A Fas-dependent Component in 5-Fluorouracil/Leucovorin-induced Cytotoxicity in Colon Carcinoma Cells

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ABSTRACT

We have shown previously (J. A. Houghton et al., Proc. Natl. Acad. Sci. USA, 94: 8144–8149, 1997) that thymineless death in thymidylate synthase-deficient (TS−) colon carcinoma cells is mediated via Fas/FasL interactions after deoxythymidine (dThd) deprivation, and that Fas-dependent sensitivity of human colon carcinoma cell lines may be dependent upon the level of Fas expressed. The objective of this study was to elucidate whether a Fas-dependent component exists in 5-fluorouracil (FUra)/leucovorin (LV)-induced cytotoxicity of colon carcinoma cells, and whether this may be potentiated by IFN-γ-induced elevation in Fas expression, using the HT29 cell line as a model. The cytotoxic activity of FUra/LV was inhibited by dThd in HT29 cells and also, in part, by NOK-1 activity of FUra/LV was inhibited by dThd in HT29 cells treated with recombinant human IFN-γ, reversed by exposure to the NOK-1 MoAb that prevents the ligation of FasL to Fas and is inhibitory to Fas signaling (1). In addition, we demonstrated that IFN-γ expression is relatively high in TS− cells (2), whereas we (2, 3) and others (4) have shown that Fas-mediated apoptosis may be limited in other colon carcinoma cell lines because of reduced expression of Fas and may be elevated following treatment with the cytokine recombinant human IFN-γ. Thus, levels of the Fas antigen varied by >1000-fold in a panel of 10 cultured human colon carcinoma cell lines and correlated with cellular sensitivity to the cytolytic anti-Fas MoAb CH-11 (3). Furthermore, after 4-fold elevation in Fas expression in HT29 human colon carcinoma cells treated with IFN-γ, a synergistic effect on Fas-mediated cytotoxicity was obtained when CH-11 and IFN-γ were combined, thereby converting a growth inhibitory response to a cytotoxic response (3).

The cell surface receptor Fas, which belongs to the tumor necrosis factor receptor superfamily, and its ligand, FasL, are known regulators of apoptosis (5), and Fas is constitutively expressed in normal colonic epithelium (6). We, therefore, wished to test the hypothesis that Fas-mediated cytotoxicity may be an important determinant of FUra/LV sensitivity of human colon carcinoma cells, and that modulation of receptor expression correlates with the induction of cell death via Fas and potentiation of FUra/LV-induced cytotoxicity. The data reported demonstrate that the cytotoxic activity of FUra/LV has a Fas-dependent component that is potentiated by IFN-γ-induced elevation in Fas expression. Using a panel of human colon carcinoma cell lines, data suggest that FUra/LV-induced DNA damage is necessary for potentiation of cytotoxicity by IFN-γ, which may form the basis for the selective action of this combination. Thus, modulation of Fas expression may enhance cellular sensitivity of colon carcinomas to FUra/LV, and Fas may be an important target to consider for exploitation in developing therapeutic strategies for the treatment of colon carcinoma in humans.

INTRODUCTION

Thymineless death is the mechanism of cell killing associated with FUra in colon cancer and remains the most effective therapy for this disease when combined with the reduced folate LV that targets FUra to the TS locus. Previously (1), we demonstrated in GC0/c1 human colon carcinoma cells selected for TS−, that thymineless death may be regulated by signaling via the Fas death receptor. Thus, apoptosis was induced in TS− cells concomitantly with up-regulated expression of FasL and blocked by exposure to the NOK-1 MoAb that prevents the ligation of FasL to Fas and is inhibitory to Fas signaling (1). In addition, we demonstrated that IFN-γ expression is relatively high in TS− cells (2), whereas we (2, 3) and others (4) have shown that Fas-mediated apoptosis may be limited in other colon carcinoma cell lines because of reduced expression of Fas and may be elevated following treatment with the cytokine recombinant human IFN-γ. Thus, levels of the Fas antigen varied by >1000-fold in a panel of 10 cultured human colon carcinoma cell lines and correlated with cellular sensitivity to the cytolytic anti-Fas MoAb CH-11 (3). Furthermore, after 4-fold elevation in Fas expression in HT29 human colon carcinoma cells treated with IFN-γ, a synergistic effect on Fas-mediated cytotoxicity was obtained when CH-11 and IFN-γ were combined, thereby converting a growth inhibitory response to a cytotoxic response (3).

The cell surface receptor Fas, which belongs to the tumor necrosis factor receptor superfamily, and its ligand, FasL, are known regulators of apoptosis (5), and Fas is constitutively expressed in normal colonic epithelium (6). We, therefore, wished to test the hypothesis that Fas-mediated cytotoxicity may be an important determinant of FUra/LV sensitivity of human colon carcinoma cells, and that modulation of receptor expression correlates with the induction of cell death via Fas and potentiation of FUra/LV-induced cytotoxicity. The data reported demonstrate that the cytotoxic activity of FUra/LV has a Fas-dependent component that is potentiated by IFN-γ-induced elevation in Fas expression. Using a panel of human colon carcinoma cell lines, data suggest that FUra/LV-induced DNA damage is necessary for potentiation of cytotoxicity by IFN-γ, which may form the basis for the selective action of this combination. Thus, modulation of Fas expression may enhance cellular sensitivity of colon carcinomas to FUra/LV, and Fas may be an important target to consider for exploitation in developing therapeutic strategies for the treatment of colon carcinoma in humans.
Fas Dependence of FUra/LV-induced Cytotoxicity

MATERIALS AND METHODS

Cell Lines. The HT29, Caco2, HCT8, and HCT116 human colon carcinoma cell lines were obtained from American Type Culture Collection. GCc/c1 and VRCc/c1 were established in these laboratories, as previously reported (3). Cells were maintained in the presence of folate-free RPMI 1640 containing 10% dFBS and 80 nm [6RS]5-methyltetrahydrofolate.

Clonogenic Assays. Cell lines were plated at a density of 1500 (HT29, HCT116), 2000 (HCT8), and 3000 (GCc/c1, VRCc/c1) cells/well in 6-well plates. After overnight attachment, cells were treated in triplicate with FUra (1–10 μM) in the presence of LV (1 μM) in either the absence or the presence of recombinant human IFN-γ (25–100 units/ml; Genentech, Inc.) and/or dThd (20 μM) for periods of up to 96 h. Alternatively, HT29 cells were plated with a combination of NOK-1 and NOK-2 MoAbs (500 ng/ml each; PharMingen) or with an IgG1 isotype-matched control MoAb (100 ng/ml, PharMingen) for 24 h before exposure to FUra/LV ± IFN-γ ± NOK-1 + NOK-2 for up to 96 h. Clonogenic survival was determined at 5–7 days (the equivalent of 7 doublings) after the removal of the drug, as previously described (3). Because Caco2 cells lacked the ability to clone, cells were plated at a density of 1 × 10³ cells/well 24 h before a 72-h exposure to drugs and were subsequently allowed to regrow for a period of 4 days before elucidation of the influence of drug treatment on cell numbers, which were enumerated using a Coulter particle counter.

For studies with the cytolytic anti-Fas MoAb CH-11 (MBL International Corp.), HT29 cells were treated with CH-11 (10–200 ng/ml) for up to 96 h in either the absence or the presence of IFN-γ (100 units/ml) and/or the MoAb ZB4 (100 ng/ml; Kamiya Biomedical Co.), and clonogenic survival was determined.

Expression of Fas and Fasl. Fas expression was determined during the treatment of HT29 cells with IFN-γ (100 units/ml) or FUra (3 μM)/LV (1 μM) ± IFN-γ at various times for up to 96 h. Alternatively, colon carcinoma cell lines were treated with IFN-γ (25–100 units/ml) for 24 h before elucidation of the level of Fas expressed. Fas was measured in cell extracts by a standard ELISA assay, as previously reported (1), that correlated with the expression of Fas mRNA as determined by RT-PCR. 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 the DX2 MoAb (PharMingen), using standard procedures.

After the treatment of HT29 with FUra (3 μM)/LV (1 μM) ± IFN-γ for periods of up to 96 h, expression of Fasl was determined by semiquantitative RT-PCR based on the number of cycles used for amplification of the cDNA template. Total RNA was extracted from 20 × 10⁶ cells in RNAzolB (Tel-test) using standard procedures. mRNA was subsequently isolated from 250 μg total RNA by using an oligotex mRNA minikit (Qia-gen). Complimentary DNA was synthesized from 50 ng mRNA in a 20-μl reaction using an oligo(dT) primer and a cDNA cycle kit (Invitrogen). RT-PCR for Fasl was performed from 2 μl of the RNA/DNA template was conducted at 95° for 1 min, 56° for 2 min, and 72° for 1 min, for 35 cycles. Forward and reverse primers used to amplify Fasl and to produce a 419-bp product were as follows:

(a) FaslF4: 5'-GGAAAGTGCCCATTATACACAG-3'; and
(b) FaslR4: 5'-CTCTTAGGCTTATATAAGCGG-3'.

β-actin was used as a control to monitor RT-PCR amplification efficiency and quality of the cDNA from 2 μl of the template at 25 cycles, as previously reported (1). PCR products were separated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining and UV light illumination. Quantitation was by optical densitometry of the reverse gel image using a Hewlett Packard Scan Jet IIC. Intensity of the signal was determined by comparison with a calibrated photographic gray scale (Kodak) in the linear range of detection.

RESULTS AND DISCUSSION

Fas Dependence of FUra/LV-induced Cytotoxicity in HT29. Previously, we demonstrated that TS—cells, which appeared sensitive to thymineless stress-induced apoptosis via Fas/Fasl interactions and were sensitive to the cytolytic anti-Fas MoAb CH-11, expressed high levels of Fas, whereas other colon carcinoma cell lines that were CH-11 insensitive, demonstrated reduced expression of the receptor (2, 3). In addition, we demonstrated that up-regulated expression of Fas after treatment of HT29 cells with recombinant human IFN-γ transformed a growth inhibitory response to CH-11 in this cell line to a cytotoxic response (3). We, therefore, used HT29 to elucidate whether FUra/LV-induced cytotoxicity demonstrated a Fas component and whether this cytotoxicity could be potentiated by IFN-γ in a Fas-dependent manner.

Initially the sensitivity of HT29 cells to varied concentrations of FUra (1–10 μM) combined with LV (1 μM) was examined by clonogenic assay following 72-h exposure in either the absence or the presence of dThd (20 μM; Fig. 1A). A dose-dependent decrease in clonogenic survival was determined, and cytotoxicity at 1 μM and 3 μM FUra was reversible by dThd, indicative of TS as the drug target. In the presence of IFN-γ (100 units/ml), not cytotoxic when administered alone, survival was reduced from 87% to 15% at 1 μM FUra and from 39% to 0% at 3 μM FUra, and this potentiation was completely reversed by dThd (Fig. 1A).

Because the greatest protection from FUra/LV-induced cytotoxicity by dThd was determined at 3 μM FUra, the influence of NOK-1 and NOK-2 MoAbs (500 ng/ml each) that inhibit Fas/Fasl interactions, on clonogenic survival during FUra (3 μM)/LV (1 μM) treatment was examined over a period of 96 h. Survival was reduced to 45% at 72 h, and to 5% at 96 h in the presence of FUra/LV, whereas some protection following simultaneous treatment with NOK-1 and NOK-2 was demonstrated. Under these conditions, survival increased to 88% and 19%, respectively, at these times (Fig. 1B), indicating a Fas/Fasl component in the mechanism of FUra/LV-induced cytotoxicity in HT29 cells. After coincubation with IFN-γ (100 units/ml), FUra/LV-induced loss in clonogenic survival was potentiated and subsequently reversed by coincubation with NOK-1 and NOK-2 MoAbs (Fig. 1B). Data demonstrate that FUra/LV-induced cytotoxicity can be potentiated by IFN-γ in a Fas-dependent manner.

Potentiation of Fas-dependent Cytotoxicity by IFN-γ. The influence of IFN-γ (100 units/ml) on anti-Fas sensitivity of HT29 cells was examined during treatment with varied concen-
trations of CH-11 (10–200 ng/ml) for 72 h (Fig. 2A). Neither CH-11 nor IFN-γ administration alone was cytotoxic to HT29 cells. However, in the presence of IFN-γ, clonogenic survival was reduced to 1% at 10 ng/ml CH-11 (Fig. 2A), and this was completely reversed by coincubation with ZB4 (100 ng/ml), which binds to Fas and prevents ligation of the cytolytic antibody to its receptor. After treatment with CH-11 (50 ng/ml) and IFN-γ in combination, clonogenic survival was reduced to 3% within 24 h (Fig. 2B), and was partially reversed to 39% by simultaneous incubation with ZB4.

**Fig. 1** Fas dependence of the potentiation of FUra/LV-induced cytotoxicity in HT29 cells by IFN-γ. A, clonogenic survival of HT29 cells after 72 h of exposure to varied concentrations of FUra+LV (1 μM), either in the presence or in the absence of IFN-γ (100 units/ml) or dThd (20 μM). Survival was determined at 6 days after drug removal. Data constitute the mean ± SD of three determinations at each FUra concentration. ■, FUra/LV; □, FUra/LV + IFN-γ; ●, FUra/LV + IFN-γ + dThd; △, FUra/LV + dThd. B, clonogenic survival of HT29 during treatment with FUra (3 μM)/LV (1 μM) ± dThd (20 μM), or ± NOK-1/NOK-2 MoAbs (500 ng/ml each), or an IgG1 isotype matched control MoAb (500 ng/ml) for periods of ±96 h. Cells were also incubated with FUra/LV in the presence of IFN-γ (100 units/ml) and either with or without NOK-1/NOK-2 MoAbs. Clonogenic survival was evaluated as described in “Materials and Methods.” Each time point, the mean ± SD of three determinations. ■, FUra/LV; □, FUra/LV + IFN-γ; ●, FUra/LV + IFN-γ + NOK-1/NOK-2; △, FUra/LV + dThd; □, FUra/LV + IgG1; ○, FUra/LV + NOK-1/NOK-2.

**Fig. 2** Sensitization of HT29 to Fas-mediated cytotoxicity by IFN-γ. A, cells were treated for 72 h with varied concentrations of CH-11 in either the absence or the presence of IFN-γ (100 units/ml) and/or the inhibitory MoAb ZB4 (100 ng/ml), and clonogenic survival was determined after a further 6 days, as described in “Materials and Methods.” Data represent the mean ± SD of triplicate determinations at each CH-11 concentration. B, clonogenic survival of HT29 cells during exposure to CH-11 (50 ng/ml) in either the absence or the presence of IFN-γ (100 units/ml) and/or ZB4 (100 ng/ml) for up to 96 h. Methods are as described in “Materials and Methods.” Each time point, the mean ± SD of three determinations.

**IFN-γ-induced Up-Regulation of Fas and FasL Expression.** In untreated HT29 cells or in cells treated with FUra (3 μM)/LV (1 μM), levels of Fas remained relatively low (100–200 pg/10⁶ cells). However, after treatment with IFN-γ (100 units/ml) in either the absence or presence of FUra/LV, total Fas expression: (a) was elevated by 3.5- to 4-fold within 24 h of the initiation of treatment; (b) was located at the cell surface as determined by FACS analysis (data not shown); and (c) correlated with IFN-γ-induced, Fas-dependent potentiation of FUra/LV cytotoxicity (Fig. 3).
Incubation of HT29 cells with FUra (3 μM)/LV (1 μM) also induced FasL expression within 24 h of the initiation of treatment, and induction was highest at 96 h when clonogenic survival was reduced to <5% (Fig. 4). In the presence of FUra/LV + IFN-γ, FasL was significantly elevated (by 4-fold) beyond what was observed with FUra/LV treatment alone at 72 h, which correlated with potentiation of FUra/LV-induced cytotoxicity (Fig. 4). No induction of FasL expression was observed after treatment of HT29 with IFN-γ alone and correlated with the maintenance of clonogenic potential under these conditions.

**IFN-γ-induced Elevated Fas Expression in Additional Colon Carcinoma Cell Lines.** Expression of total Fas was determined by an ELISA assay after treatment of five additional colon carcinoma cell lines with IFN-γ for 24 h at the maximal nontoxic concentration (25–100 units/ml; Table 1). A 31 to 63% increase in Fas expression was observed in all of the cell lines except for Caco2, which demonstrated no detectable Fas. These increases were more modest in comparison with the increase obtained in HT29. FACS analysis confirmed a similar IFN-γ-induced increase in Fas at the cell surface as determined for total Fas by the ELISA assay.

**Influence of IFN-γ on FUra/LV-induced Cytotoxicity.**

To determine the ability of IFN-γ to potentiate FUra/LV-induced loss in clonogenic survival in additional human colon carcinoma cell lines, the effect of the exposure of GC/c1, VRC/c1, Caco2, HCT8, and HCT116 cell lines to FUra/LV for 72 h in the absence or presence of dThd or of IFN-γ was examined (Fig. 5). Because Caco2 cells failed to clone, the effect of drug treatment on the numbers of cells that survived for 4 days after the end of drug treatment was determined. In addition to dThd reversibility of FUra/LV-induced cytotoxicity in HT29, FUra/LV-induced loss in clonogenic survival was reversed by dThd in GC/c1, VRC/c1, and Caco2 but not in HCT8 or HCT116, which indicated the dependence of FUra/LV-induced cytotoxicity on the inhibition of TS and effects on DNA in the former three cell lines but not in HCT8 or HCT116. FUra-induced cytotoxicity in HCT8 (7) and HCT116 (8) has been reported to be via a RNA-mediated mechanism and, in the current study even in the presence of LV, lacked dThd reversibility. In addition, cell lines with a RNA-mediated mechanism of FUra-induced cytotoxicity demonstrate higher IC50 values for FUra (9), as demonstrated by HCT8 and HCT116 in comparison with the other three cell lines. Similar to IFN-γ-induced potentiation of FUra/LV cytotoxicity in HT29, this cytotoxicity was also potentiated by IFN-γ in GC/c1 and VRC/c1, in which it was also reversible by dThd. Under normal growth conditions, VRC/c1 cells additionally express FasL, which may contribute to the enhanced cytotoxic effect produced by IFN-γ in this cell line. Although FUra/LV-induced cytotoxicity is dThd-reversible in Caco2, no potentiation was observed in the presence of IFN-γ. However, these cells also do not express Fas. In the absence of dThd-reversible, FUra/LV-induced cytotoxicity in HCT8 and HCT116, no potentiation by IFN-γ was obtained, which suggested that the induction of DNA damage by FUra/LV is necessary for potentiation of cytotoxicity to be obtained with IFN-γ.

IFN-γ is a type II IFN produced by T lymphocytes in response to specific antigenic or tumorigenic stimuli. Distinct from the type I IFNs (IFN-α and IFN-β), which may be 10- to 100-fold greater, IFN-γ exhibits antiproliferative effects against tumor cell lines and has immunomodulatory properties (10). IFNs induce hormone-like effects by binding to membrane receptors and activating a postreceptor signaling mechanism. Of the three separate classes of IFNs, α-IFNs have been the most widely used clinically; in particular, IFN-α2a has been used in both preclinical (11–14) and clinical (15, 16) studies to modulate the activity of FUra in colon carcinoma. For IFN-α2a, the mechanism of biochemical modulation of FUra cytotoxicity has been related to the mechanism of FUra action at the level of metabolism, DNA, or TS (11, 12, 14), and no up-regulated
expression of Fas has been detected in colon carcinoma cell lines treated with this cytokine. IFN-γ has also potentiated the activity of FUra against colon carcinoma cell lines (14, 17, 18), and one mechanism of drug interaction has been suggested at the level of TS (17). However, it is evident that the type of IFN that maximally enhances fluoropyrimidine cytotoxicity not only differs among cell lines but also is dependent upon the different cellular receptors for IFN-α and IFN-γ (19, 20). Ismail et al. (14) reported potentiation of FUra cytotoxicity using a combination of IFN-α + IFN-γ and concluded that the mechanism of potentiation was independent of effects at the level either of DNA or of TS. In the current study, the degree of potentiation of FUra/LV-induced cytotoxicity in HT29

by a Fas-dependent mechanism is not identical to the degree of potentiation of CH-11-induced cytotoxicity by IFN-γ, which is not surprising, inasmuch as other mechanisms are involved in FUra/LV-induced cell killing in addition to the ligation of FasL to Fas. In the presence of LV, FUra-induced cytotoxicity was dThd-reversible in three additional colon carcinoma cell lines and was potentiated by IFN-γ in the two lines that expressed Fas but was not enhanced in the absence of Fas expression. In HCT8 and HCT116 cells, in which FUra/LV-induced cytotoxicity was not dThd-reversible, no potentiation by IFN-γ was obtained. Because (a) human colon carcinoma cells that are maintained as xenografts in vivo are sensitive to FUra/LV-induced thymineless death (21) and (b) the mechanism of induction of gastrointestinal toxicity is via a RNA-mediated mechanism (22), a basis for the selective potentiation of FUra/LV cytotoxicity by IFN-γ may be established.

It is evident that IFN-γ can up-regulate components of the

Unpublished observations.

Fig. 5 Clonogenic survival of human colon carcinoma cell lines after 72 h of exposure to varied concentrations of FUra in the presence of LV (1 μM), in the absence or presence of dThd (20 μM) or IFN-γ (25–100 units/ml). Caco2 cells were enumerated 7 days after drug removal, as described in “Materials and Methods.” Data represent the mean ± SD of three determinations at each FUra concentration. ▣, FUra/LV alone; □, FUra/LV + IFN-γ; △, FUra/LV + dThd.
Fas signaling pathway including Fas itself (4, 6) and also NF-κB (23), which may directly transactivate FasL in the induction of thymineless stress-induced apoptosis in TS- colon carcinoma cells. It is also evident from the data presented in HT29 that IFN-γ can potentiate FUra/LV-induced cytotoxicity in a Fas-dependent manner and that this potentiation extends to additional colon carcinoma cell lines. Recently, von Reyher et al. (4) demonstrated that normal colonocytes were constitutively sensitive to Fas-mediated apoptosis, whereas all of the colon carcinoma cell lines examined, including HT29, were constitutively resistant but were sensitized on treatment with IFN-γ. HT29 cells express a mp53 allele (24). Because the p53 gene is mutated in 75% of colon carcinomas (25), the Fas signaling pathway may be an attractive and also an important target for therapeutic modulation in colon carcinoma. In addition, the ability to selectively modulate FURA/LV-induced cytotoxicity by IFN-γ in an expanded panel of human colon carcinoma cell lines based on the induction of thymineless death may also be important in the development of a selective therapeutic approach with FURA/LV in combination with cytokines.

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REFERENCES


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