl transferase-mediated dUTP-biotin nick end labeling and 4-6-diamidino-2-phenylindol-dihydrochloride staining

Apoptotic cells were revealed by *in situ* terminal deoxinucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay performed with the '*In situ* Cell Death Detection' fluorescent Kit (Roche Diagnostics Corporation, Indianapolis, IN) following the manufacturer's instructions. Apoptosis-associated chromatin alterations were detected by staining the cells with the DNA-labeling fluorescent dye 4-6-diamidino-2-phenylindol-dihydrochloride (1:100 in PBS/0.1% Triton X-100/4% fetal bovine serum).

Lysosomes and mitochondria integrity assessment

Lysosomal membrane integrity was assessed by the acridine orange (AO) retention test. AO is an acidotropic fluorochrome that emits red fluorescence when it accumulates in its protonated form within lysosomes, whereas it emits a green fluorescence when it localizes in the cytosol and in the nucleus. Cells adherent on coverslips were loaded with AO (1:200 from 0.5 mg/ml in distilled water for 10 min at 37°C) and immediately observed and photographed under the confocal fluorescence microscope. Lysosome leakage was quantified by cytofluorometer analysis of cells labeled with AO in suspension. The integrity of mitochondrial membrane was tested by using either the fluorescent dye mitotracker (Invitrogen Corp.) or rhodamine-123. Briefly, cells on coverslips were incubated with 0.2 μl of mitotracker solution for 15 min at 37°C, and then fixed in 3.7% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 15 min for further fluorescence staining. Alternatively, cells on coverslips were incubated with rhodamine-123 (50 nM in culture medium, 10 min) and immediately observed under the fluorescence microscope. Loss of mitochondrial membrane integrity was also assessed by cytofluorometer analysis of rhodamine-labeled cells.

Fluorescence microscope imaging

Coverslips were mounted in mowiol (1% in PBS). Images were captured with a Zeiss fluorescence microscope equipped with a digital camera or with Leica DMIRE2 confocal fluorescence microscope (Leica Microsystems AG, Wetzlad, Germany) equipped with Leica Confocal Software v. 2.61. Three coverslips were prepared for each experimental condition. Representative images, selected by two independent investigators, are shown.

Statistical analysis

All experiments were independently replicated at least three times. Data are presented as means ± standard deviation. The Instat-3 Statistical software (Graphpad Software, San Diego, CA) was used. Significance was calculated by the Mann–Whitney test.

Results

RV cytotoxicity in colorectal cancer cells is concentration and time dependent, is not mediated via ERs and is prevented by caspase inhibition

The present study was conducted in DLD1 and HT29 cells, two cell lines derived from human colon carcinomas that express ERβ, not ERα, isoforms (24,37,38). RV has been shown to bind to both α and β isoforms of ER (19,20) and to exert estrogenic activity when used at very low doses (i.e. 1–10 μM) (22). We tested the concentration and time dependency of inhibitory or stimulating effects of RV on the growth of human colorectal cancer cells. To assess whether such effects were mediated through ER, cells were treated with concentrations of RV ranging from 1 to 100 μM in the absence or the presence of 1 μM fulvestrant (ICI 182,780), which has been shown to inhibit the nuclear translocation of ERs and to promote their cytoplasmic degradation at a concentration of 100 nM (39). A treatment for 24–48 h with 1 or 10 μM RV did not affect cell vitality or final cell density in DLD1 and HT29 cultures (Figure 1A and data not shown). At 24 h treatment with 100 μM RV, some 35% reduction of cell culture growth was observed (data not shown and Figure 3C), whereas cell death, as assessed by cell counting and cytofluorometer evaluation of annexin V positivity, was apparent only at 48 h (Figure 1A and data not shown). Based on the results obtained with low doses of RV and on the lack of effects of ICI 182,780, we conclude that RV cytotoxic effects were not mediated via ER (Figure 1A). In the following experiments, RV was used at 100 μM. With this concentration, on average, annexin V-positive apoptotic cells in DLD1 cultures amounted to ∼50 and 75 % after 48 and 96 h of treatment, respectively (Figure 1B). In parallel cultures in which the potent and broad-range caspase inhibitor ZVAD-fmk was added daily along with RV, cell death was practically absent (Figure 1B). HT29 cells showed more sensitivity to RV than DLD1, the apoptotic cells amounting to 50–70 and 90% at 48 and 72 h of treatment, respectively. Also in the case of HT29 cells, ZVAD-fmk effectively prevented RV-induced cell death starting at 48 h (Figure 1B). It is to note that ZVAD-fmk, although inhibiting the occurrence of cell death at 48 h, could not prevent cell growth inhibition induced by RV in the first 24 h of treatment both in DLD1 and in HT29 cultures (data not shown).

RV cytotoxicity is associated with mitochondrial permeabilization and caspase activation

To better assess the significance of ZVAD-fmk protective effects, we monitored the time-dependent activation of caspases during RV treatment in DLD1 cells. The cleavage of a fluorogenic peptide substrate of different caspases remained at basal levels in the first 24 h of RV treatment and increased by a factor of ∼2.2 by 48 h (Figure 2A). By this time, the percentage of cells in which caspase activation occurred roughly corresponded to that of cells positive for annexin V, a biomarker of apoptotic-like programmed cell death (Figure 2B). In apoptotic cells (identified on the basis of chromatin alteration), mitochondrial integrity was lost, indicating that RV activated the intrinsic pathway of cell death (Figure 2C). The time dependency of mitochondrial permeabilization was assessed by cytofluorometry in DLD1 and HT29 cells treated with RV for up to 48 h and labeled with rhodamine. No changes in the cytofluorometric profiles were observed during the first 24 h of treatment. Mitochondrial permeabilization, as shown by loss of rhodamine retention, was observed starting at 36 h of treatment (Figure 2D). The microscope images of these cells shown in Figure 2E well reflect the phenomenon as quantified by cytofluorometry. It is to note that mitochondrial permeabilization occurred more rapidly and more extensively in HT29 than in DLD1 cells, accordingly with the highest sensitivity of these cells toward RV.

CD is an essential mediator of RV cytotoxicity in colorectal cancer cells

CB, CD and CL, the most abundant lysosomal proteases, have recently been involved in the activation of the intrinsic pathway of

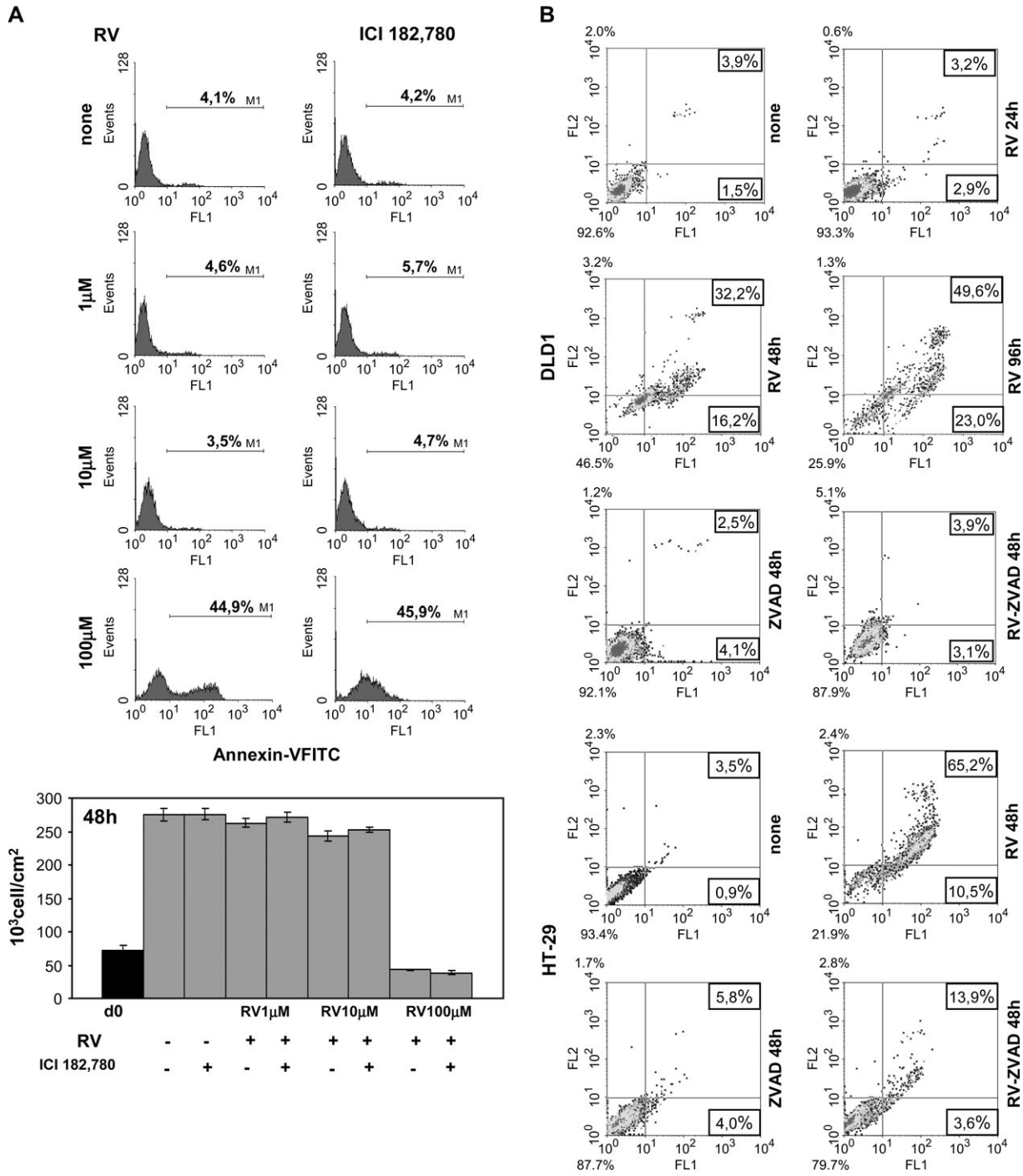

Fig. 1. RV-induced cell death in colorectal cancer cells is not mediated by ERs and is prevented by the pan-caspase inhibitor ZVAD-fmk. (**A**) DLD1 cells exposed to increasing concentrations of RV for 48 h in the absence or presence of the ER disruptor ICI 182,780. Substances were added at Time 0 and re-added in fresh medium at 24 h. Cytofluorograms of annexin V-labeled cells and histograms of adherent viable cell counts are shown (upper and lower panels, respectively). Cell counting data show that initial cell density (at Day 0) is almost halved after a 48 h exposure to 100 μM RV. This effect was not prevented by ICI 182,780. No effects on cell growth were elicited by 1 and 10 μM RV. (**B**) DLD1 and HT29 cells were incubated with 100 μM RV in the absence or presence of 30 μM ZVAD-fmk for up to 96 h. Substances were added at Time 0 and re-added in fresh medium every 24 h. Typical cytofluorograms of cells double labeled with annexin V–FITC and propidium iodide. In the cytogram, apoptotic cells (positive for annexin V) are distributed in the lower and upper right panels (values in percent are given). ZVAD-fmk prevented cell death by RV in both DLD1 and HT29 cell cultures.

programmed cell death (33,34,40,41), and therefore were considered possible mediators of RV cytotoxicity. To determine their involvement, we employed the inhibitors E64d, which is specific for the cysteine-type proteases CB and CL, and Pst, which is specific for the aspartic-type protease CD. In preliminary experiments in which cathepsin activity was assayed with specific fluorogenic substrates, 10 μM E64d and 100 μM Pst were shown to effectively inhibit (by ~85%) the activity of CB and CL and of CD, respectively, in the cells. DLD1 cells exposed to RV for up to 48 h in the absence or presence of Pst or E64d were tested for annexin V positivity and propidium iodide labeling. Cytofluorometer analysis of these samples indicated that Pst, not E64d, protected DLD1 cells from RV cytotoxicity (Figure 3A).

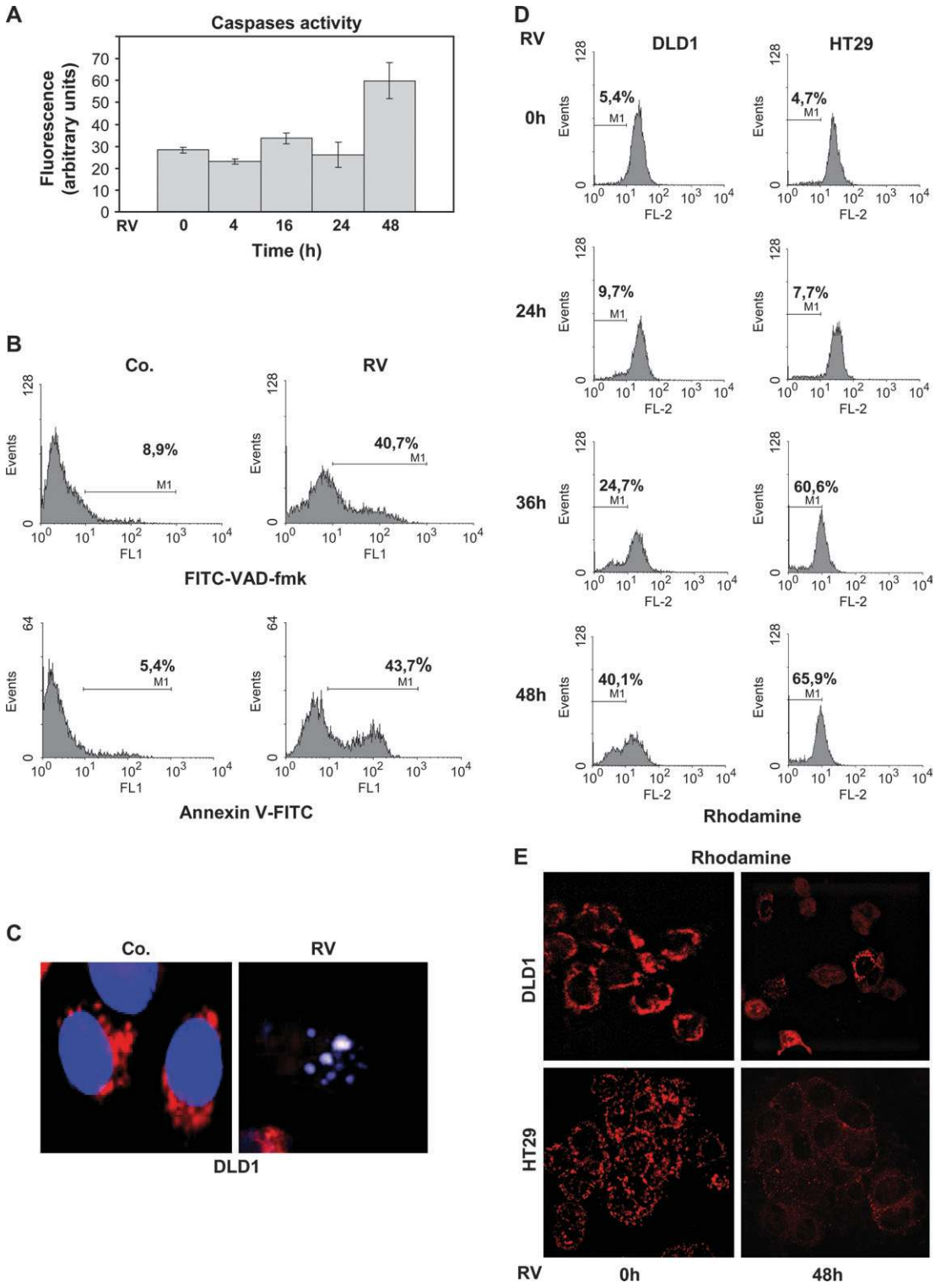

Fig. 2. Time-dependent permeabilization of mitochondria and activation of caspases by RV. (**A**) Cleavage of fluorogenic peptide substrate of different caspases at 0–48 h of RV treatment in DLD1 cells. (**B**) Caspase fluorescence assay (upper panels) and annexin V–FITC staining (lower panels) in DLD1 cells treated or not with RV for 48 h. Representative cytofluorograms are shown. (**C**) Control (Co.) and 48 h RV-treated DLD1 cells double stained for chromatin with 4-6-diamidino-2-phenylindol-dihydrochloride (blue fluorescence) and for mitochondria with mitotracker (red fluorescence). (**D**) Cytofluorograms of DLD1 and HT29 cells incubated for up to 48 h with 100 μM RV and labeled with rhodamine-123. Loss of mitochondrial retention of the fluorescent dye was apparent at 36 h of treatment. (**E**) Images at the fluorescence confocal microscope of cells treated for 48 h with RV and labeled with rhodamine.

We then checked whether the CD-dependent pathway of RV cytotoxicity was also operative in the other human colorectal cancer cell line. HT29 cells were treated for up to 48 h with RV in the absence or presence of Pst or E64d and cytotoxicity was assessed by cytofluorometer estimation of the hypodiploid (data not shown) and annexin V-positive cell population (Figure 3B). In the first 24 h of RV treatment, cell death was negligible (data not shown), but by 48 h, the treatment was toxic for >60% of the culture. At this time, inhibition of CB and

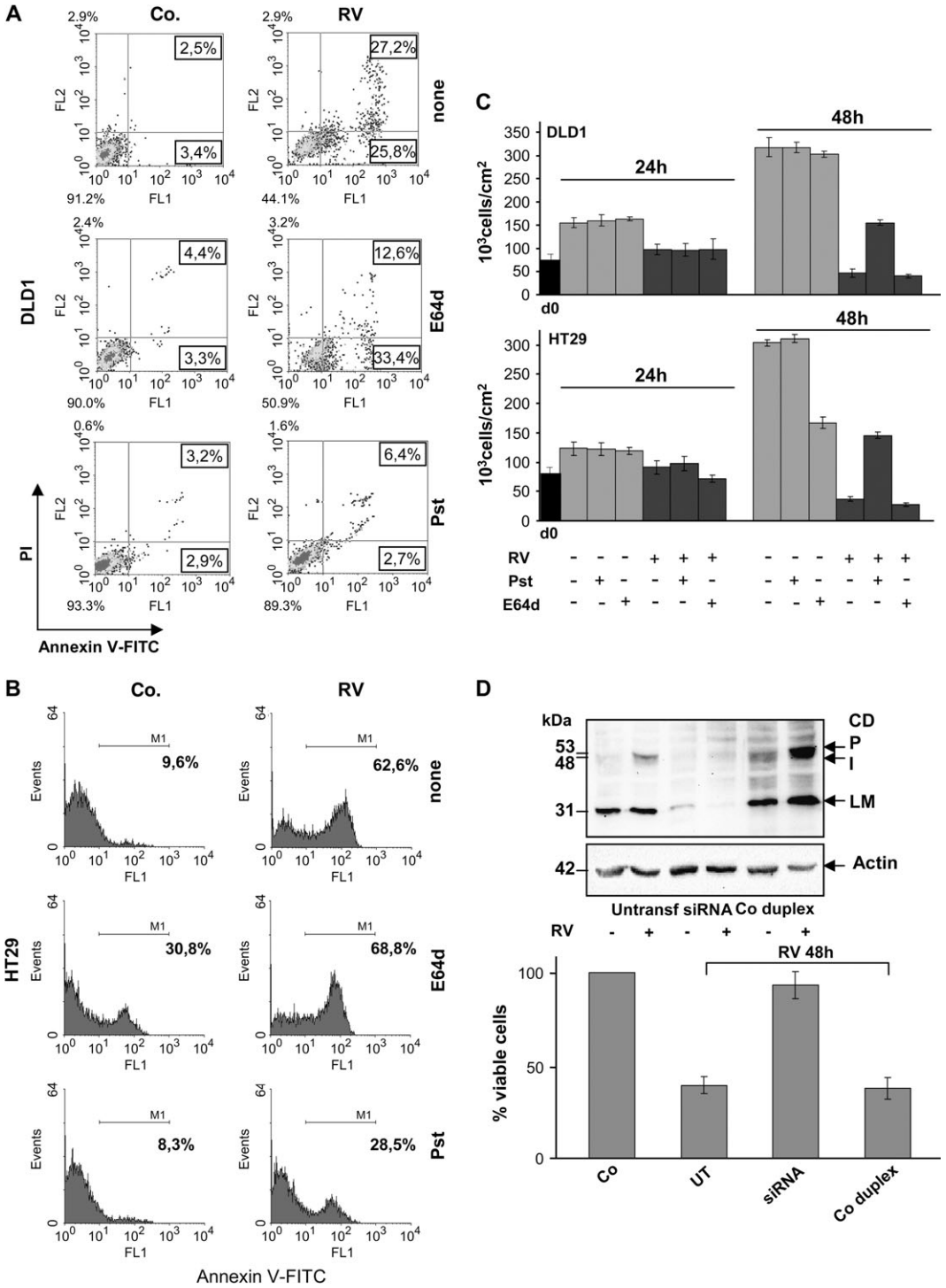

Fig. 3. CD mediates RV cytotoxicity in colorectal cancer cells. (**A**) Cells were incubated with RV in the absence or presence of Pst or E64d for 48 h. Medium was changed and substances re-added every 24 h. At the end of treatments, cells were double labeled with annexin V–FITC and propidium iodide and analyzed by cytofluorometry. Typical cytofluorograms are presented. Apoptotic cells (in lower and upper right panels of the cytofluorogram) amounted to ∼40–48% in RV- and RV plus E64d-treated cultures and to ∼10% in RV plus Pst-treated cultures. (**B**) HT29 cells were incubated with 100 μM RV in the absence or presence of 10 μM E64d or 100 μM Pst for 48 h, and then adherent and suspended cells were labeled with annexin V–FITC and analyzed by cytofluorometry. Medium was changed and substances re-added every 24 h. Representative cytofluorograms (of four experiments) are shown. At 48 h >60.0% of the cell population in RV-treated culture shows apoptotic features. RV-induced apoptosis was largely prevented by Pst, not by E64d. The latter inhibitor revealed itself cytotoxic to HT29 cells. (**C**) Histograms of cell counting in DLD1 and HT29 cultures treated or not for 24 and 48 h with RV in the absence or presence of Pst or E64d. Medium was changed and substances re-added every 24 h. Control untreated cells roughly doubled every 24 h. RV slowed down the rate of cell proliferation in the first 24 h. Compared with the initial cell density (Day 0), final cell density (48 h) in RV-treated cultures was nearly halved. Cell loss from the monolayer did not occur when RV treatment was performed in the presence of Pst. In Pst-pretreated cultures, cells also recovered from growth arrest despite the presence of RV. (**D**) Silencing of CD by siRNA prevents RV cytotoxicity. Cells were plated, untransfected (UT) or transfected with CD–siRNA or with control-duplex oligonucleotides as indicated and incubated or not with RV for 48 h. In the upper panel, a representative (of three) western blotting of CD is shown (symbols: P, precursor; I, intermediate; LM, large chain of mature form). The filter was stripped and re-probed for actin to prove equal loading of protein homogenates; in the lower panel, data on adherent viable cells in cultures exposed to RV for 48 h are given as percentage of cell number in the control culture prior to the incubation (assumed as 100%).

CL by E64d revealed itself to be toxic and did not protect from RV; in contrast, Pst protected a large fraction of the cell population from RV cytotoxicity. In parallel samples, cell survival was assessed by counting viable (trypan blue excluding) adherent cells. Data shown in Figure 3C indicate the following: (i) RV reduced the rate of cell proliferation in the first 24 h; this effect could not be prevented by Pst or E64d; (ii) at 48 h, RV induced a dramatic cell loss in the monolayer. This effect was largely prevented by Pst, not by E64d; (iii) Pst and E64d were not toxic by itself in DLD1 cells, whereas E64d only revealed to be toxic to HT29 cells after 48 h incubation. It is to note that Pst, although unable to prevent the growth inhibitory effect of RV in the first 24 h, showed to be very effective in preventing RV-induced cell death in the following 24 h both in DLD1 and in HT29 cells. In this respect, Pst behaved much like ZVAD-fmk. Pst not only prevented RV-induced cell death but also allowed to rescue cell proliferation. Pst inhibits not only CD but also cathepsin E, another aspartic protease resident in endosomes and lysosomes. To definitely prove the active role of CD in the death pathway activated by RV, we specifically down-regulated the expression of this protease by transient transfection with an siRNA 21-mer duplex. As controls, parallel cultures were not transfected or transfected with an inefficient duplex oligonucleotide not targeting CD messenger RNA (sham transfected). Proper conditions were assessed to achieve optimal down-regulation of CD expression, which was monitored by assaying the proteolytic activity at acid pH on fluorogenic substrate (data not shown) and by immunoblotting determination of CD protein level (Figure 3D, upper panel). The siRNA transfection successfully down-regulated CD expression in untreated as well as in RV-treated cells. By densitometry, in siRNA-transfected cells, CD protein level, normalized against actin protein level, was down-regulated by >95% (average of three independent experiments). A parallel set of cultures was used to estimate cell vitality. Transfection in itself was not harmful, as cell viability in control duplex-transfected cultures was not dissimilar from that reported in untransfected cultures (Figure 3D, lower panel). After exposure to RV, viable cells recovered from untransfected or sham-transfected cultures amounted to ∼50% and those recovered from CD-siRNA-transfected culture amounted to ∼90% of those initially present in the control culture, indicating that no cell loss occurred when CD expression was down-regulated (Figure 3C, lower panel). Cytofluorometric analysis of annexin V-labeled cells confirmed the protection by CD–siRNA against RV cytotoxicity (data not shown). The fact that Pst and siRNA elicited the same protective effect rules out the involvement of any aspartic protease other than CD and permits to exclude any role of CD polypeptides other than the enzymatically active one in RV cytotoxicity.

CD mediates RV-induced caspase 3 activation, Bax oligomerization on mitochondria and cytosolic release of cytochrome c

Data so far obtained demonstrate that both CD-mediated and caspase-mediated pathways are involved in the cytotoxic mechanism of RV. Whether the two proteolytic death pathways are activated independently or are recruited in series remains to be determined. Caspase 3 is a key effector protease of the apoptotic machinery. To address the link between the cathepsin-dependent and caspase-dependent proteolytic pathways, we determined the level of caspase 3 activity in cells that had been incubated or not with RV in the presence or absence of Pst for 48 h. As shown in Figure 4A, inhibition of CD largely precluded the activation of caspase 3 in RV-treated cells. To definitely prove the involvement of CD in caspase 3 activation, we further checked for the presence of TUNEL-positive cells in cultures exposed to RV along with Pst. The TUNEL technique evidences the presence of nicked DNA in apoptotic cells, which results from the caspase 3-mediated cleavage of poly-(ADP-ribose) polymerase, an enzyme involved in DNA repair. As shown in Figure 4B, Pst prevented the occurrence of TUNEL-positive cells in cultures treated with RV for 48 h. These data confirm that in RV-treated cells activation of the CD-mediated proteolytic pathway precedes that of the caspase cascade. One of the main pathways leading to chemotherapy-induced activation of the caspase cascade relies on the permeabilization of mitochondrial outer membrane and cytosolic translocation of cytochrome c. Bax, a protein belonging to the Bcl-2 family, has been involved in mitochondrial permeabilization and cytosolic relocation of cytochrome c and other pro-apoptotic proteins (32). In colon cancer cells, RV was shown to up-regulate Bax expression, to promote the exposure of its occluded N-terminus and to trigger its translocation into mitochondria (39,40). CD has been shown to be capable of inducing conformational changes and oligomerization on mitochondrial membrane of Bax in apoptotic lymphocytes (33). Therefore, it seemed of obvious interest to look at Bax localization in RV-treated colon cancer cells. Both in DLD1 and HT29 cells, a 48 h exposure to RV resulted in increased immunoreactivity and changes in cellular distribution of Bax (Figure 4C and data not shown). To clearly assess the involvement of CD in Bax-mediated permeabilization of mitochondria induced by RV, we performed a fluorescent double staining for mitotracker and Bax in cells pretreated with Pst and in cells in which CD expression had been silenced by siRNA technology. In most cells treated with RV only (Figure 4C, upper panels), mitotracker staining is weak and diffuse, whereas Bax immunoreactivity is increased and appears as spots that in some cases co-localizes with the mitotracker stain. This pattern is compatible with oligomerization of Bax on mitochondrial membrane. In contrast, in cells pretreated with Pst (middle panels) and in CD-siRNA-transfected cells (lower panels), mitochondria appear well preserved (as shown by mitotracker staining) and Bax immunostaining is diffuse in the cytoplasm, whether or not treated with RV for 48 h. We finally investigated whether RV-induced mitochondrial permeabilization was followed by cytosolic translocation of cytochrome c. The fluorescence staining of cytochrome c appears confined in the mitochondria in control cells, whereas it is diffused throughout the entire cytoplasm after 48 h treatment with RV (Figure 4D). The cytosolic relocation of cytochrome c is not observed in cultures treated with RV in the presence of Pst (Figure 4D).

RV induces the cellular accumulation and the cytosolic release of mature CD in colorectal cancer cells

How to explain the mitochondrial and cytosolic events depending on the activity of a lysosome resident protease? We reasoned that RV, like other chemotherapy drugs, could alter the permeability of lysosomal membrane without inducing the indiscriminate release of necrogenic hydrolases. We thus tested the lysosome integrity by looking at lysosomal retention of AO, an acidophilic fluorochrome that upon protonation is retained within lysosomes and becomes intensively red fluorescent. DLD1 and HT29 cells on coverslip were exposed to RV and observed under the fluorescence microscope at intervals of 6–8 h after AO staining. In control cells (Time 0), AO fluorescence appeared as intense red spots, indicating that it was confined within acid organelles (Figure 5A). No changes were observed in cells treated with RV for a period of time up to 36 h, beside an increase in size and number of red fluorescent organelles. At this time point of RV treatment, however, some cells showed a diffuse cytosolic staining of AO fluorescence, which appeared yellow colored and less intense (Figure 5A). The proportion of cells showing such staining features was increased at 48 h of RV treatment (Figure 5A). We quantified this phenomenon by cytofluorometry. Data shown in Figure 5B confirm the occurrence of lysosome leakage in cells treated with RV for ≥36 h. We then ascertained that CD indeed relocated into the cytosol under treatment with RV. Immunofluorescence studies showed that this was in fact the case in injured DLD1 and HT29 cells exposed for 48 h to RV (Figure 5C). Finally, considering the involvement of CD in RV cytotoxicity, we wondered if RV could affect its expression and maturation. DLD1 and HT29 cells were thus exposed for up to 72 h to RV and the expression of CD polypeptides in cell homogenate was analyzed at designated time points by immunoblotting. As shown in Figure 5D, RV induced a time-dependent accumulation of the mature double-chain (31 + 13 kDa) form of CD in both the colorectal cancer cell lines. Cell-associated mature CD greatly accumulated between 24 and 48 h of RV treatment: by this time, CD increased ∼2.5-fold in DLD1 and ∼3.5-fold in HT29 cells. In separate experiments we also checked

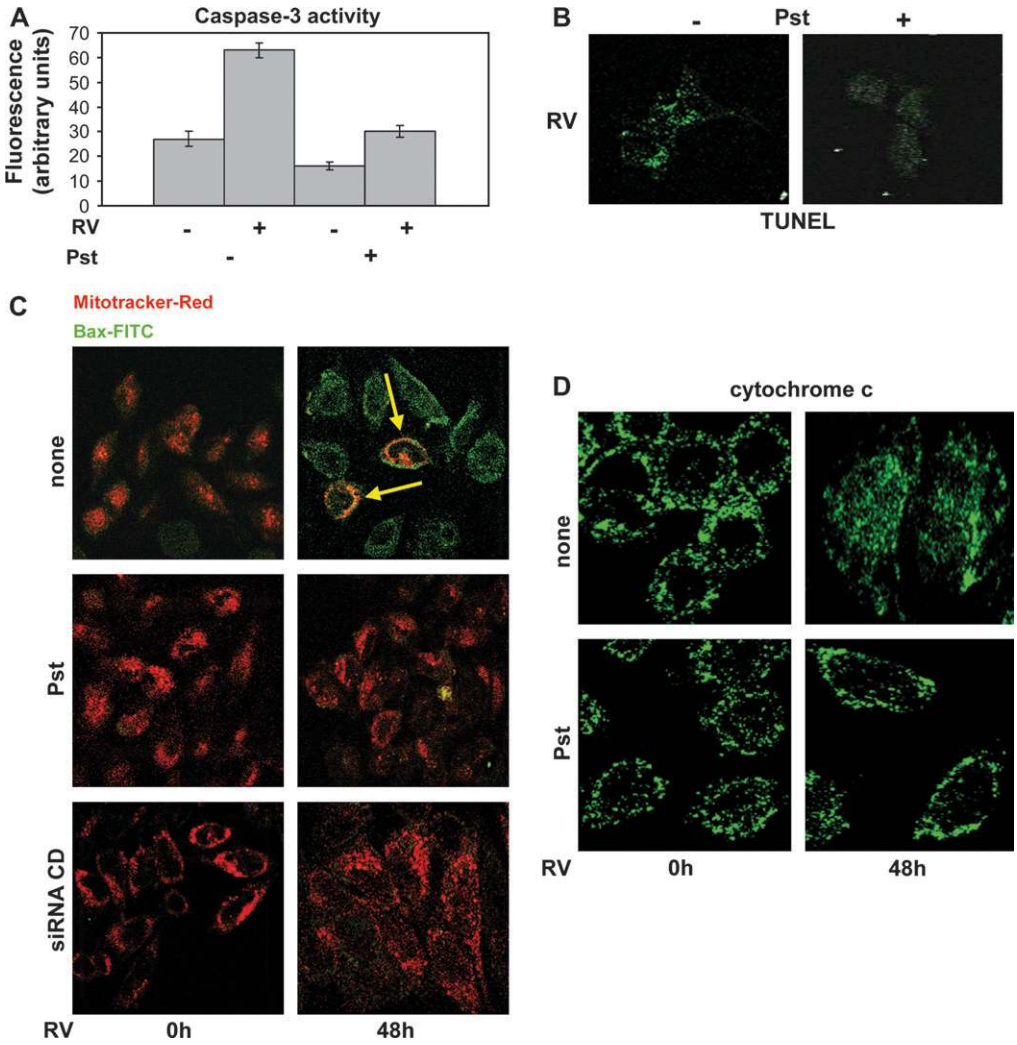

Fig. 4. CD triggers caspase 3 activation, Bax oligomerization on mitochondria and cytosolic release of cytochrome c induced by RV. (**A**) Histogram of caspase 3 activity in DLD1 cells exposed for 48 h to RV with or without Pst. (**B**) TUNEL staining of DLD1 cells plated on coverslips and incubated for 48 h with RV in the absence or presence of Pst (representative images are shown). (**C**) Control, Pst-pretreated and CD-siRNA-transfected DLD1 cells plated on coverslips were exposed to RV for 48 h. Cells were then double stained for mitotracker and Bax and photographed under the fluorescence confocal microscope. Compared with untreated cells, RV-treated cells show a weak and diffuse staining of mitotracker. In these cells, Bax immunoreactivity is increased and appears as spots that in some cases co-localizes with the mitotracker stain. This pattern, indicative of Bax oligomerization on mitochondrial membrane, is not observed in cells pretreated with Pst (middle panels) and in CD-siRNA-transfected cells (lower panels). In the latter cases, mitochondria appear well preserved and Bax immunostaining is diffuse in the cytoplasm also in the presence of RV. Cells transfected with CD-unrelated siRNA duplexes behaved as control untransfected cells. (**D**) DLD1 cells on coverslips incubated for 48 h with RV in the absence or presence of Pst and labeled for immunofluorescence detection of cytochrome c. The experiments demonstrate that Pst prevents mitochondrial permeabilization and cytosolic release of cytochrome c induced by RV. Images in (**C**) and (**D**) are representative of four independent experiments.

the expression of CD in DLD1 cells exposed for up to 48 h to 1 and 10 μM RV, doses that have been shown to induce CD synthesis in ER-positive breast cancer cells (42). However, no changes in CD expression level were observed under these conditions (data not shown). It is interesting to note that in our study cell death by (100 μM) RV became evident in cultures after 48 h of treatment and that HT29 showed more sensitivity to RV cytotoxicity than DLD1 cells. The data suggest a time-dependent correlation between the induction of CD accumulation and the toxic effect of RV.

Discussion

The naturally occurring polyphenol RV possesses potential chemopreventive and chemotherapy activities in colorectal cancer (23,43,44). Its mechanism of cytotoxicity is not fully understood, as yet. RV has been categorized as a phytoestrogen, because of its ability to act as an agonist or antagonist of ERs (19,20). Thus, its effects could be mediated via ER, as it has been shown to occur for genistein, another alimentary phytoestrogen (25). Here we show that in human colorectal cancer DLD1 and HT29 cells, which are known to express ERβ isoforms, the death pathway activated by RV is not mediated through ERs, rather it involves primarily the lysosome. The findings of the present study lead to the proposed mechanism of RV toxicity reported in Figure 6. Based on the effects of E64d, any involvement of lysosomal CB and CL in RV cytotoxic activity was excluded. In contrast, inhibition of CD by Pst or post-transcriptional silencing of CD by specific siRNA completely suppressed RV-induced cell death. In the first 24 h of treatment, RV slowed down the growth of cell cultures, and this effect could not be reverted by inhibitors of caspases or cathepsins. Consistently, during this period, caspase activity and the cellular content of CD did not change. RV-induced cell death became apparent at 48 h of treatment, and it was paralleled by induction of caspase activity and cellular accumulation of CD. These observations indicated that, although not playing an active role in

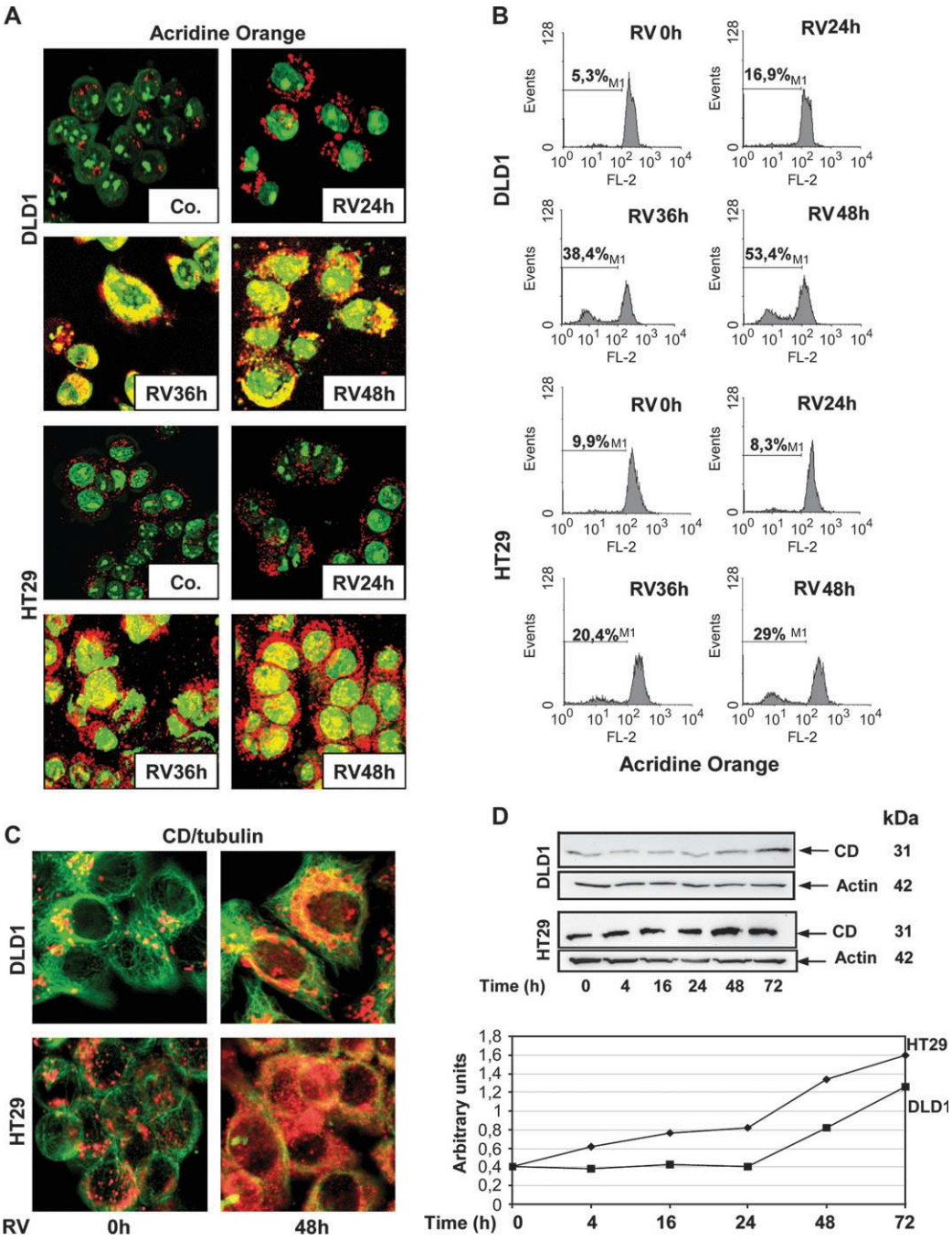

Fig. 5. RV cytotoxicity in colorectal cancer cells is preceded by lysosome leakage and up-regulation of the expression of and by cytosolic relocation of CD. (**A**) DLD1 and HT29 cells plated on coverslips were incubated for increasing time with RV and then stained with the acidotropic AO fluorochrome and immediately observed under the fluorescence microscope (representative images are shown). Intense red fluorescent spots are indicative of intra-lysosomal retention of AO. At 36 and 48 h of RV treatment, a large proportion of cells show a weak yellow-green fluorescence indicative of cytosolic diffusion of the fluorochrome. (**B**) Cells treated and labeled with AO as above and analyzed by cytofluorometry. (**C**) DLD1 and HT29 cells plated on coverslips were incubated for 48 h with RV and double labeled for immunofluorescence detection of CD (red fluorescence) and of tubulin (green fluorescence). (**D**) Western blotting analysis of mature CD (LM, large chain of the double chain) in a time course treatment with RV of DLD1 and HT29 cells and relative densitometry (representative of three experiments). Filters were stripped and re-probed for actin to quantify protein loading in the lanes.

the growth inhibitory effect, both caspases and CD were involved in the death effect of RV. Since Pst prevented caspase activation in RV-treated cells, we argued that CD was the lethal trigger and therefore we investigated its downstream targets. Cytotoxicity by chemotherapy drugs is associated with the intrinsic apoptotic pathway (45–47). This death pathway is initiated by loss of mitochondrial transmembrane potential followed by cytosolic release of pro-apoptotic molecules such as cytochrome c, second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI and apoptosis-inducing factor, which eventually results in activation of the caspase proteolytic cascade (26). CD was shown to play an active role in such mitochondrial events during cell death induced by staurosporine and chemotherapy drugs (33,34). The question still unanswered concerns the molecular targets of CD proteolysis that link this lysosomal protease with the mitochondrial release of pro-apoptotic mediators and subsequent activation of cytosolic caspase. Two candidates have been

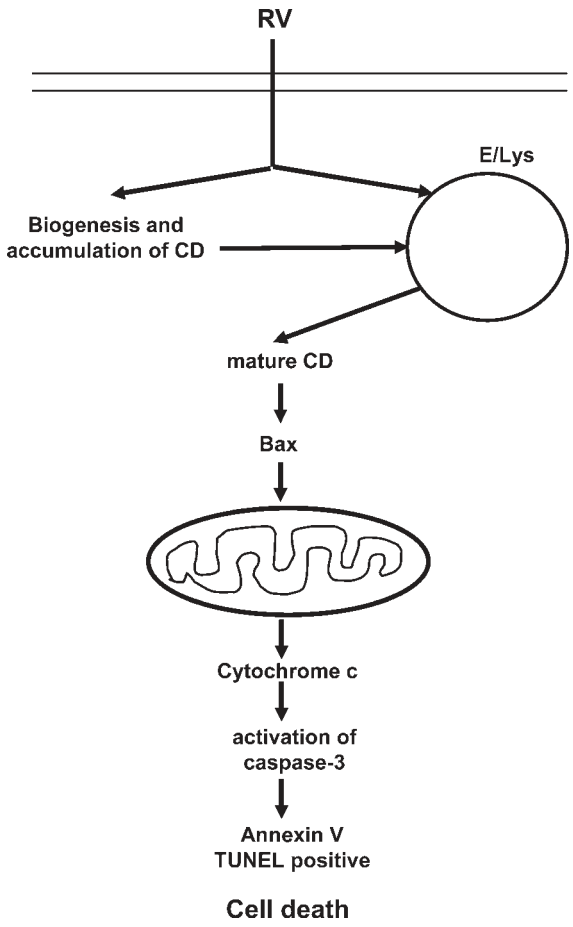

Fig. 6. Interpretative scheme of the results. RV induces the up-regulation of CD expression and the cytosolic relocation of mature active CD from lysosomes. CD was shown essential to induce mitochondria permeabilization (associated with Bax oligomerization onto mitochondrial membrane), cytochrome c release, caspase 3 activation and appearance of annexin V- and TUNEL-positive cell death.

proposed for such a role: Bax (33) and Bid (48). Several experimental evidences favor Bax as the probable target of CD in RV cytotoxicity. In an animal model of colon carcinogenesis, RV chemopreventive effect was associated with Bax hyperexpression (43), and in colorectal cancer cell lines, RV-induced activation of the apoptotic intrinsic pathway relied on Bax conformational activation (44). Moreover, Bax gene appears frequently mutated in human colorectal cancers, and it has been suggested that this may affect their responsiveness to chemotherapy drugs (49). In the present study RV caused the oligomerization and relocation onto mitochondrial membranes of Bax, the permeabilization of mitochondria and the consequent release of cytochrome c; all these events were associated with the permeabilization of lysosomes and the cytosolic relocation of CD and could be prevented by Pst or siRNA-mediated down-regulation of CD. In conclusion we have identified a novel pathway of RV cytotoxic mechanism in which the lysosomes act as death signal integrators. In colorectal cancer cells, RV cytotoxicity was associated with up-regulation of CD expression and, conversely, siRNA-mediated down-regulation of CD expression abolished the cytotoxic effect of RV, indicating that this protease was the master trigger of RV lethal activity (Figure 6). The present findings might therefore be of relevance when assessing the responsiveness or the resistance of tumors to RV-based therapy. In this respect, we notice that RV is fast and extremely metabolized in the body, so that predicted serum concentrations achieved when RV is injected for therapeutic purposes (at 100 mg/kg body wt) are much lower (probably 10 times) than those utilized in the *in vitro* experiments here described (50). Yet, in intestinal mucosa, a 30-fold enrichment of RV over serum concentration has been reported (51). These observations emphasize the need to develop RV analogues with improved bioavailability, as well as to engineer drug delivery systems that allow the efficient accumulation of RV within the target diseased tissue.

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