Advances in Brief

The Fas Signaling Pathway Is Functional in Colon Carcinoma Cells and Induces Apoptosis

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Abstract

Fas is expressed in colonic epithelial cells and is also expressed in colon carcinomas, although its functional significance in the regulation of apoptosis in cells outside of the immune system remains unknown. In this study, we determined the role of Fas signaling on cellular growth of cultured colon carcinoma cells and demonstrated apoptosis induced by a cytotoxic anti-Fas monoclonal antibody (CH-11) in cells of the GC/c1 lineage (GC/c1, TS−, Thy4) but not in HCT116 or CaCo2 cells. Growth inhibition was detected at concentrations of CH-11 as low as 1 ng/ml, and clonogenic survival studies yielded IC50 values of 3–26 ng/ml. Cytotoxicity was inhibited by ZB4, a monoclonal antibody inhibitory to Fas signaling. In addition, the survival factor Bel-2, which has demonstrated inconsistent protective effects against Fas signaling in other systems, was inhibitory to Fas-induced apoptosis in colon carcinoma cells after adenoviral transduction. Fas was expressed at the highest levels in TS− and Thy4 cells, which were the most sensitive cell lines to Fas-induced apoptosis. FAP-1, a protein tyrosine phosphatase that interacts with the cytosolic negative regulatory domain of Fas, was expressed in each cell line but did not correlate with sensitivity to Fas-mediated apoptosis. These data have therefore identified a functional Fas pathway in colon carcinoma cells when Fas is expressed at high levels. Hence, the role of Fas signaling in the regulation of apoptosis in colon carcinoma cells and its role in influencing the response to treatment with chemotherapeutic agents should be further explored.

Introduction

Fas (APO-1, CD95) is a 48-kDa integral membrane receptor protein that belongs to the tumor necrosis factor/nerve growth factor receptor superfamily. Its predominant physiologic role has been identified in the regulation of homeostatic processes in cells of the immune system, specifically by triggering apoptosis. Thus, Fas has been implicated in the peripheral deletion of autoimmune cells and in activation-induced T-cell death and is one of the major pathways of CD8+ cytolytic T cells (1). The cell death process is initiated by binding and cross-linking of the endogenous Fas ligand to Fas, followed by trimerization of the receptor and the formation of a DISC3 that ultimately activates proteases of the interleukin 1B converting enzyme family (2). Alternatively, antibodies that recognize the human cell surface antigen Fas can also induce cytolysis via Fas signaling (e.g., CH-11). Initially, it was considered that Fas expression and function might be lineage specific. However, studies conducted by RNase protection analysis or immunohistochemistry in tissues of mice (1) and humans (3, 4) demonstrated constitutive and more widespread distribution of Fas, in particular in the thymus, lung, and spleen; in a variety of epithelial cells including the small and large intestines; and also in the seminal vesicle, prostate, and uterus of the reproductive systems. In normal colonic epithelium, constitutive expression of Fas was observed within the cytoplasm and at the basolateral surface of every epithelial cell, irrespective of its localization at the crypt or the mucosal surface (4), suggesting a more general role in the regulation of normal cell turnover. We and others (5) have shown expression of Fas in cultured colon carcinoma cell lines, and Fas is also expressed in colon carcinomas (4), although whether the Fas system can regulate apoptosis in this tissue remains unknown. Our data show that Fas signaling can induce apoptosis after treatment of colon carcinoma cells of the GC/c1 lineage with a cytotoxic anti-Fas mAb (CH-11), and that cytotoxicity is inhibited by treatment with a mAb (ZB4) inhibitory to Fas signaling and also after adenoviral delivery of Bel-2. In this report, we demonstrate that an intact and functional Fas system occurs in colon carcinoma cells.

Materials and Methods

Cell Lines. The cloned human colon adenocarcinoma cell line GC/c1 has been described previously (6). A thymidine synthase-deficient mutant clone selected from GC/c1, TS−, deficient in TS mRNA and protein and auxotrophic for dThd, has been well characterized (7). In addition, a clone of TS−, Thy4, was selected for its ability to withstand prolonged periods of dThd deprivation (7). Additional colon carcinoma cell lines used for these studies, HCT116 and CaCo2, were obtained from the American Type Culture Collection. Cells were maintained as described previously (7) in the presence of 20 μM dThd.

The abbreviations used are: DISC, death-inducing signaling complex; mAb, monoclonal antibody; dThd, thymidine; Ad-Bcl-2, Bcl-2 adenovirus; MoI, multiplicity of infection; RT-PCR, reverse transcription-PCR; wt, wild-type.
Fig. 1  A, growth-inhibitory response to varied concentrations of CH-11 in cultured colon carcinoma cell lines. B, percentage of colon carcinoma cells remaining after treatment with varied concentrations of CH-11. C, clonogenic survival of TS− and Thy4 cells after treatment with CH-11. All exposures to CH-11 were for 72 h, and conditions for each assay were as described in “Materials and Methods.” Data represent the mean ± SD of three determinations at each CH-11 concentration.

Growth and Clonogenic Assays. For growth inhibition assays, cells were plated in 6-well plates (Falcon) at a density of 200,000 cells/well. After overnight attachment, cells were treated with the anti-Fas cytolytic mAb CH-11 (MBL International Corp.) or an IgG1 isotype-matched control (PharMingen) at concentrations of 1–50 ng/ml for 72 h. Cells were subsequently enumerated using a Coulter particle counter.

For clonogenic assays with TS− and Thy4 cells, plating was at a density of 3000 cells/well. In experiments using antibodies, the ZB4 mAb (Kamiya Biomedical Co.; 100 ng/ml) or an IgG1 isotype-matched control mAb (PharMingen; 100 ng/ml) was added at the time of plating. After overnight attachment, media were aspirated, and cells were treated with CH-11 (5–100 ng/ml) for 72 h. In studies using Ad-Bcl-2, Bcl-2 was transduced 24 h before treatment with CH-11 at MOIs of 10 or 50. Clonogenic survival was evaluated 11 days after removal of CH-11 (7).

Ad-Bcl-2 was prepared by subcloning a Bcl-2 cDNA (8) into the BamHI site of pSP64Cla (modified from pSP64; Promega), followed by subcloning into the ClaI site of the pAVS6.DNA adenoviral vector (Genetic Therapy Inc.). After recombination with AddI327 DNA, replication-incompetent adenovirus containing the basic vector backbone sequences (Ad-VC) or the Bcl-2 cDNA was amplified in the embryonic kidney cell line 293, purified, and titered as described previously (9).

Expression of Fas and FAP-1. The expression of Fas and a Fas-associated phosphatase (FAP-1) inhibitory to Fas signaling and Fas-mediated apoptosis was determined.

Fas expression was measured in cell extracts by RT-PCR to yield a 682-bp product (10) or by a standard ELISA assay. The capture antibody used was a purified antihuman Fas mAb (PharMingen; catalogue number 65311A), whereas the antibody used for detection was a biotin antihuman Fas mAb DX2 (PharMingen). Quantitation was by optical densitometry.

FAP-1 (11) was also determined by RT-PCR. Total RNA was extracted from cells in RNAzol B; cDNA was synthesized in a volume of 20 μl from 1 μg of total RNA using an oligodeoxythymidylic acid primer and a cDNA cycle kit (Invitrogen). PCR amplification of FAP-1 cDNA was performed as described previously (11) to yield a 607-bp product. β-actin control PCR (540 bp) was performed to monitor RT-PCR amplification efficiency (10).

Results and Discussion

Sensitivity of Colon Carcinoma Cell Lines to CH-11. To determine the influence of CH-11 and hence Fas signaling on cellular growth, GC3/c1, TS−, Thy4, HCT116, and CaCo2 cells were enumerated at time 0 and at 72 h after treatment with various concentrations of CH-11 (Fig. 1A). Growth inhibition occurred in all three cell lines of the GC3/c1 lineage, being greatest in Thy4 and least in wt GC3/c1 cells, but it was not demonstrated in either HCT116 or CaCo2. No effect on cell growth was detected in IgG1 isotype-matched controls (data not shown). Growth inhibition was observed in GC3/c1, TS−, and Thy4 at CH-11 concentrations as low as 1 ng/ml. When data were plotted as the percentage of cells at the initiation of treatment (Fig. 1B), cytotoxicity was detected in GC3/c1 cells at the highest concentration of CH-11 used (50 ng/ml). However, toxicity in TS− cells was determined at 20 ng/ml, and toxicity in Thy4 cells was determined at 5 ng/ml; IC50 values were >50, 15, and 2.3 ng/ml, respectively. To determine the influence of CH-11 on clonogenic survival, the two most sensitive lines were evaluated (TS− and Thy4; Fig. 1C). The IC50...
values for TS and Thy4 were 26 and 3 ng/ml, respectively, consistent with the cytotoxic responses to CH-11 observed in Fig. 1B. Of interest was that Thy4 was the most sensitive cell line to Fas-induced apoptosis. Previously, we demonstrated that Thy4 and not TS cells were able to sustain the accumulation of high levels of Bax, a regulator of cell death responses (7). This may be linked to enhanced sensitivity to Fas-mediated apoptosis in Thy4, because it was demonstrated that induction of Bax in breast cancer cell lines restored sensitivity to Fas-induced apoptosis (12).

Protective Effects of ZB4 and Bcl-2. The survival factor Bcl-2 has demonstrated inconsistent protective effects from Fas signaling and Fas-induced apoptosis in different systems. Thus, effects have ranged from complete inhibition (13) by Bcl-2 to partial inhibition or no protective effect (2). Therefore, in the current study, we examined the possibilities for protection against Fas-induced apoptosis by Bcl-2 in TS cells and compared the response to the protective effect of mAb ZB4, known to antagonize CH-11-induced cytolysis. In Fig. 2A, the protective effect of ZB4 was examined at a concentration of 100 ng/ml. At all CH-11 concentrations examined (10–100 ng/ml), protection from Fas-induced apoptosis was obtained during a 72-h exposure. In Fig. 2B, the protective effect of Bcl-2 was examined in TS cells after adenoviral transduction of Bcl-2 cDNA for 24 h at MOIs of 10 and 50, followed by treatment with CH-11 (50 ng/ml) for 72 h. When compared with no transduction or transduction of Ad-VC, Bcl-2 protected TS cells in particular at a MOI of 50, demonstrating that in colon carcinoma cells, Bcl-2 can protect from Fas-induced apoptosis downstream of Fas.

Expression of FAP-1 and Fas. FAP-1 has been identified as a protein tyrosine phosphatase capable of interacting with the cytosolic negative regulatory domain of the receptor and is proposed to inhibit Fas signaling and, subsequently, Fas-induced apoptosis (11). The role of FAP-1 in influencing Fas-mediated apoptosis was therefore examined by measuring levels of FAP-1 expression in the five colon carcinoma cell lines by RT-PCR. (Fig. 3). FAP-1 was expressed at low levels in both HCT116 and CaCo2 cells. Of interest were the high levels of FAP-1 expression in the most CH-11-sensitive lines, namely GC3/c1, TS, and Thy4, indicating that although FAP-1 was present, this was apparently not inhibitory to Fas-induced apoptosis. Sato et al. (11) reported a correlation between the level of FAP-1 expression and cellular sensitivity to Fas-mediated apoptosis. However, others have shown that cell lines extremely sensitive to Fas-induced apoptosis (HUT78 and SKW6.4) express high levels of FAP-1, whereas a Fas-resistant cell line was completely negative for FAP-1 mRNA (Boe,2). Our data would also support the contention that there are other factors...
independent of FAP-1 that influence Fas signaling in colon carcinoma cells.

Data from the literature suggest that expression of Fas may be reduced in certain colon carcinomas (4). We subsequently determined whether the sensitivity of the colon carcinoma cell lines to Fas-induced apoptosis correlated with levels of expression of the receptor, which must be present at sufficiently high levels to allow transduction of the signal from the cell surface. Data demonstrating expression of Fas by RT-PCR are shown in Table 1. As determined by ELISA assay and confirmed by RT-PCR, Fas expression was high in cell lines of the GC3/c1 lineage. Results indicate that the three most sensitive lines also express the highest levels of Fas in addition to the highest levels of FAP-1, with the expression of Fas being highest in TS-. However, levels of Fas are extremely high in TS- cells and must be in excess of FAP-1. The receptor was undetectable in CaCo2 cells, and this is presumably responsible for the lack of sensitivity of these cells to CH-11. Resistance of HCT116 cells to CH-11 seems unrelated to the expression of suboptimal levels of Fas. The level of Fas expression required for the induction of apoptosis has yet to be determined. It is of interest in this regard that HCT116 cells are less sensitive than GC3/c1 to treatment with 5-fluorouracil-leucovorin that targets 5-fluorouracil to the thymidylate synthase locus (data not shown) and also express lower levels of Fas. Alternatively, it also remains to be evaluated whether HCT116 cells demonstrate all of the components of the Fas signaling pathway at sufficient levels necessary for the transmission of an apoptotic response in comparison to GC3/c1, TS-, and Thy4.

There are many factors in the Fas signaling pathway that can influence cellular sensitivity to Fas-mediated apoptosis. Mutation or modification of an essential signaling molecule will prevent transduction of the death signal. It is evident that Fas must trimerize to allow formation of the DISC and to initiate a death signal (2). Cells must also express adequate levels of FADD/MORT1, which associates with Fas, contains a death domain, and is an essential component of the DISC (2), and FLICE, which associates with the death effector domain of FADD/MORT1 and induces apoptosis (14). Mutations in the death domain of Fas (15) or in Fas ligand (16) or expression of soluble Fas (17) may also result in resistance to Fas-mediated apoptosis. It will be of importance to examine the expression of these regulatory proteins in colon carcinoma cell lines with varied sensitivities to Fas-induced apoptosis. High-level Bcl-2 expression has also prevented the function of the Fas pathway, and in this regard, decreased anti-Fas sensitivity has been correlated with increased expression of Bcl-2 in colorectal carcinoma (5). Factors that regulate Fas expression and the Fas signaling pathway are currently being explored. One possibility is that Fas expression may be regulated by p53. wt p53 has induced Fas expression in malignant human cells (18), and transfection of wt p53 into human colon carcinoma cells has sensitized cells to Fas-mediated apoptosis (19). In addition, whether Bax can regulate apoptotic responses downstream of Fas has yet to be determined. Fas may be more generally applicable in the regulation of apoptosis, in particular in epithelial cells, than was originally proposed. The role of Fas signaling processes in generating apoptotic responses in colon carcinomas and other malignant cell types remains to be determined. In particular, the role of Fas signaling as a mediator of apoptosis in response to treatment with chemotherapeutic agents is worthy of further exploration.

References

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<th>Cell line</th>
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<td>727</td>
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<td>1210</td>
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<td>CaCo2</td>
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<td>1.6</td>
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* Relative optical density.
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