p53 Dependence of Fas Induction and Acute Apoptosis in Response to 5-Fluorouracil-Leucovorin in Human Colon Carcinoma Cell Lines

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ABSTRACT
We examined the patterns of induction of apoptosis, Fas expression, and the influence of the status of the p53 tumor suppressor gene, in response to treatment of human colon carcinoma cell lines to 5-fluorouracil (FUra) combined with leucovorin (LV) under conditions of both DNA-directed (HT29, VRC5/c1, and RKO) and RNA-directed (HCT8 and HCT116) cytotoxicity. Acute apoptosis was induced in cell lines expressing wtp53 (RKO, HCT8, and HCT116), independent of the mechanism of FUra action. In HT29 cells that expressed mp53, apoptosis was a delayed event. Cell lines undergoing DNA-directed FUra cytotoxicity demonstrated marked accumulation of cells in S-phase (HT29 and RKO), whereas those lines undergoing RNA-directed cytotoxicity (HCT8 and HCT116) demonstrated marked cell cycle phase arrest in G1-M, both reversible by dThd. dThd partially protected HCT8 and HCT116 cells from FUra-LV-induced apoptosis but had no influence on FUra-LV-induced loss in clonogenic survival. In cells expressing wtp53, the Fas death receptor was induced in response to FUra-LV treatment. FUra-LV sensitized RKO cells to the anti-Fas monoclonal antibody CH-11 that was completely reversed by dThd, demonstrating the involvement of DNA damage in FUra-LV-induced, Fas-dependent sensitization to CH-11. In contrast, FUra-LV sensitized HCT116 cells to CH-11-induced apoptosis, which was not dThd reversible. Transduction of HT29 cells with Ad-wtp53 induced elevated Fas expression and sensitized the cells to FUra-LV-induced apoptosis. Data indicate that the presence of a wtp53 gene determines FUra-LV-induced Fas expression, the kinetics of FUra-LV-induced apoptosis and not the extent of apoptosis induced, both being independent of the mechanism of FUra action. Therefore, in colon carcinomas that express wtp53, the approach to sensitize tumors to Fas-mediated apoptosis may be further enhanced from the effect of FUra-LV in elevating Fas expression in a p53-dependent manner.

INTRODUCTION
FUra3 combined with LV, which targets FUra to the TS locus, is the most effective therapy for the treatment of colon carcinoma. It is well known that FUra induces cytotoxicity by two predominant mechanisms:

(a) FUra inhibits TS after extensive metabolism, where the active metabolite, FdUMP, forms a covalent ternary complex with the enzyme and the reduced folate cofactor, 5,10-methylentetrahydrofolate, used in the normal catalytic reaction. The induction of a thymineless state and subsequent DNA damage follows, and the cells die by a process of thymineless death (1–3). We demonstrated previously that thymineless death is regulated via the Fas death receptor in colon carcinoma cells selected for TS deficiency (TS−) and is closely correlated with the induction of apoptosis (4). Furthermore, induction of Fas expression by the cytokine recombinant human IFN-γ in human colon carcinoma cell lines has sensitized these cells to Fas-mediated and FUra-LV-mediated cytotoxicity and apoptosis, dependent upon FUra-LV-induced DNA damage and independent of the status of the p53 tumor suppressor gene (2).

(b) The second mechanism of induction of FUra cytotoxicity is following metabolism of the drug to ribonucleotides with subsequent incorporation of FUTP into RNA, resulting in aberrant processing of RNA species. This has been associated with the toxicity of FUra to normal gastrointestinal tissues in preclinical models (5, 6). There are human colon carcinoma cell lines that demonstrate a cytotoxic response to FUra by this mechanism including HCT8 (7, 8) and HCT116 (9), supported by the finding of lack of dThd reversibility, and the inability of IFN-γ to potentiate the cytotoxic response of FUra-LV, as determined by clonogenic survival (2). Classically, cells treated with FUra are considered to undergo substantial arrest in S or at the G1-S boundary, although this is not always the case (8). In addition, the relationship between the mechanism of FUra ac-

3 The abbreviations used are: FUra, 5-fluorouracil; LV, leucovorin; TS, thymidylate synthase; dThd, thymidine; MoAb, monoclonal antibody; FACS, fluorescence-activated cell sorter; MOI, multiplicity of infection; wt, wild type.
tion, cell cycle arrest, influence of p53, and induction of apoptosis remains poorly understood.

Several reports have demonstrated that chemotherapeutic agents including cisplatin, mitomycin, methotrexate, mitoxantrone, doxorubicin, and bleomycin induce Fas expression in a variety of different cell lines, dependent on the presence of a \textit{wtp53} gene (10). This has further been substantiated in studies in which the \textit{wtp53} gene has been transfected, leading to the induction of Fas and enhancement of apoptosis (11–14). In the current study, we have demonstrated that FURA combined with LV also induces the up-regulated expression of Fas, dependent on the presence of a \textit{wtp53} gene. Furthermore, induction of Fas in the presence of wtp53 occurs in colon carcinoma cell lines that demonstrate both DNA- and RNA-directed FURA cytotoxicity. Cell lines (HT29 and RKO) in which FURA-LV cytotoxicity is DNA-directed demonstrate marked accumulation of cells in S-phase, whereas RNA-directed FURA-LV activity (HCT8 and HCT116) leads to accumulation of cells in G2-M, both reversible by dThd. After FURA-LV treatment, acute apoptosis is induced in the presence of wtp53 independent of the mechanism of FURa action. However, in the presence of a \textit{mpt53} gene, cells undergo delayed apoptosis. These data demonstrate that the presence of a \textit{wtp53} gene alters the kinetics of cell killing associated with FURA but not the overall level of cytotoxicity and apoptosis induced.

**MATERIALS AND METHODS**

**Cell Lines.** The HT29, HCT8, and HCT116 human colon carcinoma cell lines were obtained from American Type Culture Collection. VRC5/c1 was established as reported previously (15), and RKO was obtained from Dr. Michael Kastan (St. Jude Children’s Research Hospital, Memphis, TN). Cells were maintained under dThd-free conditions in the presence of folate-free RPMI 1640 containing 10% dFBS (dThd-free) and 80 nM Z-VAD-FMK; Enzyme Systems.

**Clonogenic Assays.** Cell lines were plated at a density of 1500 (HT29 and HCT116), 2000 (HCT8), and 3000 (VRC5/c1) cells/well in six-well plates. After overnight attachment, cells were treated, in triplicate, with FURA (0.1–30 μM) in the presence of LV (1 μM), either in the absence or presence of dThd (20 μM) for 72 h. Clonogenic survival was determined at 5–7 days (the equivalent of seven doublings) after removal of drug, as described previously (2). Because RKO cells lacked the ability to clone, cells were plated at a density of 1 × 10^3/well 24 h prior to a 72 h exposure to drugs and were subsequently allowed to regrow for a period of 4 days prior to elucidation of the influence of drug treatment on cell numbers, enumerated using a Coulter particle counter (2).

**Apoptosis and Cell Cycle Analyses.** All cell lines were plated at a density of 100,000–200,000 cells/well in six-well plates. After overnight attachment, cells were treated for periods up to 8 days with FURA (0.1–30 μM) + LV (1 μM), either in the absence or presence of dThd (20 μM), caspase inhibitors (10 μM Z-IETD-FMK and 100 μM Z-VAD-FMK; Enzyme Systems Products), or the anti-Fas MoAb CH-11 (200 ng/ml; MBL, International Corp.). Both the floating cells and attached cells were pooled after trypsinization, fixed in 70% ethanol, and stored at −20° prior to analysis. Apoptotic cells were detected as a sub-G1 fraction after propidium iodide staining and analysis using a Becton Dickinson FACSscan (16, 17). For evaluation of the cell cycle distribution, samples were collected as described and immediately centrifuged and resuspended in 0.005% propidium iodide containing 0.1% sodium citrate and 0.1% Triton X-100, filtered, and analyzed by FACSscan.

**p53 Sequencing.** All 11 exons of the \textit{p53} gene in the VRC5/c1 cell line were amplified by PCR of genomic DNA using specific primers as described previously (18). Sequencing of both strands of each of the PCR products was carried out using standard methodology (19). The status of the \textit{p53} gene in all other cell lines has been published (20–23).

**Expression of Fas.** Fas expression was determined during treatment of colon carcinoma cell lines at IC_{50} levels of FURA (0.3–6 μM) + LV (1 μM), either in the absence or in the presence of dThd (20 μM) for 48 h. Fas was measured in cell extracts by a standard ELISA assay, as reported previously (2), that correlated with the expression of Fas mRNA as determined by reverse transcription. Levels of the protein were linear in the range of 25–400 pg using purified Fas as a standard. Cell surface-associated Fas was also determined by FACS analysis using a phycoerythrin-conjugated DX2 anti-Fas MoAb (Phar-Mingen), using standard procedures.

**Adenoviral Delivery of wtp53.** HT29 cells were plated as described and subsequently transduced with a wtp53 adenovirus (Ad-wtp53; Genetic Therapy Inc.) at MOIs of 2, 5, 10, 50, and 100, either alone or in combination with FURA (10 μM) + LV (1 μM) for 72 h. Apoptosis was determined by FACS analysis as described. Additional HT29 cells were plated and transduced with a replication-incompetent adenoviral vector control containing the basic vector backbone sequences (Ad-VC; MOL, 100; Ref. 4) or Ad-wtp53 (MOL, 100). Fas expression was subsequently determined at 48 h by FACS analysis as described.

**RESULTS**

**Sensitivity to FURA-LV.** The sensitivity of five human colon carcinoma cell lines to FURA (0.1–30 μM) combined with LV (1 μM) is shown in Fig. 1, together with the influence of coinubcation with dThd (20 μM). As demonstrated by clonogenic survival (HT29, VRC5/c1, HCT8, and HCT116) or regrowth (RKO) assays, HT29, VRC5/c1, and RKO were sensitive to FURA at low drug concentrations, survival being reduced to <40% at concentrations of ≤3 μM. RKO was particularly sensitive to FURA-LV, with <5% survival at a FURA concentration of 0.5 μM. At these low FURA concentrations, the cytotoxic action of FURA was completely reversed in the presence of dThd in VRC5/c1 and reversed to 80% of control in HT29 and RKO. The dependence of the cytotoxic action of FURA upon DNA damage was evident in these three cell lines. At higher FURA concentrations (10 μM), clonogenic survival was not reversible by dThd in HT29, indicating a secondary mechanism of cytotoxicity under these conditions. Similar results were obtained in VRC5/c1 and RKO (data not shown). HCT8 and HCT116 were only sensitive to FURA-LV at higher FURA concentrations (≥5 μM), where FURA cytotoxicity was not dThd reversible. However, it is known that these cell lines demonstrate an RNA-
mediated mechanism of FUra cytotoxicity (7–9), and current data are consistent with these observations.

**Induction of Apoptosis by FUra-LV.** The induction of apoptosis was subsequently examined in response to varied concentrations of FUra (0.1–30 μM) combined with LV (1 μM) for 72 h, either in the absence or in the presence of dThd (20 μM; Fig. 2). Although clonogenic survival was reduced to <10% in HT29 and VRC/c1 under these conditions, apoptosis was increased only modestly (10–15%) at 72 h. In contrast, the RKO colon carcinoma cell line that also demonstrated dThd-reversible FUra-LV-induced cytotoxicity exhibited >50% apoptotic cells under these conditions. In the presence of 6 μM FUra + LV, 35–40% loss in clonogenic survival was obtained in HCT8 and HCT116 at 72 h, and this resulted in a substantial increase in the percentage of cells undergoing apoptosis, in particular in HCT116 cells, in contrast to data derived in HT29 and VRC/c1. Thus, the percentage of cells undergoing apoptosis increased from 4 to 26% in HCT8 and from 4 to ~70% in HCT116 during 72 h of FUra-LV exposure. Of interest was that in HT29, RKO, VRC/c1, and HCT8 cell lines, apoptosis induced by FUra-LV during 72 h of exposure could be reversed by dThd, and in HCT116 cells, this was partially reversible.

**Status of the p53 Tumor Suppressor Gene.** The status of p53 in the five human colon carcinoma cell lines, whether of mutant or wt sequence, was determined by PCR sequencing (VRC/c1) or was obtained from the published literature (HT29, RKO, HCT8, and HCT116; Refs. 20–23). Thus, RKO, which demonstrated dThd reversibility of FUra-LV cytotoxicity, is known to express wtp53 sequence. Furthermore, HCT8 and HCT116, which demonstrate RNA-directed FUra cytotoxicity, also contain wtp53 alleles. HT29 and VRC/c1, which demonstrate dThd reversible FUra-LV cytotoxicity, express mp53 alleles. Thus, in cell lines expressing wtp53 (RKO, HCT8, and HCT116), there was a greater induction of acute apoptosis in response to FUra-LV in contrast to cell lines expressing mp53 (HT29 and VRC/c1) that did not correlate with the mechanism of FUra action in these lines (Fig. 2).

**Effect of FUra-LV on Fas Expression.** There have been several reports demonstrating that certain chemotherapeutic agents may induce the expression of the cell surface receptor Fas when wtp53 is expressed in cells (10, 24, 25). Therefore, we examined the expression of Fas in five human colon carcinoma cell lines demonstrating either DNA- or RNA-directed mechanisms of FUra cytotoxicity and expressing either wtp53 or mp53 genes (Fig. 3). Fas expression was determined after 48 h of treatment with FUra-LV at IC50 s for each cell line. Under these conditions, FUra-LV failed to induce Fas expression in HT29 or VRC/c1 (Fig. 3). In contrast, FUra-LV treatment up-regulated Fas expression by 4–8-fold in HCT8, HCT116, and RKO. FUra-LV-induced up-regulated expression of Fas correlated with the presence of a wtp53 gene in these three cell lines, and the inability of FUra-LV to induce Fas correlated with the expression of mp53 genes in HT29 and VRC/c1.

**Induction of Acute Apoptosis.** Because human colon carcinoma cell lines differed in their ability to undergo acute apoptosis in 72 h in response to FUra-LV, which correlated with the presence of wtp53 or mp53 genes, this was examined further. The induction of apoptosis was subsequently determined for up
to 5 days by FACS analysis in HT29 (mp53) and HCT116 (wtp53) at IC$_{50}$ concentrations of FUra combined with LV (1 μM), either in the absence or in the presence of dThd (20 μM; Fig. 4). At 72 h, <20% apoptosis was induced in HT29, increasing to 34% at 5 days, and apoptosis was also reversed by dThd. In contrast, acute apoptosis was induced in HCT116 cells. The percentage of cells undergoing acute apoptosis at 72 h in response to 6 μM FUra was 48% and increased to 76% at 5 days.

**Influence of FUra-LV on Cell Cycle Distribution.** The cell cycle distribution of HT29 and HCT116 cells after 72 h of exposure to IC$_{50}$ concentrations of FUra + LV (1 μM) was subsequently examined by FACS analysis (Fig. 5). In HT29, >80% of the cells were accumulated in S-phase at 72 h and demonstrated <20% of cells in the sub-G$_1$ (apoptotic) compartment, indicating substantial S-phase arrest in the absence of acute apoptosis. However, ≈60% of HCT116 cells were arrested in G$_2$-M at this time, whereas 48% were undergoing apoptosis. Data derived in RKO were similar to those obtained in HT29 demonstrating substantial accumulation of cells in S-phase (>80%), whereas HCT8 demonstrated significant accumulation of cells (75%) in G$_2$-M after FUra-LV treatment (data not shown). S-phase accumulation of cells after FUra-LV treatment was dThd reversible in HT29 (Fig. 5) and RKO (data not shown), and G$_2$-M-phase accumulation was also dThd reversible in HCT116 (Fig. 5) and HCT8 (data not shown). Hence, the DNA-directed mechanism of FUra action (HT29 and RKO) was associated with substantial accumulation of cells in S-phase, whereas the RNA-directed mechanism of FUra action (HCT8 and HCT116) was associated with accumulation of cells in G$_2$-M.

**Delayed Apoptosis.** Because HT29 cells underwent prolonged S-phase arrest at 72 h in the absence of acute apoptosis, FUra-LV (IC$_{50}$) was removed at 72 h, and HT29 cells were subsequently incubated in drug-free medium. HCT116 cells that underwent acute apoptosis under these conditions and accumulation of cells in G$_2$-M were treated similarly, and the effect of drug removal on the induction of apoptosis was examined. It is evident that treatment of HT29 with 3 μM FUra + LV induced minimal apoptosis at 72 h (Fig. 6). However, apoptosis increased significantly, with further incubation in drug-free medium. The percentage of cells undergoing apoptosis increased from ≈14% at the time of drug removal to 32% at 6 days and to 46% at 8 days, indicating the irreversible commitment to apoptosis during S-phase arrest. In HCT116 cells, 70% of the cells were undergoing acute apoptosis at 72 h in response to 6 μM FUra + LV. When FUra-LV was removed at 72 h, the apoptotic cells were also removed. Of the cells remaining, approximately one-third underwent apoptosis 1 day after drug removal, and this level of apoptosis remained constant for the remainder of the incubation period.

**Transduction of HT29 with Ad-wtp53.** To determine whether transfection of the wtp53 gene could increase the num-

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**Figure 2.** Induction of apoptosis after 72 h exposure to FUra (0.1–30 μM), combined with LV (1 μM), either in the absence or in the presence of dThd (20 μM). Data were analyzed by FACScan and represent the mean of duplicate determinations.
bers of HT29 cells undergoing apoptosis at 72 h after treatment with FUra-LV, the following experiment was conducted. HT29 cells were transduced with Ad-wtp53 at MOIs of 0–100 in the absence or presence of dThd (20 μM). Data for HT29, VRC/c1, HCT8, and HCT116 were derived by ELISA assay and represent the means of three determinations for each treatment condition; bars, SD. For RKO, data were derived by FACS analysis and represent the means of duplicate determinations; bars, SD. Assays are as described in “Materials and Methods.”

To determine whether transduction of Ad-wtp53 allowed the induction of Fas expression in response to FUra-LV, Ad-VC (MOI, 100) or Ad-wtp53 (MOI, 100) were transduced into HT29 cells, either in the absence or presence of FUra (3 μM) + LV (1 μM). The expression of cell surface Fas was examined by FACS analysis. Introduction of the wtp53 gene allowed a 2-fold induction of Fas expression in response to FUra-LV, which was dThd reversible and correlated with enhanced apoptosis under these conditions. No increase in cell surface Fas was observed in
HT29 cells treated with Ad-VC either in the absence or in the presence of FUra-LV.

**Effect of Caspase Inhibitors on FUra-LV-induced Acute Apoptosis.** The mechanism of FUra-LV-induced cytotoxicity was DNA-mediated in RKO cells and RNA-mediated in HCT116. However, the induction of acute apoptosis was determined by the status of the $p53$ gene ($wtp53$), independent of the mechanism of FUra action. The effect of the caspase inhibitors Z-IETD-FMK and Z-VAD-FMK on acute apoptosis induced by FUra-LV in these two cell lines with differing mechanisms of FUra action was determined (Fig. 8). In both cell lines, apoptosis increased as the concentration of FUra was increased during 72 h exposure.

In RKO cells, the toxic action of FUra was completely inhibited by dThd and by Z-VAD-FMK but was not affected by coincubation with Z-IETD-FMK. Furthermore, the anti-Fas MoAb CH-11 (200 ng/ml) increased the numbers of cells undergoing apoptosis in the presence of FUra-LV, and the effect of the combination was completely reversed by dThd (Fig. 8).

In HCT116 cells, the profile was different. In contrast to RKO, apoptosis in HCT116 with increasing FUra concentrations was partially reversed by dThd, as indicated above, and reversible by both Z-IETD-FMK and Z-VAD-FMK. Furthermore, the increased apoptosis obtained when CH-11 was combined with FUra-LV was not dThd reversible in contrast to the effect observed in RKO cells (Fig. 8). Of interest was that FUra-LV-induced apoptosis was partially but not completely reversed by dThd, in contrast to FUra-LV-induced loss in clonogenic survival, which was not reversible by dThd at any FUra concentration.

**DISCUSSION**

FUra is an antimetabolite used predominantly in the treatment of epithelial cancers that has been known for many years to possess a dual mechanism of action, dependent upon the concentration of the drug used and the cellular characteristics of target cells. The panel of human colon carcinoma cell lines used in this study demonstrate predominant characteristics of DNA-directed damage attributable to the inhibition of TS and the induction of thymineless death, which are reversible by dThd at low FUra concentrations (<3 μM) in HT29, VRC/c1, and RKO cell lines. This mechanism of FUra action is independent of the status of the $p53$ tumor suppressor gene, because RKO expresses $wtp53$, whereas HT29 and VRC/c1 are mutant for $p53$. However, RKO is the most sensitive cell line of the panel to...
FUra, where clonogenic survival is eliminated at concentrations of FUra of \(1 \mu M\) when combined with LV. Other cell lines (HCT8 and HCT116) demonstrate predominant characteristics of RNA-mediated FUra cytotoxicity even in the presence of LV, which occurs only at higher FUra concentrations (\(>10 \mu M\)) in contrast to DNA-mediated FUra cytotoxicity, and is not reversible by dThd as determined by clonogenic assay. In the presence of a wtp53 gene, both FUra-LV-induced DNA damage (RKO) and RNA-mediated damage (HCT8 and HCT116) induce an acute apoptotic response. However, in the presence of a mp53 gene (HT29), DNA damage results in prolonged arrest of cells in S-phase, and apoptosis occurs as a late event after irreversible commitment during cell cycle phase arrest. Cells undergoing FUra-LV-induced DNA damage (HT29 and RKO) demonstrate substantial accumulation of cells in S-phase, whereas in cells demonstrating the RNA-mediated mechanism of FUra action (HCT8 and HCT116), accumulation occurred in G2-M; both are reversible by dThd. To date, we have not identified a human colon carcinoma cell line that undergoes RNA-mediated FUra cytotoxicity in the presence of a mp53 gene. However, it would be anticipated that such cells would not undergo acute apoptosis but would undergo delayed apoptosis, similar to HT29 in response to FUra-LV. Consistent with this hypothesis are the data reported by Bunz et al. (26), who disrupted the p53 gene through homologous recombination in a human colon carcinoma cell line. The requirement for nonpharmacological concentrations of FUra (375 \(\mu M\)) in the presence of dThd-replete conditions (10% FCS) rendered FUra-directed DNA cytotoxicity impossible to evaluate. However, FUra-induced RNA-directed cytotoxicity in p53\(^{-/-}\) and p53\(^{+/+}\) cells yielded results similar to those presented in the current study; lower accumulation of cells at the G1-S boundary and a higher level of apoptosis occurred in p53\(^{+/+}\) cells, analogous to events occurring in HCT8 and HCT116. In contrast, p53\(^{-/-}\) cells demonstrated greater G1-S-phase arrest and did not undergo apoptosis at 72 or 96 h. Whether apoptosis was delayed was not addressed. However, the substantial G1-S-phase arrest and lack of acute apoptosis in p53\(^{-/-}\) cells undergoing RNA-mediated FUra cytotoxicity in the presence of p53 is similar to data derived in HT29 cells undergoing FUra-LV-induced DNA-directed cytotoxicity in the presence of mp53. Furthermore, intestinal cells in p53\(^{+/+}\) mice underwent acute apoptosis in response to FUra treatment (27), where FUra induced RNA-mediated toxicity (5, 6), in contrast to p53\(^{-/-}\) mice, where intestinal cells demonstrated reduced FUra-induced apoptosis.

The pattern of rescue from acute apoptosis induced by

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**Fig. 7** Transduction of HT29 cells with Ad-wtp53 at increasing MOI from 0 to 100, either in the absence or in the presence of FUra (10 \(\mu M\)) + LV (1 \(\mu M\)). Apoptosis was determined at 72 h by FACS analysis, and data represent the means of three determinations at each virus concentration; bars, SD. In addition, HT29 cells were transduced with Ad-VC (MOI, 100) or Ad-wt-p53 (MOI, 100) for 48 h, either with or without FUra (3 \(\mu M\) + LV (1 \(\mu M\)), and either in the absence or in the presence of dThd (20 \(\mu M\)). Fas expression was examined by FACS analysis, and data represent the means of duplicate determinations; bars, SD.

**Fig. 8** Induction of acute apoptosis in RKO and HCT116 cells after treatment for 72 h with varied concentrations of FUra combined with LV (1 \(\mu M\)). Cells were also coincubated with the caspase inhibitors Z-IETD-FMK (10 \(\mu M\)) or Z-VAD-FMK (100 \(\mu M\)), dThd (20 \(\mu M\)), or the anti-Fas MoAb CH-11 (200 ng/ml). Data were derived by FACS analysis and represent the means of duplicate determinations; bars, SD.

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DNA-directed (RKO) or RNA-directed (HCT116) FUra-LV-induced cytotoxicity differed in the presence of Z-IETD-FMK, Z-VAD-FMK, or CH-11 + dThd. Z-IETD-FMK is considered to have higher specificity for the inhibition of caspase-8 activation, whereas Z-VAD-FMK is a general caspase inhibitor. In DNA-directed damage, Z-IETD-FMK was not inhibitory to FUra-LV-induced apoptosis, whereas Z-VAD-FMK was completely inhibitory in RKO cells. In contrast, both caspase inhibitors prevented FUra-LV-induced RNA-mediated toxicity in HCT116 cells and may reflect the different pathways by which apoptosis is induced by FUra-LV under these conditions. Furthermore, FUra-LV treatment enhanced the cytotoxic action of CH-11 in both RKO and HCT116 cells, which was reversible by dThd in RKO but not in HCT116. These data suggest that Fas is an integral component of the mechanism by which FUra-LV induces apoptosis after DNA damage in RKO cells. However, in HCT116, up-regulated expression of Fas after treatment with FUra-LV in the presence of wtp53 sensitized cells to CH-11, but this was independent of the mechanism of FUra action in RNA-directed cytotoxicity.

The relationship between apoptosis, p53, and the sensitivity of cancer cells to chemotherapeutic agents has received much attention in the recent literature (28). The relationship between wtp53 and induction of apoptosis after DNA damage has been well established, particularly in oncogenically transformed normal cells, which appear to have a lower threshold for apoptosis induction after drug treatment, as well as in tissues of lymphoid origin (reviewed in Refs. 28 and 29). The presence of wtp53 can enhance sensitivity to DNA-damaging agents, and in general restoration of wtp53 function enhances drug responses (30). However, it is evident in HCT8 and HCT116 cells that FUra-LV-induced RNA-mediated cytotoxicity also induced acute apoptosis in the presence of wtp53. Data derived in these two cell lines demonstrated that acute apoptosis could be partially reversed by dThd, indicating a partial effect of FUra-LV in inducing DNA damage in these cell lines. The accumulation of cells in G2-M was also reversible by dThd, which may therefore contribute to this effect. However, DNA damage was clearly not the predominant mechanism of FUra-LV-induced cytotoxicity, because the overall survival of the population as determined by clonogenic assay was not influenced by dThd, and hence any DNA-directed damage was not of permanent significance to the overall survival of the population. Furthermore, FUra-LV-induced sensitization to CH-11 in HCT116 was not dThd reversible, in contrast to the dThd reversibility obtained in RKO.

There is increasing evidence that mp53 can affect the rate of onset but not the overall extent of apoptosis induced (28, 31). Brown and Wouters (28) demonstrated that p53 status may determine the threshold and kinetics of drug-induced apoptosis but not overall survival in a treated cell population. Thus, mouse embryo fibroblasts from p53+/+ or p53−/− mice that were transformed with E1A and Ras, treated with VP-16 for 1 h, and assayed by 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt assay at 1 day or by clonogenic survival after 8 days demonstrated enhanced survival in p53−/− cells at 1 day but not at 8 days. Data derived in HT29 and VRCJC1 human colon carcinoma cell lines expressing mp53 alleles and treated with FUra-LV are consistent with this model, in which apoptosis occurs as a late event after irreversible commitment to apoptosis during S-phase arrest. However, RKO, which also undergoes DNA damage in response to FUra-LV, undergoes acute apoptosis at a lower threshold of FUra in the presence of a wtp53 gene. Hence, all three cell lines undergo apoptosis induced by thymineless stress, although the kinetics are more rapid in RKO, accelerated by the presence of a wtp53 gene. (A schematic representation of FUra-LV-induced apoptotic responses in human colon carcinoma cell lines is shown Fig. 9.)

There have been several reports demonstrating that transfection of a wtp53 gene into mammalian cells elevates the expression of the Fas death receptor (12–14) and sensitizes the cells to Fas-mediated apoptosis (11, 13). Fas is a transmembrane protein bearing a death domain that belongs to the tumor necrosis factor receptor superfamily. After specific engagement of Fas ligand, a signal is generated that induces apoptosis (32). p53-mediated cytotoxicity has correlated with p53-mediated Fas induction (13). Furthermore, adenoviral transfer of Fas to MCF-7 cells that are resistant to the transduction of Ad-wtp53 was not toxic alone but sensitized cells to p53-mediated apoptosis (13). Additional data demonstrate that in human vascular smooth muscle cells, p53 activation has increased Fas expression at the cell surface by transport from the Golgi complex, and that disruption of this complex blocked both p53-induced sur-
face Fas expression and apoptosis (33). These findings suggest that Fas induction may be a rate-limiting step in p53-mediated apoptosis. They are also consistent with the observation that high levels of Fas per se do not induce Fas-dependent apoptosis, but that an additional stimulus is required to trigger the apoptotic signaling cascade. The current data derived in HT29 cells support these hypotheses. In this study, transduction of HT29 cells with Ad-wtp53 that elevated the expression of Fas was not cytotoxic when administered alone. However, when combined with FUra-LV that induced thymineless stress and subsequent DNA damage, apoptosis increased considerably as the MOI of Ad-wtp53 was increased. Treatment of neoplastic cell lines with agents that damage DNA have up-regulated both the expression of Fas (10, 24, 25) and Fas ligand (10, 24), dependent upon the presence of a wtp53 gene. Furthermore, differential phosphorylation of the p53 protein, as observed after treatment of human leukemic cells with Ara-C or ionizing radiation, may account for different levels of induction of Fas expression (25). Although it is well known that DNA damage signals to induce wtp53 activity and the induction of acute apoptosis, our studies and those of others (6, 26) demonstrate that RNA-directed damage can also induce acute apoptosis in the presence of a wtp53 gene.

In summary, we have demonstrated that the target of FUra-LV-induced cytotoxicity, whether DNA- or RNA-directed, induces acute apoptosis in human colon carcinoma cell lines in the presence of a wtp53 gene. When toxicity is DNA directed, acute apoptosis occurs at very low concentrations of FUra (1 μM; RKO), cells are arrested in S-phase; however, in the presence of mp53, cells undergo prolonged S-phase arrest followed by delayed apoptosis (HT29 and VRC5/c1). When toxicity is RNA directed, cells accumulate in G2-M and undergo acute apoptosis in the presence of wtp53, both of which are dThd reversible. However, loss in clonogenic survival induced by FUra-LV is not dependent on rapid apoptosis or cell cycle block and was not reversible by dThd. Furthermore, FUra-LV has induced wtp53-dependent Fas expression, which has sensitized colon carcinoma cell lines to anti-Fas treatment. We have demonstrated previously that IFN-γ induces the expression of Fas in human colon carcinoma cell lines, dependent upon FUra-LV-induced DNA damage, independent of the status of the p53 tumor suppressor gene, and sensitizes these cells to Fas-mediated and FUra-LV-induced/DNA damage-mediated cytotoxicity (2). Because p53 is mutated in >75% of colon carcinomas (34), which die by the process of thymineless death, IFN-γ has the potential to yield a selective therapeutic approach in combination with FUra-LV for the treatment of colon carcinomas. In those colon carcinomas that express wtp53, the approach to sensitize tumors to Fas-mediated apoptosis by up-regulating the expression of Fas may be further enhanced from the effect of FUra-LV in elevating Fas expression in a p53-dependent manner.

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p53 Dependence of Fas Induction and Acute Apoptosis in Response to 5-Fluorouracil-Leucovorin in Human Colon Carcinoma Cell Lines

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