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Expression of genes that regulate Fas signalling and Fas-mediated apoptosis in colon carcinoma cells

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Abstract

The expression of genes that regulate Fas-induced apoptosis has been examined in 10 human cultured colon carcinoma cell lines with defined and varied sensitivity to the cytolytic anti-Fas MoAb CH-11. Four lines demonstrated sensitivity to CH-11 (HT29, GC_3/c1, TS^-, Thy4), and six were resistant to the induction of apoptosis vis Fas. In nine lines expressing Fas, PCR-sequencing indicated that the death domain contained wt sequences. Downstream of Fas, expression of FADD/MORT1 and FLICE, essential components of the DISC, and negative regulators of Fas signalling including sFas, FAP-1 and Bcl-2, showed no correlation between levels of expression and sensitivity to Fas-mediated cytotoxicity. However, levels of the Fas antigen varied by >1000-fold, and correlated with CH-11 sensitivity. Following fourfold elevation in Fas expression in HT29 cells treated with interferon-γ, a synergistic effect on Fas-mediated apoptosis was obtained when CH-11 and interferon-γ were combined.

Keywords: Fas signalling; gene expression; apoptosis; colon carcinoma cells

Abbreviations: TS, thymidylate synthase; DISC, death-inducing-signalling-complex; ICE, interleukin-1β converting enzyme; FAP, fas-associated phosphatase; TNF, tumor necrosis factor

Introduction

Fas, a type I transmembrane protein, comprises a cell surface receptor and is a member of the TNF receptor superfamily. When activated by crosslinking either with the natural ligand FasL (Suda *et al*, 1993) or with an agonistic Ab (Trauth *et al*, 1989), apoptosis can be induced. Fas has been considered to be primarily involved in the regulation of apoptosis in cells of the immune system during normal tissue homeostasis (French *et al*, 1996). However, the Fas antigen is expressed in a wide variety of normal cell and tissue types, including cells of the thymus, lung, spleen, small and large intestines, seminal vesicle, prostate and uterus, which demonstrate high rates of cell turnover and apoptotic cell death (French *et al*, 1996; Leithauser *et al*, 1993). The role of Fas in regulating apoptotic responses in cells outside of the immune system remains unknown. However, Fas-dependent apoptosis has been identified in several malignant cell types, including malignant cells of the colon (Houghton *et al*, 1997a; Rokhlin *et al*, 1997; Weller *et al*, 1994).

In the normal colon, Fas is constitutively expressed in every epithelial cell of the crypt and mucosal surface (Moller *et al*, 1994), suggesting that it may be involved in the regulation of normal cell turnover. We (Houghton *et al*, 1997a) and others (Meterissian and Kontogiannea, 1996; Moller *et al*, 1994) have shown Fas to be expressed in cultured colon carcinoma cell lines, as well as in colon carcinomas (Moller *et al*, 1994). Previously we have shown that the cytolytic anti-Fas MoAb CH-11 can induce apoptosis in colon carcinoma cells of the GC_3/c1 lineage, although Fas-mediated apoptosis does not occur in all malignant cells of this histiotype (Houghton *et al*, 1997b). In addition, TS^- cells that were selected from wt GC_3/c1 cells for thymidylate synthase (TS)-deficiency undergo apoptosis via thymineless death when dThd is withdrawn from the medium, an event which is mediated via autocrine signalling through Fas/FasL interactions following the induction of DNA damage (Houghton *et al*, 1997a).

For Fas-dependent apoptosis to occur in colon carcinomas, all components of the Fas signalling pathway must be present. Following trimerization of the receptor after stimulation, several proteins (CAP1-4) have been identified that bind to the intracellular death domain of Fas to form the death-inducing-signalling-complex (DISC). CAP1 and CAP2 comprise the death domain signalling molecule FADD/MORT1 (Boldin *et al*, 1995; Chinnaiyan *et al*, 1995), which recruit FLICE (CAP4), a FADD/MORT1-homologous ICE/CED-3-like protease to the DISC (Boldin *et al*, 1996; Medema *et al*, 1997; Muzio *et al*, 1996). Following release of active FLICE from the DISC, a cascade of ICE-like proteases is activated. However, modification of any one of the components of the Fas signalling pathway either by mutation or reduced expression, or elevated expression of proteins that are inhibitory at various steps in the pathway, may reduce or eliminate Fas-mediated apoptosis.

Thus, mutations in the death domain of Fas (Itoh and Nagata, 1993), expression of soluble Fas (sFas; Cheng *et al*, 1994), or reduced expression of the Fas antigen may result in resistance to Fas-dependent apoptosis, and reduced expression of Fas has been identified in certain colon carcinomas (Moller *et al*, 1994). Cells must also express sufficient levels of all components of the DISC to induce apoptosis. Inhibitory factors that may be expressed include FAP-1 (a Fas-associated phosphatase), which associates with the negative regulatory domain of the receptor to inhibit Fas signalling (Sato *et al*, 1995), and has been found to be expressed in colon carcinoma cell lines

(Yanagisawa *et al*, 1997). In addition, high-level Bcl-2 expression has also prevented the function of the Fas pathway, and in this regard, decreased anti-Fas sensitivity has correlated with increased expression of Bcl-2 in colorectal carcinomas (Meterissian and Kontogiannea, 1996), and colorectal carcinoma cell lines (Houghton *et al*, 1997a,b).

In the current study we have sequenced the death domain of Fas, which comprises a defined area encoding 65 amino acids responsible for promoting the death signal (Itoh and Nagata, 1993). In addition we have examined the expression of Fas, FADD/MORT1, FLICE, sFas, FAP-1 and Bcl-2 in a series of 10 cultured colon carcinoma cell lines with defined and varied sensitivity to the cytolytic anti-Fas MoAb CH-11, and hence to Fas-mediated apoptosis. Data demonstrate no mutations in the death domain of Fas and while some variability exists in the expression of all genes examined, the greatest differential (>1000-fold) has occurred in the expression of the Fas antigen itself. Thus, cell lines with the greatest sensitivity to CH-11, despite the presence of FAP-1, Bcl-2 and even sFas, also express the highest levels of Fas. In HT29 colon carcinoma cells with reduced Fas expression, levels of the receptor were elevated fourfold by treating cells with the cytokine interferon-γ, resulting in significantly enhanced sensitivity to Fas-mediated apoptosis. Results suggest that the most important determinant of Fas-dependent apoptosis in colon carcinoma cells may be the actual level of Fas expression.

Results

Fas-mediated apoptosis in colon carcinoma cell lines

Previously we determined that Fas-mediated apoptosis could be induced in GC_3/c1, TS^- and Thy4 cells by the cytolytic anti-Fas MoAb CH-11 (Houghton *et al*, 1997b). Subsequently we have compared the sensitivity of a series of 10 human colon carcinoma cell lines to CH-11 at concentrations of 150 ng/ml and 750 ng/ml using both growth inhibition and clonogenic assays (Figure 1), and have found considerable variability in their responsiveness. CH-11 inhibited the growth of HT29 and GC_3/c1 cells to $<60\%$ of control, and in TS^- and Thy4 to $<10\%$ of control following a 72 h exposure (Figure 1A). Little influence of CH-11 on the growth of the remaining six cell lines was detected. Subsequent evaluation of the cytotoxic response to CH-11 following 72 h exposure by clonogenic assay indicated that the MoAb was growth inhibitory in HT29 cells, but was cytolytic in GC_3/c1, TS^- and Thy4 (Figure 1B). At 150 ng/ml CH-11, clonogenic survival was 21% in GC_3/c1 and $<3\%$ in TS^- and Thy4, and at 750 ng/ml, survival was $<1\%$ in all three cell lines. No sensitivity to the IgG1 isotype-matched control MoAb was detected in any of the cell lines (data not shown).

Morphologic evidence of apoptosis was also obtained (Figure 2). Apoptosis induced in TS^-, Thy4 and GC_3/c1 cells treated with CH-11 (15 ng/ml) is shown at 11 h (TS^-, Thy4) or 24 h (GC_3/c1). Cells characteristically demonstrated chromatin condensation, nuclear fragmentation,

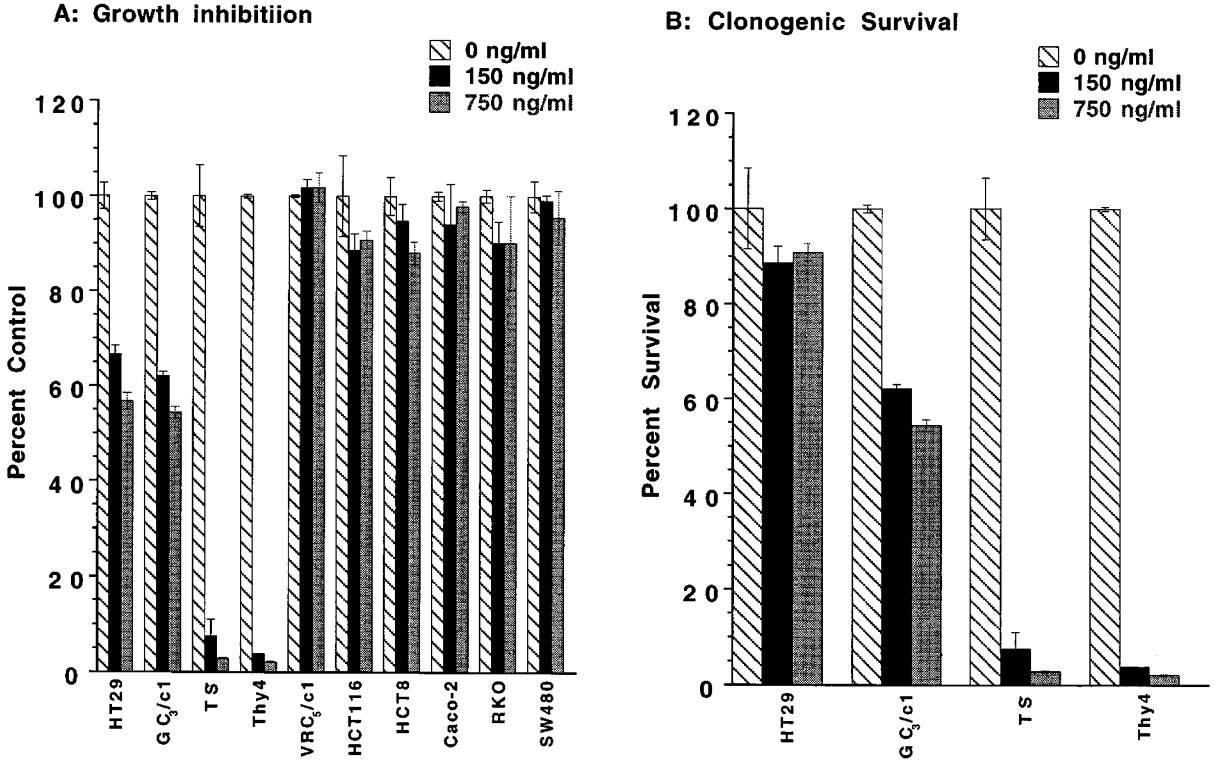

Figure 1 Growth inhibitory response (**A**) and clonogenic survival (**B**) of human colon carcinoma cell lines following 72 h exposure to CH-11. Experimental conditions are as described in Materials and Methods. Results are the mean $\pm$ S.D. of triplicate determinations for each condition

cytoplasmic vacuoles and membrane blebbing. However, in HT29 cells which did not demonstrate a cytotoxic response to CH-11, no morphologic evidence of apoptosis was obtained following treatment with 150 ng/ml CH-11. Morphologic evaluation of apoptosis correlated well with results obtained from clonogenic assays.

Expression of Fas

Fas expression was determined by RT–PCR (Figure 3) and further quantitated by ELISA (Table 1). Considerable variability in levels of receptor expression was demonstrated among the ten cell lines, where levels ranged from no expression in Caco-2 cells to 1262 pg/10^6 cells in TS^-. Levels of Fas were highest in the three cell lines where Fas-mediated apoptosis had been induced by CH-11 (GC_3/c1, TS^-, Thy4), being highest in TS^- cells and lowest in GC_3/c1 (540 pg/10^6 cells). In all other cell lines that were Fas-resistant, levels of Fas expressed were <350 pg/10^6 cells. To determine whether Fas expression or the ability to induce Fas-dependent apoptosis may be due to modification or mutation in the death domain of the Fas antigen, the cDNA comprising the death domain was sequenced. In all cell lines except Caco-2 where no Fas expression was detected, wt sequence for Fas was demonstrated (Table 1).

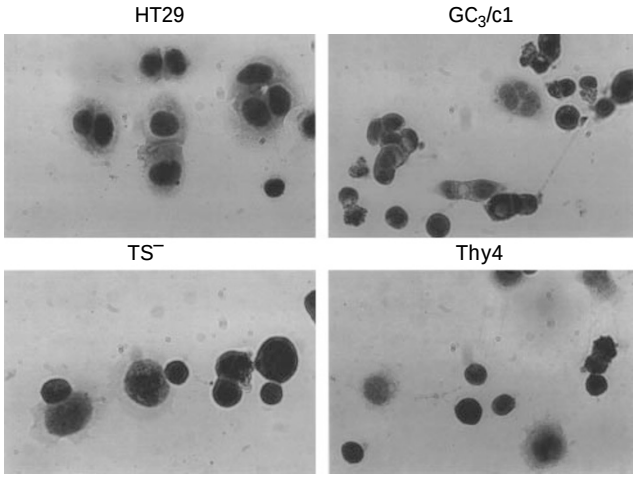

Figure 2 Morphologic evaluation of apoptosis in HT29, GC_3/c1, TS^- and Thy4 cells. Experimental conditions are as described in Materials and Methods. No evidence of apoptosis was detected in HT29 cells, shown treated with 150 ng/ml CH-11 for 11 h. However apoptotic cells were demonstrated in GC_3/c1 following exposure to CH-11 (15 ng/ml) for 24 h, and in TS^- and Thy4 cells following exposure to CH-11 (15 ng/ml) for 11 h. Slides were examined by light microscopy using $\times 100$ magnification

Expression of FADD/MORT1 and FLICE

To determine whether other components of the DISC were present in Fas-resistant cell lines in comparison to Fas-sensitive cells, the expression of FADD/MORT1 and FLICE was examined by RT–PCR (Figure 4; Table 1). Levels of expression of both cell death regulatory factors varied by 8–9-fold (Table 1); both were lower in Fas-sensitive GC_3/c1 cells than in e.g., RKO or SW480 cells where no Fas-mediated apoptosis was detectable. In Fas-resistant Caco-2 cells, not only was the expression of Fas absent, but this cell line also demonstrated the lowest expression of FLICE. However, other than for FLICE in Caco-2 cells, there was no apparent relationship between the expression of the two cell death regulatory factors and cellular sensitivity to CH-11 in the nine additional colon carcinoma cell lines.

Expression of factors inhibitory to Fas signalling

Expression of sFas, FAP-1 and Bcl-2 have, in several different systems, been found to be capable of inhibiting the ability of the Fas signalling pathway to mediate an apoptotic response.

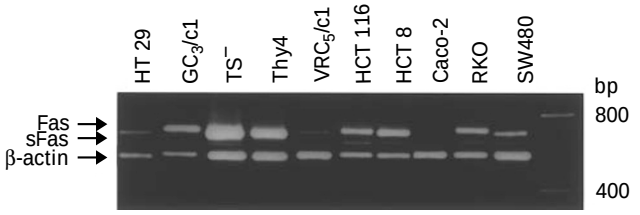

Figure 3 Expression of Fas determined by RT–PCR analysis. Cell extracts were prepared as described in Materials and Methods. PCR amplification was conducted following production of Fas cDNA to yield a 682 bp product. sFas was also amplified to yield a 619 bp product. β-actin control PCR (540 bp product) was simultaneously performed to monitor RT–PCR amplification efficiency

Table 1 Relative levels of expression of factors that influence Fas-mediated apoptosis in colon carcinoma cell lines

	Fas		Relative O.D.					CH-11
Cell line	(pg/10^6 cells)	Fas seq	sFas	FAP-1	FADD/MORT	FLICE	Bcl-2	sensitivity
HT29	85	wt	–	3.1	5.1	4.9	–	+/–
GC_3/c1	540	wt	1.0	5.9	1.0	1.2	3.2	+
TS	1262	wt	3.8	16.0	7.4	7.8	4.9	++
Thy4	743	wt	1.6	12.7	9.2	6.9	7.7	+++
VRC_5/c1	75	wt	–	–	5.5	2.2	2.1	–
HCT116	350	wt	8.8	1.8	3.5	5.8	1.0	–
HCT8	180	wt	2.9	8.9	5.4	8.4	3.4	–
Caco-2	0	–	–	3.0	3.4	1.0	–	–
RKO	261	wt	3.3	–	4.9	6.8	2.5	–
SW480	175	wt	2.1	1.0	8.3	6.7	1.1	–

Their relative levels of expression were therefore examined in this series of ten human colon carcinoma cell lines, and compared to the sensitivity of the lines to Fas-induced apoptosis.

There was a ninefold range in expression of sFas (Figure 3; Table 1), which was highest in Fas-resistant HCT116 cells and lowest in Fas-sensitive GC_3/c1 and Thy4 cell lines. However, TS^- cells expressed an intermediate level. SFas was not detected in VRC_5/c1 or Caco-2 cells, although these lines were Fas-insensitive.

Expression of FAP-1, which binds to the negative regulatory domain of Fas, varied by 16-fold among the ten cell lines, being highest however in the two lines most sensitive to Fas-mediated apoptosis (TS^-, Thy4) and very low or absent in Fas-resistant VRC_5/c1, RKO and SW480 cell lines (Figure 5; Table 1). Of interest was that TS^- and Thy4 also expressed the highest levels of the receptor in addition to the highest level of FAP-1.

Levels of Bcl-2 expression varied by eightfold (Figure 6; Table 1), and of interest Bcl-2 was highest in Thy4, the line most sensitive to Fas-induced apoptosis. Bcl-2 levels were lowest in Fas-resistant cells (HCT116, SW480), and data suggested no correlation between Bcl-2 expression and Fas-induced apoptosis.

Taken together, data indicated that levels of expression of the negative regulators of Fas signalling examined including sFas, FAP-1 and Bcl-2, were not correlative with Fas-mediated apoptosis in colon carcinoma cell lines.

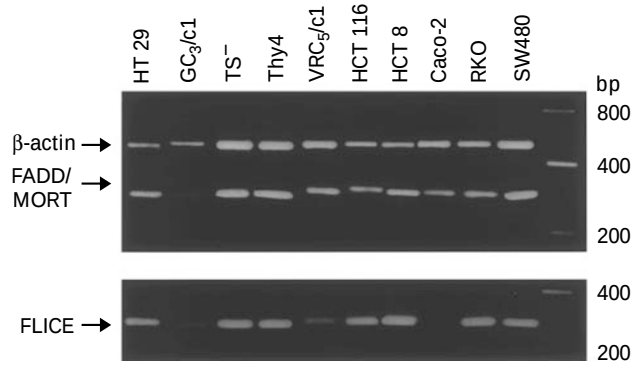

Figure 4 Expression of FADD/MORT1 and FLICE, components of the DISC and β-actin as internal control, in cell extracts. Conditions were as described in Materials and Methods

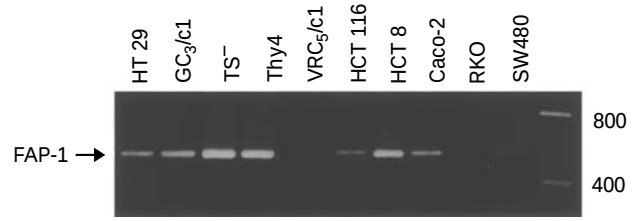

Figure 5 Levels of FAP-1 expressed in colon carcinoma cell lines following analysis by RT–PCR. β-actin expression is shown in Figure 2

Upregulated expression of Fas by interferon-γ increases sensitivity to Fas-mediated apoptosis

From the data presented, it is evident that the strongest correlation between relative levels of expression of components of the Fas signalling pathway and Fas-mediated apoptosis in colon carcinoma cell lines, comes from the levels of expression of the Fas antigen itself. Several reports have indicated that the expression of Fas may be modulated and upregulated either following transfection of human Fas antigen cDNA (Itoh *et al*, 1991) or following treatment of cells with certain cytokines including interferon-γ or TNF-α (Leithauser *et al*, 1993; Moller *et al*, 1994; Ossina *et al*, 1997; Weller *et al*, 1994). To examine whether modulation of Fas expression could be achieved in colon carcinoma cells by cytokines, and if so, could enhance the sensitivity to Fas-dependent apoptosis, cells with relatively low (HT29) or intermediate (HCT8, HCT116) levels of expression of the receptor were initially treated with interferon-γ (100 U/ml) for a period of 48 h, followed by examination of the level of elevation in Fas expression by ELISA assay (Table 1). Exposure to interferon-γ for 48 h had been shown previously to maximally elevate Fas expression in HT29 cells (Moller *et al*, 1994). In each of the three cell lines, elevated Fas expression was obtained, where a modest 1.6-fold increase was found in HCT8 and HCT116 cells. However, the greatest elevation of 4.3-fold occurred in HT29, where Fas expression was increased to 417 pg/10^6 cells.

To determine whether increased expression of Fas could increase cellular sensitivity to Fas-induced apoptosis, the influence of interferon-γ (100 U/ml) on the sensitivity of HT29 cells to CH-11 (50–200 ng/ml) was examined by both growth inhibition and clonogenic assays

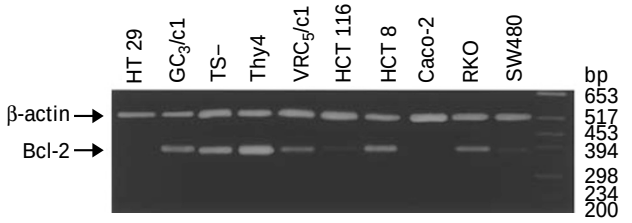

Figure 6 Bcl-2 expression in cultured colon carcinoma cells determined by RT–PCR analysis and expression of β-actin

Table 2 Expression of Fas in colon carcinomaf cell lines after treatment with IFN-γ

Cell line	IFN-γ (U/ml)	Fas (pg/10^6 cells $\pm$ S.D.)	% Control
HT29	0	97.2 $\pm$ 25.0	100
	100	416.6 $\pm$ 32.2	429
HCT8	0	200.0 $\pm$ 25.0	100
	100	325.0 $\pm$ 25.0	163
HCT116	0	358.4 $\pm$ 29.2	100
	100	569.4 $\pm$ 28.8	159

following exposure to the cytokine prior to (24 h) and during (72 h) CH-11 exposure (Figure 7). Interferon-γ and CH-11 each induced a 15–40% inhibition of growth when administered alone. However, in combination, the growth inhibitory response was >95%, indicating a greater than additive effect for the cytokine and cytolytic Ab administered in combination. When examined by clonogenic assay, the growth inhibitory response of HT29 cells was found to be due to the induction of a cytotoxic response. Interferon-γ and CH-11 each administered alone exerted no effect on the clonogenic survival of HT29 cells. However, in combination, they induced 90% reduction in cellular survival at 50 ng/ml CH-11, and >95% at a CH-11 concentration of 200 ng/ml, clearly demonstrating greater than additive effects. Therefore, the initial growth inhibitory response to CH-11 observed in HT29 cells was enhanced and converted to an apoptotic response when upregulated expression of Fas was induced by treatment with interferon-γ, thereby sensitizing cells to Fas-mediated apoptosis.

Discussion

It is well established that Fas is expressed and has a functional role in the induction of apoptosis and maintenance of cellular homeostasis in cells of the immune system. Specifically, Fas has been implicated in the peripheral deletion of autoimmune cells, activation-induced T cell death, and is one of the major pathways of $CD8^+$ cytolytic T cells (French *et al*, 1996). However, Fas is constitutively expressed in a variety of epithelial cell types including all cells of the colon, although whether Fas exerts a similar functional role in the maintenance of normal cell turnover and homeostasis in epithelial tissues still remains to be explored. We have shown previously that Fas-dependent apoptosis can occur in malignant cells of the colon (Houghton *et al*, 1997a,b). However, malignant colonic epithelial cells frequently demonstrate reduced capabilities of responding to apoptotic stimuli either by mutation of genes involved in promoting responses downstream of DNA damage including the p53 tumor suppressor gene (Fearon and Vogelstein,

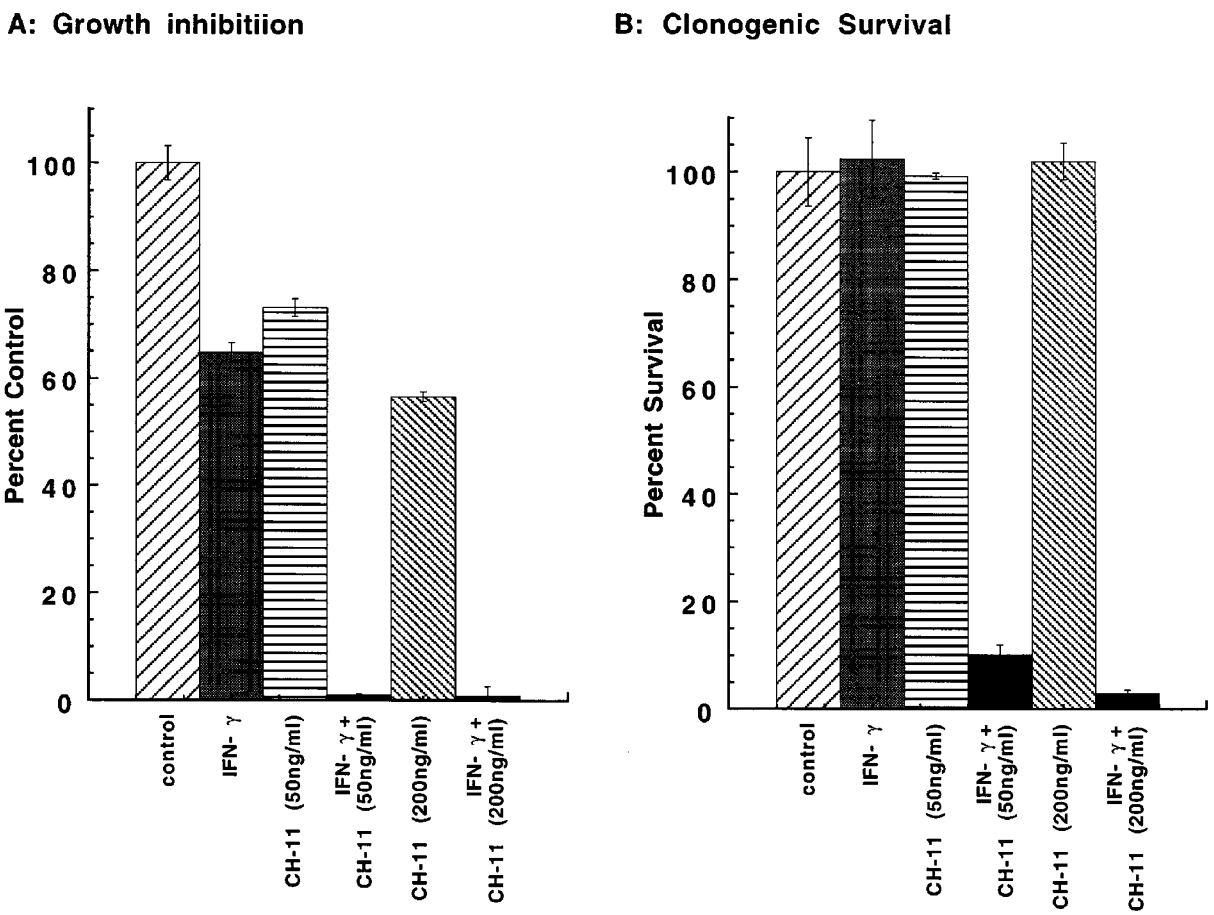

Figure 7 Sensitivity of HT29 cells to CH-11 and interferon-γ administered singly or in combination. (**A**) Growth inhibition induced by interferon-γ (100 U/ml) administered for 96 h or CH-11 (50 ng/ml, 200 ng/ml) administered for 72 h. Alternatively, HT29 cells were treated with interferon-γ 24 h prior to a 72 h exposure to CH-11 simultaneously with interferon-γ for a further 72 h. Cells were enumerated at the end of the treatment period. (**B**) Clonogenic survival of HT29 cells following administration of interferon-γ and CH-11 either alone or in combination. Plating conditions were as described in Materials and Methods, and treatment conditions were as described in **A**. Colonies were enumerated 6 days following the removal of CH-11

1990), reduced expression of genes required to promote a cell death signal (e.g. reduced expression of the Fas antigen; Moller *et al*, 1994), or elevated expression of genes that promote cellular survival (Bcl-2; Bedi *et al*, 1995) or inhibit essential signalling pathways (e.g. FAP-1; Yanagisawa *et al*, 1997). We have therefore defined the cellular sensitivity to Fas-mediated apoptosis in 10 human colon carcinoma cell lines and have determined the expression of genes known to regulate the function of the Fas signalling pathway in the induction of Fas-dependent apoptosis.

Four of the ten cell lines demonstrated some sensitivity to the cytolytic CH-11 MoAb and of these, cell death was induced in three of the four, as determined by reduced clonogenic survival and morphologic evaluation of the presence of apoptotic cells. However, six lines were resistant to the induction of Fas-dependent apoptosis, suggesting that the ability to tightly regulate this apoptotic pathway had been reduced or eliminated. The importance of the death domain for Fas signalling has been well established (Itoh and Nagata, 1993). Lpr^{cg} mice carry a point mutation located within the Fas death domain resulting in signalling deficiency (Watanabe-Fukunaga *et al*, 1992), and mutations in Fas are associated with human lymphoproliferative disorders and autoimmune disease (Rieux-Laucat *et al*, 1995). However, in the panel of colon carcinoma cell lines investigated, sequencing analyses indicated that the Fas death domain in nine of the lines was of wt sequence, indicative of the ability to maintain promotion of an apoptotic stimulus. Fas expression was undetectable in Caco-2 cells, although the reason for this loss of expression is at present unknown.

Downstream of Fas, the 28 kd cytosolic protein FADD/MORT1, an essential component of the DISC, also contains a death domain and induces apoptosis when over-expressed in different cell types (Boldin *et al*, 1995; Chinnaiyan *et al*, 1995). Its importance to Fas signalling was established when transfection of a dominant negative FADD/MORT1 (FADD-DN) prevented recruitment of the other CAP proteins to the DISC, thereby preventing the induction of apoptosis (Chinnaiyan *et al*, 1996). In the current study, all ten colon carcinoma cell lines appeared to demonstrate adequate FADD/MORT1 expression, since the cell line with the lowest level of expression (GC_3/c1) was one of the lines most sensitive to Fas-mediated apoptosis. Similarly FLICE, another key component of the DISC, has been identified as an important regulator of the induction of apoptosis downstream of Fas (Boldin *et al*, 1996; Muzio *et al*, 1996). The recent cloning of a naturally occurring structural homolog of FLICE (I-FLICE) which exerts dominant negative function, was found to inhibit Fas-dependent apoptosis (Hu *et al*, 1997). Although the lowest level of FLICE expression was found in Caco-2 cells, which also demonstrated no Fas expression, levels of FLICE appeared adequate in all of the other cell lines, being low in GC_3/c1 which was Fas-sensitive.

Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule has been identified (Cheng *et al*, 1994). SFas lacks the transmembrane domain due to the deletion of an exon encoding the region, and has been identified in patients with systemic lupus erythematosus (Cheng *et al*, 1994) and in the serum and tumors of patients presenting with solid malignancies (Midis *et al*, 1996). The expression of sFas was detected in Fas-resistant HCT116 colon carcinoma cells, but was also present in TS^- cells, which were sensitive to Fas-mediated apoptosis. Levels of sFas appeared insignificant in comparison to the higher levels of Fas expressed in the same cell lines, suggesting that the presence of sFas did not confer Fas resistance. The expression of other proteins that interfere with Fas signalling include FAP-1, which is an inhibitory Fas-binding protein that interacts with the C-terminal 15 amino acids of the regulatory domain of the Fas receptor (Sato *et al*, 1995). FAP-1 has been identified in cultured colon carcinoma cells (Yanagisawa *et al*, 1997), and its expression was reported to be highest in cell lines and tissues that are relatively resistant to Fas-mediated cytotoxicity (Sato *et al*, 1995). However, others have shown that cell lines extremely sensitive to Fas-induced apoptosis (HUT78, SKW6.4) expressed high levels of FAP-1, whereas a Fas-resistant line was completely negative for FAP-1 mRNA (Boe^R; Peter *et al*, 1996). In agreement with the latter report, colon carcinoma cell lines in the current study that were highly sensitive to the induction of apoptosis via Fas also expressed the highest levels of FAP-1. Expression of Bcl-2, which has been reported to negatively correlate with anti-Fas sensitivity in colorectal carcinomas (Meterissian and Kontagiannea, 1996) and cell lines (Houghton *et al*, 1997a,b), was also examined. However, cell lines that were the most sensitive to CH-11 (TS^-, Thy4) also expressed the highest levels of Bcl-2, indicating no direct correlation between high Bcl-2 expression and low anti-Fas sensitivity.

It should be emphasized however, that while no relationship has been found between relative expression of certain key regulatory factors as determined by RT–PCR, and Fas response, relative levels of expression of the respective proteins have yet to be determined. The activities of these proteins may also be influenced by their intracellular location, phosphorylation state and/or other posttranslational modifications, and hence may also influence Fas signalling processes beyond the level of RNA expression.

Of particular interest in the current study was the large diversity in levels of expression of the Fas antigen itself in the ten human colorectal carcinoma cell lines, where levels of the receptor varied by >1000-fold. TS^-, Thy4 and GC_3/c1 cell lines with the highest sensitivity to Fas-induced apoptosis also demonstrated the highest levels of Fas expression (>540 pg/10^6 cells); cell lines with Fas levels of <350 pg/10^6 cells were all Fas-resistant. In TS^- and Thy4, high-level Fas expression may be responsible for negating the inhibitory effects of high levels of expression of FAP-1 or Bcl-2 in these cell lines. Variability in levels of Fas expression did not appear to be due to mutation in the death domain of Fas. Previous reports indicated that cytokines including interferon-γ can elevate the expression of Fas in epithelial cell lines (Leithauser *et al*, 1993; Moller *et al*, 1994; Ossina *et al*, 1997). Our studies demonstrated that modest elevation in Fas expression was achieved in HCT8 and HCT116 cells following treatment of cells with

interferon-γ, but a significant elevation (4.3-fold) was achieved in HT29. These data are consistent with a previous report indicating varied inducibility of Fas expression in colon carcinoma cell lines (Moller *et al*, 1994). However, of particular interest, was that pretreatment of HT29 cells with interferon-γ prior to CH-11 was synergistic in the induction of Fas-dependent cytotoxicity. In this cell line, a growth inhibitory response was converted to a cytotoxic response, simultaneous with upregulated expression of Fas. Other cytokines including TNF-α have also been found to be capable of inducing Fas expression (Leithauser *et al*, 1993; Moller *et al*, 1994; Weller *et al*, 1994), and a combination of cytokines has been shown to exert greater effects than single agents in this regard (Moller *et al*, 1994). Our study has demonstrated that the level of Fas expression may be a major determinant of Fas-dependent apoptosis in colon carcinoma cells, and that modulation of receptor expression can significantly influence the ability of these cells to respond to Fas signalling, and to the induction of apoptosis via Fas.

Previously we have shown that autocrine signalling via Fas/FasL interactions promotes thymineless death following the induction of DNA damage by dThd withdrawal in TS^- cells, thereby linking DNA damage to the apoptotic machinery of colon carcinoma cells (Houghton *et al*, 1997a). Hence the Fas signalling pathway may be an essential functional pathway for the induction of apoptotic responses in colon carcinoma cells, and may be important in the regulation of apoptosis in malignant epithelial cells treated with agents that target thymidylate synthase. As a mediator of apoptosis in response to treatment with chemotherapeutic agents the role of Fas is worthy of further exploration. The significance of the level of Fas in colon carcinoma cells and the ability to upregulate Fas expression using cytokines to promote apoptosis indicate that Fas may be an important target to explore further in the modulation of apoptotic responses in colon carcinomas.

Materials and Methods

Cell lines

The cloned human colon adenocarcinoma cell lines GC_3/c1 (Houghton *et al*, 1995) and VRC_5/c1 (Houghton *et al*, 1991) have been described previously. A thymidylate synthase-deficient mutant clone selected from GC_3/c1, TS^-, deficient in TSmRNA and protein and auxotrophic for dThd, has been well characterized (Harwood *et al*, 1996). A clone of TS^-, Thy4, was selected for its ability to withstand prolonged periods of dThd deprivation (Harwood *et al*, 1996). RKO cells were a gift from Dr Michael Kastan, Johns Hopkins Medical Center. All additional colon carcinoma cell lines used for these studies were obtained from ATCC. Cells were maintained as previously described (Harwood *et al*, 1996), in the presence of 20 μM dThd.

Growth and clonogenic assays

For growth inhibition assays, cells were plated in 6-well plates (Falcon) at a density of 100 000 – 200 000 cells/well. Following overnight attachment, cells were treated with the anti-Fas cytolytic MoAb CH-11 (MBL International Corp.), or an IgG1 isotype-matched control (Pharmingen) at concentrations from 150 – 750 ng/ml for 72 h. Alternatively, cells were treated with interferon-γ (100 U/ml; Genentech Inc.) for 24 h prior to exposure to CH-11 (50 – 200 ng/ml) for a further 72 h. Cells were subsequently enumerated using a Coulter particle counter.

For clonogenic assays, GC_3/c1, TS^- and Thy4 cells were plated at a density of 3000 cells/well, and HT29 at 1000 cells/well. Following overnight attachment, media was aspirated and cells were treated with CH-11 (150 – 750 ng/ml) for 72 h. Clonogenic survival was evaluated 6 days (HT29), 7 days (GC_3/c1) or 11 days (TS^-, Thy4) after removal of CH-11 (Harwood *et al*, 1996).

Morphologic identification of apoptosis

HT29, GC_3/c1, TS^- and Thy4 cells which demonstrated varied sensitivity to Fas-mediated apoptosis induced by CH-11, were examined for morphologic evidence of apoptosis. Cells were plated in glass chamber slides each containing two chambers (Nunc Lab-Tek#154461) under standard conditions at plating densities of 100 000 or 400 000 cells/well in a volume of 2 ml. Following overnight attachment, cells were treated with CH-11 (15 – 150 ng/ml) diluted in sterile PBS or PBS alone (20 μl) for 11 h or 24 h. Cells were subsequently fixed in paraformaldehyde and stained with hematoxylin and eosin using standard procedures.

Expression of Fas, FADD/MORT1, FLICE, sFas, FAP-1 and Bcl-2

The expression of components of the Fas signalling pathway (Fas, FADD/MORT1, FLICE) and factors inhibitory to Fas-mediated apoptosis (sFas, FAP-1, Bcl-2), were examined in ten human colon carcinoma cell lines.

Fas expression was determined in cell extracts either by RT – PCR to yield a 682 bp product (O'Connell *et al*, 1996), or by a standard ELISA assay (Houghton *et al*, 1997a). The capture Ab used was a purified anti-human Fas MoAb (Pharmingen; cat.#65311A), whereas the Ab used for detection was a biotin anti-human Fas MoAb DX2 (Pharmingen). Quantitation was by optical densitometry using recombinant soluble human Fas (Pharmingen) as the standard, and normalized for cell number as previously described (Houghton *et al*, 1997a).

FADD/MORT1, FLICE, FAP-1 and Bcl-2 were also determined by RT – PCR. Total RNA was extracted from cells in RNAzol B; cDNA was synthesized in a volume of 20 μl from 2 μg total RNA using an oligo dT primer and a cDNA cycle kit (InVitrogen). B-actin control PCR (540 bp product) was performed to monitor RT – PCR amplification efficiency (O'Connell *et al*, 1996). PCR amplification of FAP-1 (Sato *et al*, 1995) and Bcl-2 (Mori *et al*, 1996) cDNAs was performed as described to yield 607 bp and 389 bp products, respectively. Forward and reverse primers used to amplify FADD/MORT1 and FLICE to produce 291 bp and 273 bp products, respectively, were: FADDF, 5′-GAAGAA-GACCTGTGTGCAGC-3′; FADDR, 5′-ACTCCTGTTCTGGAGGTCAC-3′; FLICEF, 5′-GAGGAGTTGTGTGGGGTAATGA-3′; FLICER, 5′-CTCTACTGTGCAGTCATCGTG-3′. In addition, the death domain of Fas was sequenced by dideoxynucleotide sequencing of a Fas F3-R3 PCR product synthesized from cDNA prepared from each cell line (Sato *et al*, 1995).

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