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Inhibition of Fas expression by RNAi modulates 5-fluorouracil-induced apoptosis in HCT116 cells expressing wild-type p53

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Abstract

Drug resistance to 5-fluorouracil (5-FU) is still a major limitation to its clinical use. In addition, the clinical value of p53 as a predictive marker for 5-FU-based chemotherapy remains a matter of debate. Here, we used HCT116 human colorectal cancer cells expressing wild-type p53 and investigated whether inhibition of Fas expression by interference RNA modulates 5-FU-induced apoptosis. Cells were treated with 5-FU (1, 4 or 8 μM) for 8–48 h. Cell viability was evaluated by trypan blue dye exclusion. Apoptosis was assessed by changes in nuclear morphology and caspase activity. The interference RNA technology was used to silence Fas expression. Caspase activation, p53, Fas, cytochrome *c*, and Bcl-2 family protein expression was evaluated by immunoblotting. 5-FU was cytotoxic in HCT116 cells ($p<0.001$). Nuclear fragmentation and caspase-3, -8 and -9 activities were also markedly increased in HCT116 cells after 5-FU ($p<0.001$). In addition, wild-type p53 and Fas expression were 25- and 4-fold increased ($p<0.05$). Notably, when interference RNA was used to inhibit Fas, 5-FU-mediated nuclear fragmentation and caspase activity were markedly reduced in HCT116 cells. Finally, western blot analysis of mitochondrial extracts from HCT116 cells exposed to 5-FU showed a 6-fold increase in Bax, together with a 3-fold decrease in cytochrome *c* ($p<0.001$). In conclusion, 5-FU exerts its cytotoxic effects, in part, through a p53/Fas-dependent apoptotic pathway that involves Bax translocation and mitochondrial permeabilization.

Keywords: Apoptosis; Fas; 5-fluorouracil; Mitochondria; p53; siRNA

1. Introduction

The main goal in cancer therapy is the abrogation of tumor cell growth and proliferation, and ultimately the complete elimination of tumor cells. This can be achieved by either activation of cell death programs, correction of non-functional metabolic pathways, or reduction of drug efflux from cancer cells. Further, it is commonly accepted that tumor cells treated with anticancer agents undergo apoptosis, and that cells resistant to apoptosis often do not respond to anticancer therapy [1]. However, other determinants of drug resistance exist, including loss of cell surface receptors or transporters, altered metabolism, or mutation of specific target [2].

5-Fluorouracil (5-FU) has been a key drug in the treatment of colorectal cancer for more than 40 years. However, tumor cell resistance remains a significant limitation to the clinical use of 5-FU [3]. The mechanism of 5-FU cytotoxicity has been attributed to misincorporation of fluoronucleotides into RNA and DNA and to inhibition of the nucleotide synthetic enzyme thymidylate synthase [3]. Further, 5-FU induces apoptosis in normal intestinal cells [4] and in a number of colon cancer cell lines [5,6]. Other reports have demonstrated that 5-FU-induced apoptosis is dependent on the tumor suppressor p53 protein [5,7,8], although apoptosis can also occur in mutant p53 cell

Abbreviations: 5-FU, 5-fluorouracil; siRNA, short interference RNA

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lines [9,10]. In contrast, defects in apoptosis have been implicated in chemoresistance of colon cancer cells and, therefore, alterations in the mediators of 5-FU-induced apoptosis may account for 5-FU resistance [11]. Thus, understanding its molecular mechanisms of action is pivotal to overcoming tumor cell resistance and improving overall chemotherapy efficacy.

The tumor suppressor p53 gene plays a major role in preventing tumorigenesis, by responding to both cellular stress and DNA damage [12]. In fact, p53 is stabilized by inhibition of its degradation in response to a wide range of stresses, including DNA damage, aberrant proliferative signals and exposure to chemotherapeutic drugs. However, increased p53 protein levels are not sufficient for its activation. Rather, the modulation of p53 activity occurs via phosphorylation, acetylation, glycosylation, ribosylation, ubiquitination or sumoylation, of p53, thereby altering its DNA-binding ability or affinity for negative regulators [13]. Post-translational modifications that increase the ability of p53 to bind specific gene sequences and activate gene transcription, ultimately lead to either cell cycle arrest or apoptosis [13,14]. Numerous studies have demonstrated that p53 directly activates the transcription of a number of genes including $p21^{WAF1/CIP1}$ [15], the major mediator of p53 cell-cycle inhibitory capacity, and the apoptotic genes *BAX* [16], *PUMA* [17], *NOXA* [18], *p53AIP* [19] and *FAS/APO1* [20]. It has also been shown to transcriptionally repress *BCL-2* [21]. It is not entirely clear how p53 modulates apoptosis and the role of transcriptional activation and repression [22]. In fact, p53 has been shown to translocate to the mitochondria where it is directly apoptogenic, forming complexes with anti-apoptotic Bcl-2 family proteins. This allows the release of sequestered pro-apoptotic proteins and subsequent permeabilization of the outer mitochondrial membrane [23,24].

p53 is the most commonly mutated tumor suppressor gene in human cancers. However, the relationship between p53 status and sensitivity to chemotherapeutic drugs, including 5-FU, is still controversial. 5-FU- and cisplatin-induced apoptosis was significantly increased after wild-type p53 transfection in otherwise resistant cell lines [25]. In addition, p53 disruption rendered colon cancer cells resistant to 5-FU, both *in vitro* and *in vivo* [8]. However, both pre-clinical and clinical studies have failed to associate p53 status with survival after 5-FU adjuvant chemotherapy [26], or surgery+5-FU-based chemotherapy [27]. Thus, p53 mutational status and/or increased p53 immunostaining does not appear to be an independent predictor of poor prognosis in colon cancer [28]. In contrast, other studies have shown an association of normal p53 with improved colon cancer prognosis. In a well-defined population of 391 patients with stage III colon cancer treated with 5-FU adjuvant chemotherapy, mutant p53 was associated with shorter disease-free survival, both in univariate and multivariate analysis [29]. Another study using tumor tissue from phase III surgical adjuvant trials also showed that normal p53 and 5-FU-based treatment were significantly associated with improved overall survival and also disease-free survival [30].

Despite the controversy, the majority of human tumor mutations decrease the sequence-specific DNA binding affinity and the transcriptional activity of p53 protein [31]. This may account for acquired chemoresistance to anticancer drugs due to failure of cells to undergo apoptosis [13,32]. In contrast to the mitochondrial pathway, inactivation of the death receptor apoptotic pathway is not frequent nor a prerequisite for tumor development. In fact, tumor cells frequently present intact death receptor signaling pathways [33]. Several chemotherapeutic agents have been shown to up-regulate Fas death receptor expression in cancer cell lines of different origin, including colorectal cancer [34,35]. Loss of p53 function has also been reported to affect the ability of cancer cells to up-regulate the Fas receptor in response to chemotherapy [5,6].

In this study, we further investigated the apoptotic pathways activated by 5-FU in a particular HCT116 cancer cell line. Our results confirm that Fas is up-regulated by 5-FU in a p53-dependent manner. Silencing Fas expression abrogated the sensitivity to 5-FU-induced apoptosis. Further, 5-FU increased p53 and Bax mitochondrial translocation, thus amplifying apoptosis via the mitochondrial pathway.

2. Materials and methods

2.1. Cell culture and induction of apoptosis

HCT116 cells were grown in DMEM culture medium (Invitrogen Corp., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen Corp.), 1% L-glutamine 200 mM (Merck and Co. Inc., NJ, USA) and 1% antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO, USA), and maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were plated at either 1×10^5 cells/cm^2 for morphological assessment of apoptosis and viability assays, or 2×10^5 cells/cm^2 for protein extraction and transfection assays. Cells were then incubated in culture medium containing 1, 4 or 8 μM 5-FU (Sigma Chemical Co.) in dimethyl sulfoxide (final concentration<0.1%), or no addition (control) for 8, 24 or 48 h. Cells were fixed for microscopic assessment of apoptosis or processed for cell viability assays. In addition, total and cytosolic proteins were extracted for immunoblotting and caspase activity.

2.2. Evaluation of cell death and apoptosis

Cell death was measured by trypan blue dye exclusion. Hoechst labeling of cells was used to detect apoptotic nuclei. Briefly, the medium was gently removed at the indicated times to prevent detachment of cells. Attached cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 10 min at room temperature, incubated with Hoechst dye 33258 (Sigma Chemical Co.) at 5 μg/ml in PBS for 3 min, washed with PBS and mounted using PBS:glycerol (3:1, v/v). Fluorescent nuclei were scored blindly by laboratory personnel and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed non-condensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin. Three random microscopic fields per sample of ~100 nuclei were counted and mean values expressed as the percentage of apoptotic nuclei.

2.3. Caspase activation

Caspase activity was determined in cytosolic protein extracts after harvesting and homogenization of cells in isolation buffer, containing 10 mM Tris–HCl buffer, pH 7.6, 5 mM MgCl2, 1.5 mM potassium acetate, 2 mM dithiothreitol, and protease inhibitor mixture tablets (Complete; Roche Applied Science, Mannheim, Germany). General caspase-3, -8 and -9-like activities were determined by enzymatic cleavage of chromophore *p*-nitroanilide (*p*NA) from the substrate *N*-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-*p*NA), *N*-acetyl-Ile-Glu-

pro-Asp-*p*NA (IEPD-*p*NA) and *N*-acetyl-Leu-Glu-His-Asp-*p*-NA (LEHD-*p*NA) (Sigma Chemical Co.), respectively. The proteolytic reaction was carried out in isolation buffer containing 50 μg cytosolic protein and 50 μM of specific caspase substrate. The reaction mixtures were incubated at 37 °C for 1 h, and the formation of *p*NA was measured at 405 nm using a 96-well plate reader.

2.4. Short interference RNA and transfections

To evaluate the role of Fas membrane receptor in 5-FU-induced cell death, we performed Fas gene silencing assays. Briefly, we used a 25-nucleotide duplex short interference RNA (siRNA) sequence targeting human Fas mRNA, designed to knock down human Fas gene expression (Santa Cruz Biotechnology, Santa Cruz, CA). At 24 h after plating, cells at ~70% confluence were transfected with Fas siRNA using JetSI Transfection Reagent for siRNA (Polyplus-Transfection, Illkirch, France), at 50 nM, according to the manufacturer's instructions. Twenty four hours after transfection, cells were incubated with 8 μM 5-FU or no addition for 24 h, after which all floating and attached cells were harvested for cytosolic and total protein extraction. Hoechst labeling of cells was used to detect apoptotic nuclei as described above. To assess gene silencing, Fas protein levels were evaluated by immunoblotting.

2.5. Mitochondrial fractionation

Mitochondrial levels of cytochrome *c*, p53 and Bax were determined using mitochondrial protein extracts from cells treated with 8 μM 5-FU for 24 h, or no addition. Cells were harvested and centrifuged at 600×*g* for 5 min at 4 °C. The pellets were washed once in ice-cold PBS and resuspended with 3 volumes of isolation buffer containing 20 mM HEPES/KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and supplemented with protease inhibitor cocktail tablets (Roche Applied Science), in 250 mM sucrose. After chilling on ice for 15 min, cells were disrupted by 40 strokes on a glass homogenizer, and homogenates centrifuged twice at 2500×*g* for 10 min at 4 °C to remove unbroken cells and nuclei. The mitochondrial fraction was then centrifuged at 12,000×*g* for 30 min at 4 °C, and the pellet was resuspended in isolation buffer.

2.6. Immunoblotting

Steady-state levels of p53, Bcl-2, Bax, poly (ADP-ribose) polymerase (PARP), Fas, and caspase-3 proteins were determined by immunoblot analysis. Briefly, 150 μg of total, 40 μg of cytosolic or 150 μg of mitochondrial protein extracts were separated on 8, 12 or 14% SDS-polyacrylamide electrophoresis gels. Following electrophoretic transfer onto nitrocellulose membranes, immunoblots were incubated with 15% H_2O_2 for 15 min at room temperature. After blocking with 5% milk solution, the blots were incubated overnight at 4 °C with primary mouse monoclonal antibodies reactive to p53, Bax, Bcl-2, Fas (Santa Cruz Biotechnology), cytochrome *c* (BD Biosciences PharMingen, San Diego, CA) and cytochrome *c* oxidase subunit II (Molecular Probes, Eugene, OR), or primary rabbit polyclonal antibodies for PARP and caspase-3 (Santa Cruz Biotechnology). Finally, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA) for 3°h at room temperature. The membranes were processed for protein detection using Super Signal™ substrate (Pierce, Rockford, IL, USA). β-actin was used as a loading control. Protein concentrations were determined using the Bio-Rad protein assay kit according to the manufacturer's specifications.

2.7. Densitometry and statistical analysis

The relative intensities of protein bands were analyzed using the ImageMaster 1D Elite v4.00 densitometric analysis program (Amersham Biosciences, Piscataway, NJ, USA). All data were expressed as mean±SEM from at least three separate experiments. Statistical analysis was performed using GraphPad InStat v3.00 (GraphPad Software, San Diego, CA) for the Student's *t*-test. Values of $p<0.05$ were considered significant.

3. Results

3.1. Sensitivity of colon cancer cells to 5-FU

We initially evaluated 5-FU cytotoxicity in wild-type p53 HCT116 cells. Our results showed that general cell death, as measured by trypan blue dye exclusion, increased up to 2-fold after supplementation with 5-FU for 48 h ($p<0.001$). This effect was dose-dependent, with a maximum response with 8 μM 5-FU (Fig. 1, left panel). Similarly, a time-response assay showed that 8 μM 5-FU induced significant cell death in HCT116 cells at 24 h ($p<0.01$), and this continued to increase at least up to 48 h ($p<0.001$) (Fig. 1, right panel). Exposure to 0.1% dimethyl sulfoxide solvent alone did not significantly alter cell death (data not shown).

3.2. Induction of apoptosis by 5-FU

It is well established that tumor cells exposed to anticancer agents die from apoptosis; and those resistant to apoptosis are able to survive anticancer treatment [1]. Coincubation experiments with the pan-caspase inhibitor z-VAD.fmk strongly prevented HCT116 cell death, suggesting that induction of apoptosis plays an important role in 5-FU-injury to HCT116 cells (data not shown). In fact, high levels of apoptosis were observed in HCT116 cells after 48 h incubation with 8 μM 5-FU as evaluated by Hoechst staining. The number of apoptotic cells increased by >2-fold as compared with control cells ($p<0.001$) (Fig. 2A).

3.3. 5-FU induces caspase-dependent apoptosis

Drug-induced apoptosis generally occurs through caspase-dependent pathways [3,36]. We therefore evaluated caspase activation after 5-FU treatment in HCT116 cells, using specific colorimetric caspase substrates. Caspase-2 and -6 were not significantly activated (data not shown). Caspase-8 activity was almost 3-fold increased and caspase-3 and -9 activities were ~2-

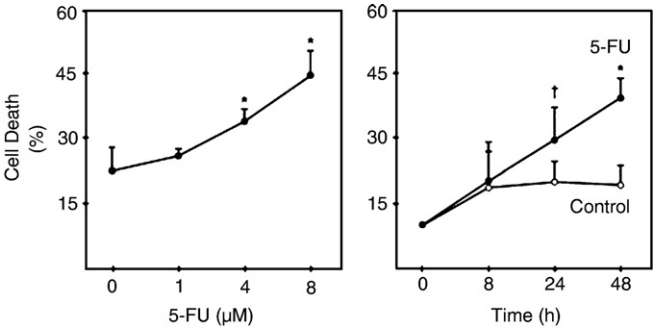

Fig. 1. 5-FU-induced cytotoxicity in the HCT116 colon cancer cell line. Cells were harvested for general cell death evaluation by trypan blue dye exclusion. Percentage of cell death after incubation with 8 μM 5-FU, or no addition (0 μM; control) for 48 h (left). Percentage of cell death after incubation with 8 μM 5-FU, or no addition (control) for 8, 24 and 48 h (right). The total number of HCT116 cells before and after 8 μM 5-FU for 48 h was 1×10^5 and $0.58\times10^5/cm^2$, respectively. Results are expressed as mean±SD of at least three independent experiments. *$p<0.001$ and $^{\dagger}p<0.01$ from controls.

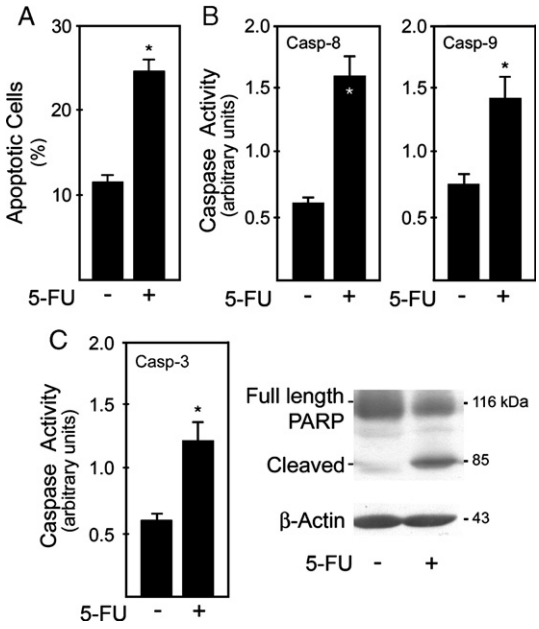

Fig. 2. 5-FU induces apoptosis in HCT116 cells. Cells were fixed and stained for morphological evaluation of apoptosis. Cytosolic and total proteins were extracted for caspase activity assays and immunoblot analysis, respectively. (A) Percentage of apoptosis in cells exposed to 8 μM 5-FU, or no addition (control) for 48 h. (B) Caspase-8 and -9 activities in cytosolic protein extracts after incubation with 8 μM 5-FU, or no addition (control) for 24 h. (C) Caspase-3 activity (left) and PARP cleavage (right) in protein extracts after incubation with 8 μM 5-FU, or no addition (control) for 24 h and 48 h, respectively. The results are expressed as mean±SEM percentage or arbitrary units of at least three independent experiments. *$p<0.001$ from controls. β-actin was used as a loading control.

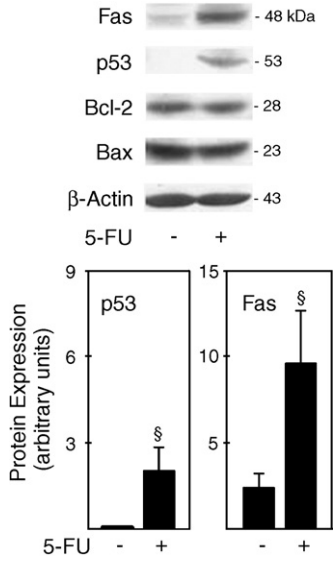

Fig. 3. 5-FU increases p53 and Fas expression in p53-proficient cells. HCT116 cells were exposed to 8 μM 5-FU or no addition (control) for 48 h. Total proteins were extracted for immunoblot analysis. Representative immunoblots of p53 and Fas expression and corresponding protein levels. Representative immunoblots of Bcl-2 and Bax are also shown. The results are expressed as mean±SEM arbitrary units of at least five independent experiments. §$p<0.05$ from controls. β-actin was used as a loading control.

fold increased in cells incubated with 8 μM 5-FU for 24 h ($p<0.001$) (Fig. 2B and C). In addition, the endogenous substrate of active caspase-3, PARP, was cleaved in HCT116 cells. These results demonstrate that 5-FU induces caspase-dependent apoptosis through multiple classical apoptotic pathways.

3.4. 5-FU-induced apoptosis is associated with changes in p53 expression

Anticancer drugs including 5-FU promote DNA damage [3,14]. The tumor suppressor p53 protein is normally up-regulated in response to DNA insults, inducing cell-cycle arrest and apoptosis via multiple different pathways [37]. We next evaluated the effect of 5-FU on steady-state p53 levels in HCT116 cells. Our results show that p53 expression was ~25-fold increased in cells treated with 8 μM 5-FU for 48 h compared with untreated cells ($p<0.05$) (Fig. 3).

To further ascertain the mechanism by which 5-FU induces apoptosis, we evaluated the steady-state levels of known transcriptional targets of p53 involved in apoptosis, including the Fas death receptor, pro-apoptotic Bax and anti-apoptotic Bcl-2 (Fig. 3). Fas expression was >4-fold elevated in 5-FU-treated HCT116 cells compared with untreated cells ($p<0.05$). Bax and Bcl-2 protein levels did not change after 5-FU incubation of HCT116 cells. This is not in itself sufficient to rule out the involvement of the mitochondrial pathway in 5-FU-induced apoptosis. In fact, caspase-9 activity was increased by 5-FU in HCT116 cells.

3.5. Fas is required in 5-FU-induced apoptosis

To evaluate the functional role of the Fas death receptor in 5-FU apoptotic signaling, we performed Fas siRNA-mediated

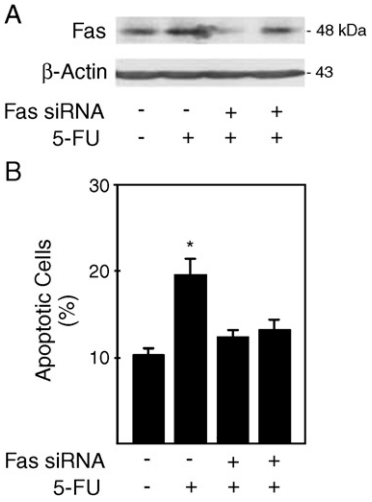

Fig. 4. Fas silencing reduces 5-FU-induced apoptosis in HCT116 cells. Cells were transfected with Fas siRNA or mock transfected at 24 h after platting. Twenty-four hours after transfection, 8 μM 5-FU or no addition (control) were included for additional 24 h. Total proteins were extracted for immunoblot analysis. In addition, cells were fixed and stained for morphological evaluation of apoptosis. (A) Representative immunoblot of total Fas expression. (B) Percentage of apoptosis. The results are expressed as mean±SEM percentage of at least three independent experiments. *$p<0.001$ from control. β-actin was used as a loading control.

gene silencing experiments. HCT116 cells were transfected with human Fas siRNA duplex or with a nonspecific control for 24 h, after which cells were incubated with 8 μM 5-FU or no addition for another 24 h. Transfection with the nonsilencing duplex did not significantly affect Fas protein levels relative to untransfected controls (data not shown). Steady-state levels of Fas protein expression were determined by western blot analysis of total protein extracts. Fas gene silencing resulted in a reduction of Fas protein expression by at least 70% in 5-FU-treated HCT116 cells (Fig. 4A).

Having confirmed the effectiveness of Fas gene silencing, we next evaluated nuclear fragmentation after Fas siRNA transfection. Fas siRNA had negligible inherent cytotoxicity (Fig. 4B). Importantly, Fas siRNA transfection prevented 5-FU-induced nuclear fragmentation by 85% in HCT116 cells. To determine the impact of Fas silencing in downstream molecular effectors of apoptosis, caspase activities were also evaluated. Transfection with Fas siRNA significantly prevented caspase-8 and -9 activities after 5-FU incubation by ~80 and 40%, respectively (Fig. 5A). The increase in caspase-3 activity induced by 5-FU was also prevented in 65% by Fas siRNA (Fig. 5B). Thus, the Fas death receptor is essential for the onset of 5-FU-induced apoptosis in p53-proficient cells. Finally, depletion of Fas by siRNA partially inhibited induction of p53 after 5-FU exposure in HCT116 cells (Fig. 5C).

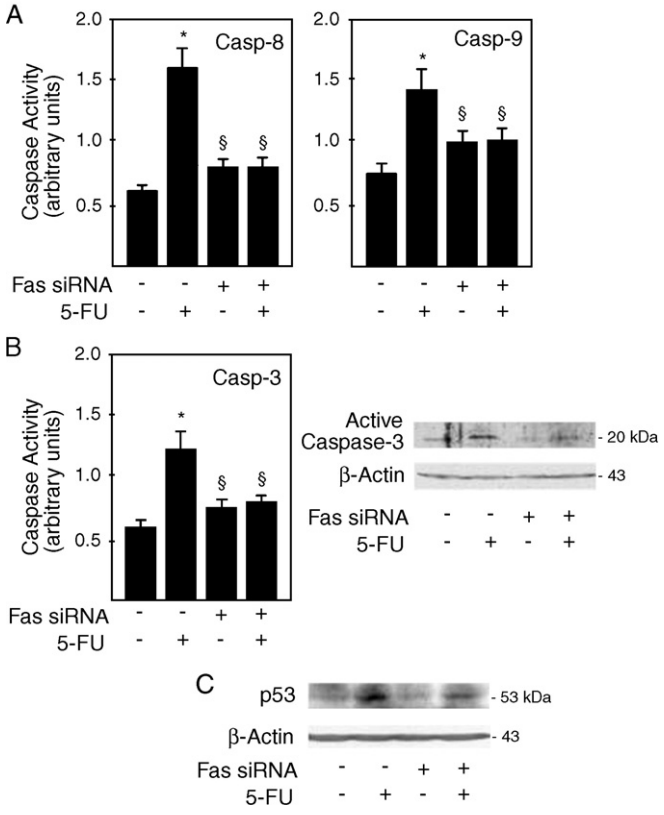

Fig. 5. Fas silencing reduces 5-FU-induced caspase activation in HCT116 cells. Cells were transfected with Fas siRNA or mock transfected at 24 h after plating. Twenty-four hours after transfection, 8 μM 5-FU or no addition (control) were included for additional 24 h. Cytosolic proteins were extracted for caspase activity and processing assays. (A) Caspase-8 and -9 activities. (B) Caspase-3 activity (left) and representative immunoblot of active caspase-3 (right). (C) Representative immunoblot of total p53 expression. The results are expressed as mean±SEM arbitrary units of at least three independent experiments. *$p<0.001$ and $^{\S}p<0.05$ from controls. β-actin was used to control for lane loading.

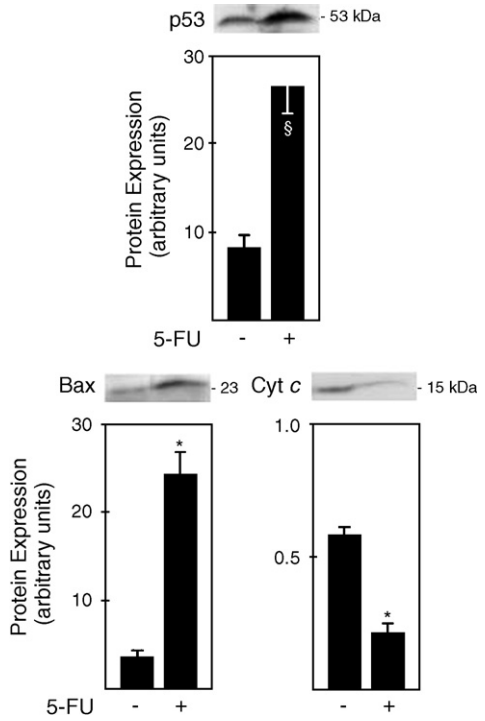

Fig. 6. 5-FU induces cytochrome *c* release and translocation of p53 and Bax to the mitochondria in HCT116 cells. Cells were exposed to 8 μM 5-FU or no addition (control) for 24 h. Mitochondrial proteins were extracted for immunoblot analysis. Representative immunoblots of p53, Bax and cytochrome *c* (Cyt *c*) and corresponding protein levels. The results are expressed as mean± SEM arbitrary units of at least three independent experiments. *$p<0.001$ and $^{\S}p<0.05$ from controls.

3.6. 5-FU induces cytochrome c release and translocation of p53 and Bax to the mitochondria

Drug-induced apoptosis has been reported to be dependent on activation of the mitochondrial pathway [32]. Further, p53 may have a direct non-transcriptional effect in activating this pathway [23,24,38]. To determine the role of the mitochondrial pathway in 5-FU-induced apoptosis, we evaluated cytochrome *c* in mitochondrial protein extracts from HCT116 cells incubated with 8 μM 5-FU for 24 h. Our results showed that 5-FU reduced mitochondrial cytochrome *c* content by 3-fold (Fig. 6) ($p<0.001$). Moreover, cytochrome *c* oxidase II was not significantly changed in mitochondrial protein extracts and, therefore, remained undetectable in the cytosolic fraction, consistent with the absence of mitochondrial disruption (data not shown).

It is thought that a portion of wild-type p53 lacking transactivation activity, localizes to the mitochondria, where it is capable of forming complexes with Bcl-2 and Bcl-X_L, thereby releasing sequestered pro-apoptotic members of the Bcl-2 family, such as Bid and Bax. This is achieved through direct physical interaction of p53 protein with anti-apoptotic

members of the Bcl-2 family [24]. Indeed, following 5-FU incubation, mitochondrial p53 levels were increased in HCT116 cells ($p<0.05$). Further, consistent with cytochrome *c* release, Bax mitochondrial levels were >6-fold increased ($p<0.001$).

These results show that 5-FU is capable of activating the mitochondrial pathway of apoptosis. p53 may play a crucial transcription-independent pro-apoptotic role in this cellular context, probably interacting with anti-apoptotic mitochondria localized proteins and allowing Bax to translocate to the mitochondria, where it exerts its pro-apoptotic function.

4. Discussion

5-FU has been the most widely used drug for colorectal cancer adjuvant chemotherapy for more than 4 decades. However, chemoresistance remains a significant drawback to its clinical success. Here, we provide further insight into the molecular pathways activated by 5-FU in a HCT116 colon cancer cell line. In these cells, most of the apoptotic activity is mediated via Fas signaling in association with wild-type p53 as evidenced by the use of siRNA Fas molecules. Moreover, p53 and Bax pro-apoptotic function at the organelle level may also account for activation of the mitochondrial pathway of apoptosis.

5-FU is a pro-apoptotic agent which can interfere with both RNA and DNA metabolism. Further, its cytotoxicity has been correlated with the presence of wild-type p53 protein [5,7,8]. However, some controversy exists regarding the predictive value of p53 status on 5-FU-sensitivity as well as patient survival. In the present study, we used HCT116 cells, expressing both wild type p53 and Bax [39]. 5-FU resulted in significant nuclear fragmentation of wild-type p53 HCT116 cells. Further, 5-FU increased caspase-8, -9 and -3 activities, while markedly up-regulating p53 in HCT116 cells. These results confirm that 5-FU-induced apoptosis is caspase-dependent and associates with functional expression of wild-type p53; however, they do not exclude that there might be non-p53 dependent apoptosis in mutant p53 cells.

p53 plays a pivotal role in the cellular environment due to its ability to "sense" cellular stresses and activate several molecular signaling pathways. p53 can lead to cellular repair of the sustained damage by induction of cell cycle arrest, or ultimately to apoptotic cell death. Indeed, p53 is known to transactivate several genes involved in apoptosis [16–20]. The results presented here show that Fas was up-regulated after 5-FU exposure in HCT116 cells. In contrast, Bax and Bcl-2 proteins were not modulated. This indicates that 5-FU exerts its cytotoxicity mainly through activation of the death receptor pathway of apoptosis. However, increased caspase-9 activity by 5-FU suggests that some apoptotic signaling is proceeding through a mitochondrial pathway. A known cross-talk between the extrinsic and intrinsic pathways of apoptosis depends on proteolytic activation of pro-apoptotic Bid protein by caspase-8 [40,41].

Fas is a well-known p53 transcriptional target. Further, it has been suggested that 5-FU up-regulates Fas expression in p53-proficient cells [5], but not in cells with mutant p53. However, it has not been shown that Fas up-regulation by 5-FU is essential for 5-FU-induced apoptosis in colon cancer cell lines. To evaluate the role of Fas in 5-FU-induced apoptosis, we silenced Fas gene expression at the post-transcriptional level using siRNA technology, and exposed cells to 5-FU. Our results showed that Fas gene silencing resulted in an almost complete abrogation of apoptosis in HCT116 cells. Caspase-8 and -3, and to a lesser extent caspase-9, activities were also largely prevented. This indicates that Fas signaling is responsible for most of 5-FU apoptotic signaling in p53-proficient colon cancer cells. However, the results do not entirely exclude involvement of the mitochondrial pathway in mediating 5-FU apoptotic signaling; and, in fact, suggest an alteration in cross-talk between the pathways.

It has recently been shown that p53 is able to translocate to mitochondria both *in vitro* [23] and *in vivo* [38,42]. Thus, p53 appears to play a direct pro-apoptotic role associated with permeabilization of the mitochondria and release of apoptogenic factors, such as cytochrome *c* [38,43]. In this study, 5-FU increased the fraction of mitochondrial p53 and induced cytochrome *c* release in HCT116 cells, indicating activation of the mitochondrial pathway. Interestingly, 5-FU was also able to increase the translocation of pro-apoptotic protein Bax to the mitochondria. These results suggest that p53 might also play a transcription-independent function in HCT116 cells exposed to 5-FU, probably by interacting with anti-apoptotic members of the Bcl-2 family. Released Bax then permeabilizes the outer mitochondrial membrane. Thus, the activation of the mitochondrial pathway of apoptosis is dependent on p53- and Bax-proficiency. This supports previous reports showing that disruption of both *TP53* or *Bax* alleles in a colon cancer cell line resulted in resistance to 5-FU-induced apoptosis [8,44]. However, it has also been demonstrated that 5-FU only partially depends on Bax for induction of apoptosis via mitochondrial membrane perturbation. Further, a significant amount of apoptosis was observed in Bax-negative DU145 human prostate carcinoma cells, following exposure to 5-FU [33].

Taken together, our results indicate that 5-FU-induced cell death proceeds via activation of extrinsic and intrinsic pathways of apoptosis in HCT116 cells. Further, the death receptor Fas is pivotal in 5-FU-induced, wild-type p53-dependent apoptosis. Silencing Fas significantly reduces tumor cell sensitivity to 5-FU in the presence of wild-type p53 competence. Activation of the mitochondrial pathway by 5-FU depends on p53 and Bax and appears to have a lesser contribution to the overall cell death rate. Finally, approaches that are directed at increasing 5-FU activation of the intrinsic pathway of apoptosis might improve the response to this colorectal cancer chemotherapy.

Acknowledgments

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