Bax and Bak are the critical complementary effectors of colorectal cancer cell apoptosis by chemopreventive resveratrol

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Resveratrol (RS) exerts a large number of cell-protective and anti-tumor effects, among them the induction of tumor cell apoptosis. Since the bioavailability of ingested RS at distant organs is low and apoptosis induction often requires relatively high RS levels (above 20 μmol/l), this polyphenolic food ingredient might be particularly effective as a chemopreventive in the digestive tract. Previous studies have suggested that chemoprevention by non-steroidal anti-inflammatory drugs (NSAIDs) is blunted by the loss of a single component of the apoptotic machinery – the Bax protein. Here, we report that RS efficiently provokes apoptosis in human colorectal carcinoma cells deficient for Bax, although at a reduced rate compared to the parental cells, through the activation of the mitochondrial death pathway. Knockdown of pro-apoptotic Bak by RNA interference reduced the apoptotic response to a similar extent as Bax deficiency in the parental cells and completely abolished apoptosis in Bax-null cells. Notably, although negative for RS-induced, mitochondria-mediated apoptosis, Bax + Bak double-deficient cells were sensitized by RS to ligand-induced, death receptor-mediated apoptosis. Thus, in contrast to NSAIDs, RS may remain effective as a pro-apoptotic chemopreventive as long as Bax and Bak have not both been inactivated during clonal selection. *Anti-Cancer Drugs* 17:471–478 © 2006 Lippincott Williams & Wilkins.

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Introduction

Colorectal carcinomas (CRCs) account for approximately 10% of the at least 6 million cancer deaths each year worldwide. Substantial research is therefore currently focusing on the efficacy of chemopreventive agents such as the non-steroidal anti-inflammatory drugs (NSAIDs) [1] and natural food constituents against CRC. Resveratrol (RS; 3,5,4′-trihydroxy-*trans*-stilbene) is a non-toxic polyphenolic phytoalexin (plant antibiotic) produced in at least 70 species of plants that may reach, in Pinot noir grapes, concentrations of up to 5 mg/l. RS exerts a multitude of effects on very different biological pathways which ultimately result in the observed cancer chemopreventive, cardioprotective and neuroprotective activities of the compound. Among the well-studied effects are the inhibition of signal transduction pathways, cyclin-dependent kinases and reactive oxygen species (ROS) production, the stimulation of cell differentiation, the down-regulation of pro-inflammatory cytokines, and the modulation of androgen and estrogen responses, and of drug-metabolizing, cyclooxygenase, nitric oxide, DNA synthesis and apoptosis pathways (reviewed in [2–4]). RS may specifically trigger apoptosis in tumor cells, sparing normal proliferating tissue [3,5].

The mitochondrial (intrinsic) pathway of apoptosis seems to be the major pathway of drug-induced death [6]. Although several reports have implicated a role of the death receptor pathway in RS-provoked apoptosis [7,8], the failure of dominant-negative inhibition of this pathway to block apoptosis upon RS treatment [9] and the identification of the involvement of mitochondrial death effectors [10,11] point to mitochondria as the prime mediators of RS-induced cell death. RS can lead to apoptosis in dependence of p53 or independently of the tumor suppressor [12,13], and perhaps through the inhibition of survival factors such as NF-κB [14,15]. Drug-induced mitochondria-mediated apoptosis usually involves mitochondrial outer membrane permeabilization (MOMP), and the release of pro-apoptotic effectors such as cytochrome *c*, HtrA2/Omi and apoptosis-inducing factor (AIF). MOMP is most frequently caused by the two major BH (Bcl-2 homology group) 123 multi-domain proteins Bax and Bak, and cells from Bax + Bak double-deficient mice fail to respond to many apoptotic stimuli [16]. We have previously documented that low to moderate concentrations of RS can induce co-localization of Bax with mitochondria in human CRC cells and that Bax-deficiency reduced, but failed to abrogate, apoptosis

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by RS [10]. Further work has recently indicated that RS can sensitize the same tumor cell type to mitochondria-independent (Bcl-2-insensitive), death receptor-mediated apoptosis [8]. The present study has been designed to help provide an insight into which apoptosis effectors have to be lost during tumor progression to blunt resveratrol's pro-apoptotic anti-tumor activity.

Materials and methods

Chemicals and antibodies

Resveratrol, tumor necrosis factor (TNF)-α and TNF-related apoptosis-inducing ligand (TRAIL) were purchased from Alexis Biochemicals (San Diego, California, USA); resveratrol was dissolved to 1 mol/l in molecular biology-grade DMSO. Propidium iodide (PI) and DCFH-DA were from Sigma (St Louis, Missouri, USA). Trypsin was from PAA (Linz, Austria). The monoclonal anti-cytochrome *c* antibody 7H8.2C, monoclonal antibody E1 recognizing AIF, and the polyclonal anti-Bak anti-serum and cleaved-caspase-9 antibody were purchased from Pharmingen (San Diego, California, USA). Sigma provided both the monoclonal anti-β-actin antibody AC-IS, and the peroxidase-conjugated polyclonal anti-rabbit and anti-mouse secondary antibodies. The monoclonal anti-cytochrome oxidase IV antibody was from Molecular Probes (Eugene, Oregon, USA), the anti-p53 monoclonal DO-1 was from Calbiochem (San Diego, California, USA) and the anti-CD95 antibody CH11 was from Immunotech (Marseille, France). Qiagen (Hilden, Germany) provided the transfection reagent RNAiFect, the standard FITC-labeled small interfering (si) RNA 5′-aaUUCUCCGAACGUGUCACGU-3′, the control siRNA 5′-aaUUCUCCGAACGUGUCACGU-3′ and the Bak siRNA 5′- aaCCGACGCUAUGACUCAGAG-3′.

Cell culture, transfection and flow cytometry

HCT116 cells (ATCC CCL-247) and derivatives were grown in McCoy's 5A medium supplemented with 10% FCS in a humidified 7% CO_2 atmosphere at 37°C. siRNA was transfected according to the manufacturer's recommendation (Qiagen); transfection efficiencies were routinely between 85 and 95%. For flow cytometry analysis of apoptosis, cells were seeded in six-well dishes to approximately 30% confluency, mock-treated or treated with RS (100 μmol/l) and harvested by trypsinization. The cells were stained with PI as described elsewhere [17]. The DNA fluorescence was measured with a Becton Dickinson FACScan (Bedford, Massachusetts, USA) and data were analyzed with the CellQuest software from Becton Dickinson. Superoxide production was monitored by trypsinizing and resuspending the cells in medium, followed by a 30-min incubation at 37°C in the presence of the ROS-sensitive fluorescence probe DCFH-DA (10 μmol/l) [18].

Cell fractionation, trypsin digestion and immunoblot analysis

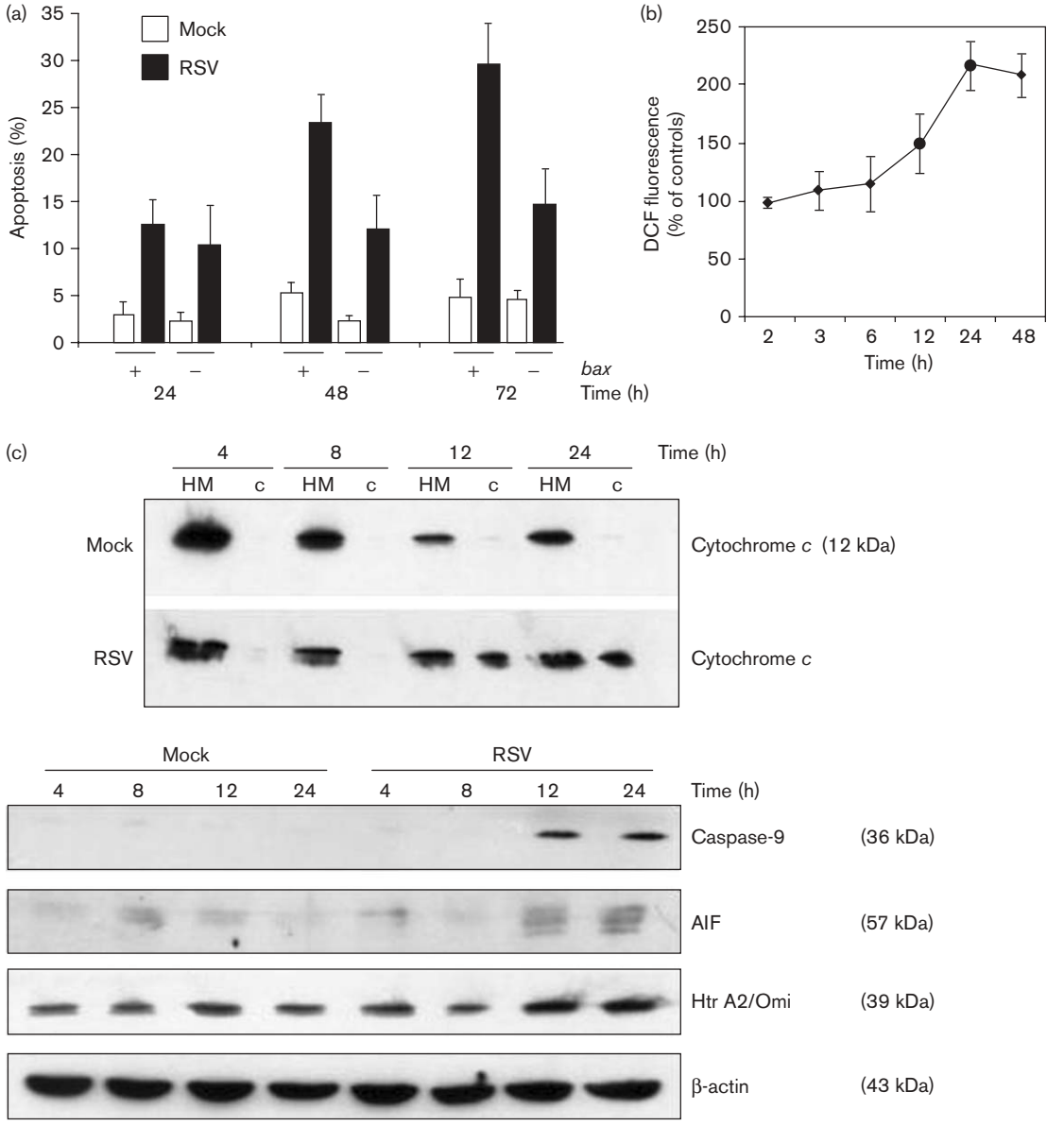
Mock-treated and drug-treated cells on 10-cm dishes were Dounce-homogenized, and cell extracts were separated by sucrose-gradient centrifugation into a cytosolic and high-membrane fraction containing the mitochondria, as described before [17]. Trypsin-sensitivity was tested by incubation of the mitochondria with trypsin (PAA; 125 μg/ml) for 20 min, as detailed elsewhere [19]; trypsin digestion was stopped by the addition of 2 × Laemmli buffer and heating to 94°C for 5 min. For total protein preparation, cells were lysed in 150 μl of a lysis buffer heated to 85°C, and containing 50 mmol/l Tris–HCl (pH 6.8), 100 mmol/l DTT, 2% SDS and 20% glycerol. Samples containing 15 μg of total cellular protein were subjected to 8 or 12% SDS–PAGE and transferred to a PVDF membrane (Immobilon-P; Millipore, Bedford, Massachusetts, USA). Signals were detected upon overnight incubation of the membranes with the indicated relevant antibodies followed by a final incubation with a peroxidase-conjugated secondary anti-mouse or anti-rabbit antibody and Renaissance Enhanced Luminol Reagents (NEN, Boston, Masschusetts, USA), performed as specified by the supplier.

Results

Human HCT116 CRC cells have been widely employed for the study of the molecular mechanisms of chemotherapeutic and chemopreventive drug action. These cells harbor an intact p53–p21Cip–Rb–E2F tumor-suppressor pathway, and are responsive to many stresses including DNA damage and spindle disruption (excluding oncogene expression; probably mostly due to the lack of p14ARF expression in these cells). Many isogenic derivatives are available in which individual genes of interest have been knocked out by targeted homologous recombination [1,20] or knocked down by antisense expression [21]. HCT116 cells are growth factor insensitive, poorly differentiated and mismatch repair deficient, and predominantly harbor a stable *2n* chromosome set. Moreover, the cells experience apoptosis in response to the mainstay CRC chemotherapeutic 5-fluorouracil as well as upon treatment with chemopreventive NSAIDs [1,22]. Since the *bax* gene is frequently inactivated in CRCs and *bax* deficiency blunts the pro-apoptotic activity of NSAIDs [1], we asked (a) whether the Bax relative Bak can complement for Bax deficiency in the RS-induced apoptosis of HCT116 $bax^{-/-}$ derivatives and (b) whether both effectors combined are the critical components of the intrinsic (not death receptor ligand-induced) cell death.

HCT116 and the isogenic *bax*-null cells proliferated in culture with an approximately equal population doubling time (28–30 h) and the $bax^{-/-}$ cultures showed a slightly reduced basal apoptosis rate (approximately 2 versus 4%).

Fig. 1



Resveratrol induces apoptosis in human HCT116 CRC cells deficient for *bax*. (a) Exponentially growing cultures of the parental HCT116 cells (+bax) and the *bax*$^{-/-}$ derivatives (–bax) were mock-treated or treated with RS (100 μmol/l) for 24-72 h, and then fixed and stained with PI (25 μg/ml in PBS) for 1 h for flow cytometric analysis of DNA content. Cells with a sub-2*n* DNA content were apoptotic. Error bars denote SDs of at least three experiments. (b) Increase with time of DCF fluorescence in HCT116 *bax*$^{-/-}$ cells following oxidation of the ROS-sensitive fluorescent probe DCFH-DA by peroxides in response to RS, compared with mock-treated cultures. SDs were calculated from three experiments. (c) Cultures of HCT116 *bax*$^{-/-}$ cells were mock-treated or exposed to RS for different times. Cells were Dounce-homogenized and cell extracts were separated by sucrose-gradient centrifugation into a cytosolic (c) and high-membrane (HM) fraction containing the mitochondria. Immunoblot analysis was performed on the high-membrane and cytosolic fractions (upper panel), or the cytosolic fractions (lower panel), on 15 μg protein with a monoclonal anti-cytochrome *c* antibody (1:1000), the polyclonal cleaved caspase-9 antibody (1:1000), as well as monoclonal antibody E-1 recognizing AIF (1:300), polyclonal antibody detecting HtrA2/Omi (1:1000) and, finally, monoclonal anti-β-actin antibody (1:5000). Peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies were used at 1:1000 and 1:2000, respectively.

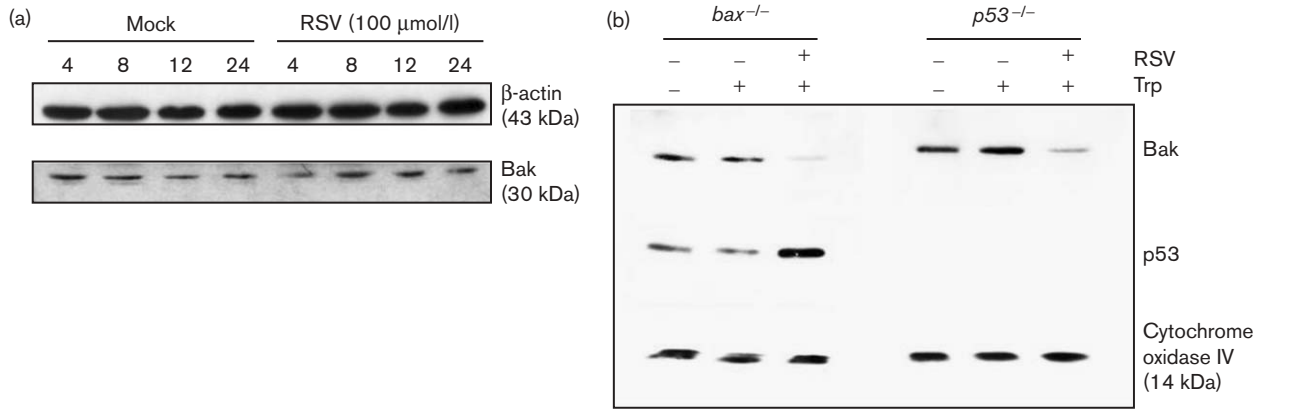
When cells were cultured in six-well dishes, in McCoy's 5A medium supplemented with 10% FCS, and the cultures were then mock-treated or treated with RS (100 μmol/l) for the indicated times and analyzed by flow cytometry for the presence of cells with a sub-2*n* DNA content indicative of apoptosis, RS provoked apoptosis in both the parental and *bax*-deficient cultures, although significantly less in the latter (Fig. 1a). In accordance with our previous observations [10], this shows that apoptosis by RS is only in part dependent on the presence of the major pro-apoptotic multidomain protein Bax.

Mitochondrial apoptosis favors the production of ROS and the appearance of ROS usually accompanies rather than precedes mitochondria-mediated cell death. Monitoring superoxide production in the presence or absence of RS with the ROS-sensitive fluorescent probe DCFH-DA revealed that, in accordance with mitochondrial cell death, ROS is generated in both the parental and *bax*-null cells (Fig. 1b [18]). To corroborate the occurrence of mitochondria-mediated cell death in the *bax*-negative cells, immunoblot analyses for apoptosis effectors were performed. RS was able to induce MOMP and thereby the release of the apoptosome constituent cytochrome *c*, AIF, and the inhibitor of caspase inhibitors and pro-apoptotic serin protease HtrA2/Omi. Concomitant with ROS production and cytochrome *c* release, the apoptosis-initiating caspase of the apoptosome, caspase-9, was activated (Fig. 1c). Thus, *bax*-deficient HCT116 cells can respond to RS treatment with mitochondria-mediated apoptosis, albeit at a reduced level when compared to the parental cells.

Cells from mice deficient for both major BH123 proteins Bax and Bak fail to undergo mitochondria-mediated apoptosis provoked by many different insults [16]. We have previously shown that the expression of the BH123 protein antagonist Bcl-X_L was not detectably altered in HCT116 cells in response to RS [10], and we and others have observed that the other important antagonist, Bcl-2, is not produced in this cell type [1,10]. It was therefore asked whether the Bax relative Bak is responsible for (some or all of) the residual apoptosis by RS in HCT116 $bax^{-/-}$ cells. Immunoblot analysis revealed that the steady-state levels of Bak were similar in mock-treated and RS-treated cultures (Fig. 2a). Our previous work has shown that, in the parental cells, the levels of Bax were also unchanged [10]. Bak, however, like Bax, is an inducible protein that experiences conformational changes upon activation [23]. Since Bak protein is constantly present at the mitochondria, these changes can be detected by mitochondrial Bak becoming more sensitive to trypsin digestion [19]. Figure 2(b) shows that mitochondria prepared from HCT116 $bax^{-/-}$ cells that had been treated with RS or not for 24 h and subsequently probed for trypsin sensitivity harbor trypsin-sensitive (i.e. active) Bak protein only when treated with RS. Furthermore, RS-treated mitochondria harbor p53 tumor-suppressor protein, in accordance with reports that p53 can translocate to mitochondria in response to apoptotic stimuli [24]. Notably, the activation of Bak by RS was not affected by the presence of p53 as Bak protein was similarly sensitive to trypsin in mitochondria from HCT116 $p53^{-/-}$ cells (Fig. 2b). This suggests that, in contrast to some recent work showing that mitochondrial p53 can bind to and activate Bak [19], Bak activation in RS-treated HCT116 cells is independent of p53. Accordingly, p53-proficient and -deficient cells were equally sensitive to RS [18]. Thus, Bak is activated in HCT116 $bax^{-/-}$ cells upon RS exposure.

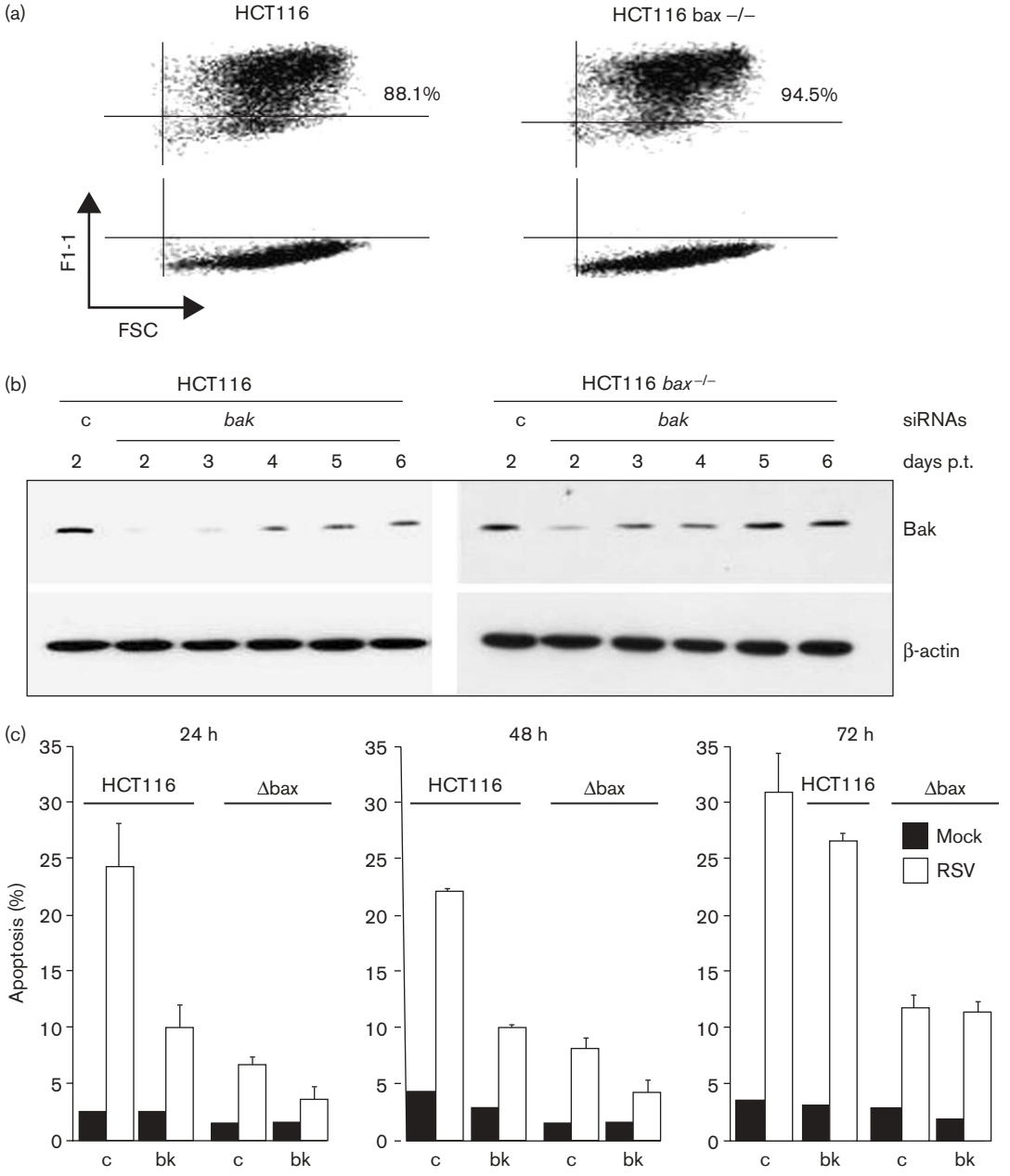
To investigate whether Bak activation can account for the apoptosis observed in RS-treated *bax*-null cells, siRNA knockdown studies were carried out. Initial tests revealed that siRNA can be transfected into HCT116 and HCT116 $bax^{-/-}$ cells via the RNAiFect procedure (Qiagen) to levels of 85–95% (Fig. 3a). Transfection of

Fig. 2



Steady-state levels and trypsin-sensitive conformational change of Bak protein in HCT116 $bax^{-/-}$ cells in response to RS (100 μmol/l). (a) Immunoblot analysis on 15 μg protein from cells that had been mock-treated or RS-treated for 4–24 h. Monoclonal anti-β-actin antibody was used at 1:5000; polyclonal anti-Bak anti-serum at 1:1000. (b) HCT116 $bax^{-/-}$ and $p53^{-/-}$ cells were treated for 24 h with RS or not. Cells were Dounce-homogenized and the mitochondria-containing high-membrane fraction was collected from a 1.5-mol/l sucrose cushion after centrifugation. The enrichment for mitochondria and equal loading of mitochondrial proteins were tested by immunoblotting with monoclonal anti-cytochrome oxidase IV antibody (1:500). The p53 monoclonal antibody DO-1 was used at 1:1000. Trypsin sensitivity was tested by incubating mitochondria with trypsin as detailed in Materials and methods.

Fig. 3



Effect of Bak knockdown on the sensitivity of HCT116 cells and HCT116 *bax*$^{-/-}$ cells to RS. (a) Transfection of exponentially growing HCT116 and HCT116 *bax*$^{-/-}$ cultures with a standard FITC-labeled siRNA, using the RNAiFect reagent from Qiagen as recommended by the manufacturer, was performed in parallel with every knockdown approach and regularly revealed transfection efficiencies of 85–95%. This indicates that approximately 10% of the cells in each knockdown approach produced a knockdown-negative background. Transfection efficiencies were measured by flow cytometry. (b) Cultures of the indicated cell types were transfected with the control siRNA or *bak* siRNA for 2–6 days. Total cellular protein (15 μg) was analyzed by immunoblotting with the polyclonal anti-Bak anti-serum at 1:1000 and the monoclonal anti-β-actin antibody at 1:5000 dilution. *bak* siRNA transfection caused a strong knockdown at day 2, and a gradual recovery of the Bak levels back to normal between days 3 and 5. (c) Cultures of HCT116 cells or *bax*$^{-/-}$ cells (Δbax) were transfected with control siRNA (c; 30 nmol/l) or *bak* siRNA (bk; 30 nmol/l) and, upon verification of knockdown by immunoblotting, were treated with RS (100 μmol/l) or not (mock) for the indicated times. Transfection was performed with RNAiFect from Qiagen, as recommended. Apoptosis was measured by counting the numbers of cells with a sub-2*n* DNA content following methanol fixation and PI staining. Error bars show SDs of at least six assays.

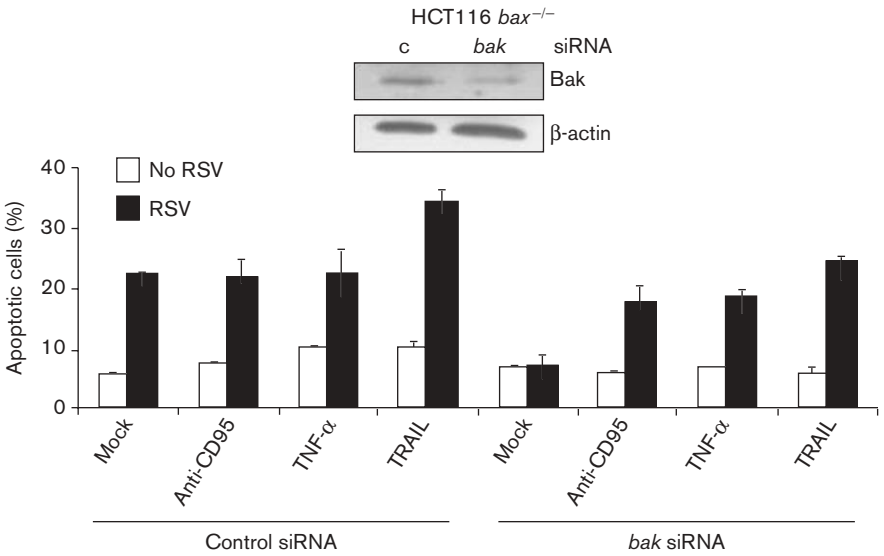
30 nmol/l of *bak* siRNA into either cell type resulted in the reduction of Bak protein levels to approximately 20% of the levels obtained in control siRNA transfected cultures at day 2 after transfection and a gradual recovery of the Bak levels back to normal during the following days (Fig. 3b). When HCT116 cultures were transfected with

control or *bak* siRNA and either mock-treated or treated with RS, the Bak-knockdown cultures were significantly impaired for the induction of apoptosis ($P < 0.05$; Student's *t*-test), to an extent comparable to the apoptosis inhibition observed in the *bax*-null cultures (Fig. 3c). Notably, when HCT116 *bax*$^{-/-}$ cultures were transfected similarly, the apoptotic response was abolished in the absence of Bax + Bak ($P = 0.07$, Student's *t*-test, 24 h time point; note that approximately 10% of the cells were non-transfected; Fig. 3a). In accordance with the gradual recovery of the Bak levels in this transient RNA interference approach (Fig. 3b), apoptosis impairment was no longer detectable at 72 h (Fig. 3c). Thus, the two major pro-apoptotic BH123 proteins are the critical complementing effectors of the intrinsic (not death receptor ligand-dependent), RS-provoked apoptosis in HCT116 CRC cells.

Certain ligands such as TRAIL and CD95 ligand can induce tumor cell apoptosis via pro-apoptotic receptors, either through the extrinsic caspase-8-dependent (type I) pathway or through amplification of the death signal via the intrinsic mitochondria-mediated (type II) pathway. HCT116 cells often behave like type II cells in that drug-induced, receptor-mediated death requires Bax and is blocked by Bcl-2 [25,26]. However, recent work has documented that certain drugs including RS may sensitize these cells to the extrinsic type I pathway, e.g. through the induction of death receptor accumulation in plasma membrane microdomains on the cell surface [8,27,28]. Notably, while the mitochondria-mediated apoptosis by RS reported above required no further trigger, the apoptosis through redistribution of death receptors depended upon the additional presence of ligands [8]. Against this background, we asked whether RS, in synergy with death receptor ligands, would be able to attack HCT116 carcinoma cells even in the face of a mitochondrial pathway incapacitated by Bax + Bak depletion.

As has been reported before by Delmas *et al.* [8] for the parental HCT116 cells, treatment of the *bax*-deficient derivatives with RS for 48 h resulted in the formation of large patches of CD95 protein on the cell surface that constituted *bona fide* lipid rafts. When HCT116 *bax*$^{-/-}$ cultures were transfected with control siRNA or *bak* siRNA for 24 h and then either mock-treated or treated with RS for a further 48 h, the Bax + Bak-depleted cells again showed apoptosis only at background levels (Fig. 4, compare "mock" of the control and *bak* siRNA-treated cells). Importantly, addition to the medium, for the final 24 h, of death receptor-activating CD95 antibody, TNF-α or TRAIL entailed a significant increase in the apoptotic response of the Bax + Bak-depleted cultures (Fig. 4; anti-CD95: $P = 0.01$; TNF-α and TRAIL: $P < 0.001$; Student's *t*-test). This indicates that even under the condition of inhibition of RS-induced apoptosis, following the absence of sufficient Bax and Bak proteins, the cells can be sensitized by RS for a receptor-mediated cell death.

Fig. 4



Sensitivity of Bax + Bak-deficient HCT116 cells to death receptor ligands in dependence of RS. HCT116 *bax*$^{-/-}$ cultures were transfected with RNAiFect with control siRNA or *bak* siRNA (30 nmol/l) for 24 h. Inset: immunoblotting of 15 μg of total protein with the polyclonal anti-Bak anti-serum at 1:1000 and the monoclonal anti-β-actin antibody at 1:5000 dilution, documenting Bak knockdown. At 24 h after transfection, cells were mock-treated or received RS (100 μmol/l) for another 24 h. Where indicated, the cultures were then exposed for 24 h to the death receptor activating anti-CD95 antibody CH11 (100 ng/ml), or to TNF-α (25 ng/ml) or TRAIL (100 ng/ml). Apoptosis was measured by flow cytometry upon methanol fixation and PI staining. The SDs were calculated from four experiments.

Discussion

Selective apoptosis induction is thought to be critical for the elimination of cancers as well as for interventions aimed at arresting the process of carcinogenesis in premalignant lesions. The non-toxic plant polyphenol RS has caused apoptosis not only in tumor cell lines *in vitro* [7,11,13,18,29], but also in a rat model of azoxymethane-induced colon carcinogenesis [30] and a mouse model of familial adenomatous polyposis [31]. Solid tumors including those of the colon seem to be primarily affected through the stimulation of the intrinsic mitochondrial death pathway [11,18] and at least in part independently of the p53 tumor suppressor that is often inactivated [18]. The mitochondrial apoptosis pathway intimately involves the pro-apoptotic Bax and Bak proteins; the engagement of cytosolic Bax following RS exposure requires a conformational change and mitochondrial translocation [10], whereas the constantly mitochondrial Bak is activated by conformational alterations [23]. Remarkably, deficiency of Bax alone is sufficient to completely abolish the apoptotic response of HCT116 cells to the chemopreventive drug sulindac and other NSAIDs [1]. In contrast, RS at concentrations easily attainable in the colon through ingestion was able to provoke apoptosis in HCT116 $bax^{-/-}$ cells and *bax*-proficient HCT116 cells with knocked-down Bak expression, albeit at a reduced level (this paper). This underscores that RS is a potentially highly effective chemopreventive and therapeutic agent. The absence of both Bax and Bak abolished RS-induced apoptosis, indicating that these two constitute the critical complementary effectors of CRC cell apoptosis by this compound. However, RS can function as a sensitizer for death receptor pathway-mediated apoptosis triggered by the death receptor ligands CD95, TNF-α and TRAIL [8,27]. We have demonstrated here that this sensitization effect can be retained in the absence of Bax and Bak. Thus, in contrast to NSAIDs, RS may remain effective as a pro-apoptotic agent as long as Bax and Bak have not both been inactivated during clonal selection, and may even then be able to help eliminate tumor cells through sensitization to the extrinsic death receptor pathway.

Conclusion

The non-toxic phytoalexin and polyphenol RS may act as a pro-apoptotic chemopreventive most effectively in the digestive tract where the relatively high concentrations required to provoke apoptosis can be easily attained. RS itself induces apoptosis via the mitochondrial pathway and can sensitize cells to ligand-induced, death receptor-mediated apoptosis. In contrast to common NSAIDs, whose pro-apoptotic chemopreventive effects are blunted by the loss of expression of the Bax protein, RS remains effective in the absence of Bax or Bak, and can sensitize cells to death receptor-mediated apoptosis even upon loss of both Bax and Bak. Thus, RS may remain longer active as a powerful chemopreventive even under conditions of clonal selection.

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