c-myc/p53 Interaction Determines Sensitivity of Human Colon Carcinoma Cells to 5-Fluorouracil in Vitro and in Vivo

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ABSTRACT

Colon carcinoma cells overexpress c-myc due to defective Wnt signaling, but only patients whose tumors have an amplified c-myc gene show improved disease-free and overall survival in response to 5-fluorouracil (5FU). Here we show that in two colon carcinoma cell lines that do not have an amplified c-myc gene but differ in their p53 status, high c-myc levels can be further elevated by introducing a c-myc expression vector. Whereas sensitivity to low serum-induced apoptosis was imposed on the parental lines independent of p53 status and was unaffected by further elevation of c-myc, sensitivity to 5FU-induced apoptosis was dependent on both the higher c-myc levels due to the expression vector and wild-type p53 function. The elevated c-myc levels led to higher c-myc transactivating activity in the p53 wild-type cell line, but not in the mutant p53 cell line. The requirement for both elevated c-myc and p53 for 5FU sensitivity was confirmed using antisense to c-myc and pifithrin-α, a specific inhibitor of p53. Finally, the in vitro data predicted that only patients with both amplified c-myc and wild-type p53 in their primary tumors would be responsive to 5FU-based therapy, which was borne out by analysis of tumors from 135 patients entered into a Phase III clinical trial of 5FU-based adjuvant therapy. The data provide significant insight into mechanisms that establish colon tumor cell sensitivity to 5FU, clearly demonstrate the necessity of exercising caution in considering combining novel strategies that target elevated c-myc with standard 5FU-based therapy, and suggest alternative therapeutic strategies that target c-myc and/or p53 mutations in the treatment of colon cancer.

INTRODUCTION

A role for c-myc in the development of colon tumors was first suggested by the report that c-myc was amplified and overexpressed in a chemically induced mouse colon tumor (1). Subsequent work showed that in humans, high c-myc levels are present in approximately 70% of colon tumors (2), and that this is due, at least in part, to the fact that c-myc is a target gene of Wnt, or β-catenin-TCF, signaling. β-Catenin is usually degraded in a complex with APC, GSK3β, and axin (3), but mutations in APC, which initiate the development of almost all human colon cancer, abrogate this degradation (4). This elevates β-catenin levels, β-catenin-TCF complex formation and signaling, and the expression of c-myc and other target genes (5, 6). However, in addition to this altered regulation of c-myc expression, low-level amplification of the c-myc gene is also present in approximately one-third of human colon tumors (7, 8).

Elevated c-myc expression has been shown to sensitize colon cancer cells, as well as other cell types, to apoptosis induced by different stimuli, including low serum conditions, hypoxia, and deprivation of specific growth factors (9–11). However, the relationship between c-myc expression and the apoptosis-promoting effects of more clinically relevant chemotherapeutic agents on colon cancer cells overexpressing c-myc has not been investigated. This is important because our group (8) reported that amplification of c-myc identified stage II and III colon cancer patients with increased disease-free and overall survival after 5FU-based adjuvant therapy, and amplification of c-myc has been shown to result in further up-regulation of c-myc in vitro (12).

We therefore addressed whether colon cancer cell lines that have elevated c-myc expression due to defective Wnt signaling can express even higher levels of c-myc and further elevate c-myc transactivating activity. Moreover, we investigated the role that c-myc plays in sensitizing these cells to apoptosis induced by both low serum, a well-characterized inducer of apoptosis in c-myc-overexpressing cells, and 5FU, the principal agent used in chemotherapy of colon cancer. Because the product of the p53 tumor suppressor gene can either promote DNA repair by inducing cell cycle arrest or cause cell death via apoptosis (13–16), and p53 function has been shown to be fundamental in the response to 5FU (17, 18), we also investigated the interaction between c-myc and p53 in sensitization to 5FU-induced apoptosis.

We found that sensitivity to 5FU in vitro was dependent on both elevated c-myc expression and transactivating activity above the high levels due to the increased β-catenin-TCF activity and expression of wild-type p53. This was confirmed using antisense to c-myc and PFT-α, a specific inhibitor of p53 function. This contrasted with the fact that sensitivity to low serum-induced apoptosis was unaffected by further elevation of c-myc above the high endogenous levels and was also independent of p53 status or function. Moreover, the in vitro data predicted that only patients with tumors showing amplified c-myc and wild-type p53 would benefit from 5FU-based adjuvant therapy. Analysis of the data on c-myc amplification and p53 mutation in patient tumors from a multi-institutional Phase III clinical trial of adjuvant therapy for stage II and III colon cancer confirmed this prediction.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. LoVo and DLD1 colon carcinoma cells were maintained in MEM supplemented with 10% FBS, 1× antibiotic/antimycotic (100 units/ml streptomycin sulfate, 100 units/ml penicillin G sodium, and 0.25 μg/ml amphotericin B), 100 μM nonessential amino acids, and 10 mM HEPES buffer solution (all from Life Technologies, Inc.) in a humidified incubator at 37°C with 5% carbon dioxide. FuGene 6 (Boehringer Mannheim) was used to transfect a vector carrying the c-myc sequence in the sense or antisense orientation into LoVo or DLD1 cells. The vector p290-myc(2,3) contains the c-myc genomic sequence in the sense orientation (19). An EcoRI/BamHI c-myc cDNA fragment corresponding to exons 2 and 3 was excised from pMV-mycER (20) and inserted in the antisense orientation in the BamHI/EcoRI site of pcDNA3 (Invitrogen). Clones of LoVo and DLD1 cells stably transfected with the p290-myc(2,3) vector were selected with 500 μg/ml hygromycin (Life Technologies, Inc.), whereas clones carrying the pcDNA-mycANS vector were selected with 1500 μg/ml Geneticin (Life Technologies, Inc.).
c-myc mRNA Levels and Transactivity. mRNA levels in LoVo and DLD1 derivative clones were measured by Northern blot analysis. Briefly, 20 μg aliquots of total RNA isolated with Trizol (Life Technologies, Inc.) were lyophilized, resuspended in standard loading buffer, denatured by boiling, and fractionated in a 1% agarose gel containing 2.2 mM formaldehyde. RNA was transferred onto a nitrocellulose membrane (Schleicher and Schuell) and hybridized to a c-myc probe corresponding to a Pho3/BglII fragment from the pMC41-5p plasmid (21). The DNA probes were radiolabeled by the random priming method (Life Technologies, Inc.) with [α-32P]dCTP and [α-32P]dATP (Amersham). The nitrocellulose membrane was stripped by incubation at 95°C a plasmid containing the gal vector with either the min-CAT or the M4-min-CAT vector. pCMV-gal is using the signal from the GAPDH probe to account for loading artifacts. ImageQuant software (Molecular Dynamics). c-myc levels were standardized using a Storm PhosphorImager (Molecular Dynamics) and quantified using radiolabeled as described above. Visualization of hybridization was carried out using a Storm PhosphorImager (Molecular Dynamics) and quantified using ImageQuant software (Molecular Dynamics). c-myc levels were standardized using the signal from the GAPDH probe to account for loading artifacts.

c-myc transactivity was measured by transient cotransfection of a pCMV-gal vector with either the min-CAT or the M4-min-CAT vector. pCMV-gal is a plasmid containing the β-galactosidase gene under the control of the CMV promoter, whereas the M4-min-CAT vector contains a four-tandem repeat of the CACGTG consensus c-myc binding site upstream of a minimal TK promoter controlling the expression of the CAT gene (22). The min-CAT vector is identical to M4-min-CAT but lacks the c-myc binding sites. Either 5 or 3.5 × 10^4 cells (LoVo and DLD1 cells or derivatives, respectively) per well were seeded in triplicate in 24-well plates, and for each cell line, three experimental conditions were assayed: untransfected control cells and cells cotransfected with pCMV-gal and either min-CAT or M4-min-CAT. To achieve optimal transfection efficiencies, different transfection agents were used for LoVo and DLD1 cells and their derivatives. LipofectAMINE PLUS (Life Technologies, Inc.) or GenePORTER 2 Gene Therapy Systems, respectively. It should be noted that all transfections included internal controls. The relative levels of CAT activity in each cell line obtained from the M4-min-CAT were adjusted for background levels using the data from the min-CAT cotransfection and adjusted for transfection efficiency using the β-galactosidase levels from pCMV-gal.

Growth Curves. Growth kinetics of LoVo, DLD1, and stable transfectants were determined as follows: 100 μl of medium containing 2 × 10^3 cells were added to six replica wells in seven different Falcon 96-well plates (Becton Dickinson). After 24 h, 100 μl of fresh medium were added to each well. Plates were harvested 2, 3, 4, 5, 6, 7, or 8 days after seeding. The relative levels of total protein were assessed using sulforhodamine B staining as described previously (23). All experiments were repeated at least three times, in six replica wells each time. Representative experiments are shown.

Apoptotic Response to Serum Starvation and 5FU Treatment. Falcon T75 tissue culture flask (Becton Dickinson) were used to seed 2 × 10^6 LoVo-myc-3 cells, and 24 h later, growth medium was replaced by medium containing either low serum (0.1%) or 3 μM 5FU. To demonstrate the presence of apoptotic death, floating cells from these cultures were harvested, pelleted, and resuspended in 1 μM DAPI (Sigma Chemical Co.) in PBS. Micrographs of DAPI-stained nuclei were captured with a SPOT RT Diagnostic Instruments charge-coupled device camera (Diagnostic Instruments) attached to a BX60 Olympus fluorescence microscope. Quantification of the apoptotic response to either low serum conditions or 5FU treatment was done as described previously (24, 25). Cells (1 × 10^4) were seeded in triplicate in Falcon 6-well plates (Becton Dickinson) and allowed to attach for 24 h before medium was aspirated and replaced by fresh medium containing either 10% FBS, 0.1% FBS, or 10% FBS/3 μM 5FU. This dose of 5FU was found in preliminary experiments to result in approximately 50% growth inhibition after 6 days of treatment for both LoVo and DLD1 cells (data not shown). In some experiments, PFT-α, a specific p53 inhibitor (26), was also added (15 or 30 μM). After 72 h of treatment, both attached and floating cells were harvested, washed twice with 2 ml of PBS, and resuspended in PBS containing 50 μg/ml propidium iodine, 0.1% sodium citrate, and 0.1% Triton X-100. Cells were stained overnight at 4°C and analyzed for DNA content within 24 h using a cell sorter (FACScan; Becton Dickinson). The cellular debris was gated out, and the proportion of cells with a subdiploid content of DNA, characteristic of apoptotic cells, was quantified using WinList 2.0 (Verity Software House, Inc.). All experiments were repeated at least three times in triplicate, and representative experiments are shown.

Clinical Studies. DNA was extracted from paraffin sections of tumor samples from a subset of patients entered into EST2284, an intergroup trial of adjuvant therapy for stage II and III colon cancer as described previously in detail (8). The c-myc amplification status for the subset of patients used in this study has been reported previously (8). We carried out single-strand conformational polymorphism analysis and sequence analysis of p53 mutational status at the hot spots for mutations in human colon cancer (exons 5–8) in a subset of 135 patients in EST2284, as described previously (8). Analysis of both c-myc and p53 status was carried out blinded from the clinical outcome below the c-myc band is the fold increase in c-myc relative to the parental level adjusted for GAPDH.

Fig. 1. c-myc mRNA levels in DLD1 and LoVo transfectants. Stable transfection of both LoVo and DLD1 cells with plasmid p290-myc(2,3) resulted in a constitutive up-regulation of c-myc mRNA at different levels in different selected clones. The number below the c-myc band is the fold increase in c-myc relative to the parental level adjusted for GAPDH.

Fig. 2. c-myc transactivation activity in LoVo and DLD1 transfectants. a, c-myc transfection resulted in increased c-myc transactivation activity only in LoVo transfectants. b, both LoVo and DLD1 cells transfected with antisense c-myc showed reduced c-myc transactivation. Transactivation activity was adjusted for both background levels and transfection efficiency of the reporter plasmids, as described in "Materials and Methods."
data. Analysis of the clinical data in relation to the genetic markers studied was done by the Eastern Cooperative Oncology Group Biostatistical Center. Survival curves for time-to-event data were estimated by the method of Kaplan and Meier, with differences assessed by the log-rank test. Proportional hazards regression models were used for multivariable comparisons of time-to-event end points. All computations were done in SAS. All \( P \) values are from two-sided tests of significance.

RESULTS AND DISCUSSION

Alterations in c-myc Levels Can Affect c-myc Transactivation Activity and Cell Growth. To investigate whether c-myc levels could be further elevated in cells with high c-myc due to defective Wnt signaling and whether this would affect cell phenotype, we introduced a c-myc expression vector into LoVo and DLD1 colon carcinoma cells, neither of which exhibit amplification of the c-myc gene \( (8) \). Moreover, LoVo cells have wild-type p53, whereas DLD1 cells have a nonfunctional mutated p53 protein \( (27) \). Clones from both cell lines that showed higher levels of c-myc expression were then chosen for further analysis (Fig. 1). For the p53 wild-type LoVo cells, the additional expression of c-myc in the transfectants led to increased c-myc transactivation activity \( (\text{Fig. 2a}) \) and faster growth \( (\text{Fig. 3a}) \). In contrast, for the p53 mutant DLD1 cells, the elevated c-myc expression after transfection did not alter either transactivation activity \( (\text{Fig. 2a}) \) or cell growth kinetics \( (\text{Fig. 3b}) \). To further confirm the role of c-myc in the growth of these cells, an antisense c-myc expression vector was introduced into both LoVo and DLD1 cells. This was efficient in reducing c-myc transactivation activity and growth in both parental cell lines \( (\text{Figs. 2b} \text{ and 3, a and b}) \).

\textbf{c-myc Is Necessary for the Apoptotic Response to Both Serum Starvation and 5FU Treatment.} The nuclear morphology of DAPI-stained cells was characteristic of cells undergoing apoptosis after either serum starvation or treatment with 5FU \( (\text{Fig. 4}) \). Quantification of the number of apoptotic cells in both LoVo and DLD1 cells grown under low serum \( (0.1\%) \) conditions showed an equivalent 7–10-fold increase in the number of apoptotic cells for both cell lines when compared with normal growth conditions \( (\text{Fig. 5}) \). This is consistent with the fact that both cell lines exhibit high levels of c-myc as a consequence of a mutant APC protein \( (28, 29) \) coupled with previous reports that c-myc overexpression sensitizes colon cancer cells and other cell types to apoptosis induced by serum starvation \( (9–12) \). Moreover, the elevated c-myc levels present in the parental cell lines resulted in a maximal response since further up-regulation of c-myc in the transfected clones did not lead to increased apoptosis in response to serum deprivation \( (\text{Fig. 5}) \). Finally, the role of c-myc in sensitizing cells to apoptosis induced by serum starvation was confirmed by the fact that c-myc...
antisense transfection, which reduced transactivation activity and growth (Figs. 2b and 3a and b), reduced the number of apoptotic cells in both LoVo and DLD1 cells (Fig. 5).

In contrast, the apoptotic response of LoVo and DLD1 cells to 5FU differed markedly from the response to low serum. First, wild-type p53 parental LoVo cells treated with 5FU exhibited a 5-fold higher level of apoptosis than did mutant p53 parental DLD1 cells treated similarly (Fig. 5). Second, the c-myc transfectant derivatives of LoVo cells, which have a wild-type p53 protein and showed increased c-myc transactivity and faster growth, are markedly sensitized to the apoptotic effects of 5FU compared with parental LoVo cells (Fig. 5). Moreover, for the DLD1 transfectants (mutant p53), which showed increased levels of c-myc mRNA but did not show increased transactivity or faster growth, there was no such sensitization to the effects of 5FU. Dependence of 5FU-induced apoptosis on c-myc expression was further demonstrated by the fact that c-myc antisense transfection, which was effective in reducing c-myc transactivity and growth (Figs. 2b and 3a), protected LoVo cells from the effects of 5FU (Fig. 5).

Apoptosis Induced by Serum Starvation is p53 Independent, but p53 Function Is Required for the c-myc-mediated Sensitization to 5FU. Both wild-type p53 LoVo cells and mutant p53 DLD1 cells underwent apoptosis under low serum conditions, suggesting that this process was p53 independent. We tested this directly by abrogating p53 function in wild-type p53 LoVo cells with the specific p53 inhibitor PFT-α (26). Inhibition of p53 function had no effect on apoptosis induced by serum starvation, confirming that this process is p53 independent (Fig. 6a). However, in the case of 5FU treatment, only the wild-type p53 LoVo cells overexpressing c-myc were sensitized to the effects of 3 μM 5FU, suggesting the necessity for p53 in this process. This too was tested with the p53 inhibitor PFT-α. For the LoVo-derived cell lines overexpressing c-myc above the parental level (LoVo-myc-2 and LoVo-myc-3), there is a PFT-α dose-dependent abrogation of the apoptotic response to low serum and 5FU difference in their requirement for p53 function. Inhibition of p53 function by PFT-α had no effect on the apoptotic response of LoVo cells to serum starvation. However, PFT-α treatment resulted in a dose-dependent abrogation of the enhanced apoptotic effects of 5FU treatment on c-myc-overexpressing LoVo cells.

Fig. 5. Quantification of the apoptotic response to serum starvation and 5FU treatment. a shows that c-myc transfection of LoVo cells (LoVo-myc-2 and LoVo-myc-3) resulted in a 3-4-fold sensitization to the effects of 5FU, whereas c-myc antisense transfection (LoVo-mycANS-9) protected LoVo cells against the effects of both serum deprivation and 5FU treatment. b. DLD1 transfection with sense c-myc (DLD1-myc-4, DLD1-myc-11, and DLD1-myc-1) had no effect on apoptosis induced by either serum starvation or 5FU treatment. Antisense transfection (DLD1-mycANS-7) led to a significant protection from the effects of serum removal.

Fig. 6. The apoptotic responses to low serum and 5FU differ in their requirement for p53 function. Inhibition of p53 function by PFT-α had no effect on the apoptotic response of LoVo cells to serum starvation. However, PFT-α treatment resulted in a dose-dependent abrogation of the enhanced apoptotic effects of 5FU treatment on c-myc-overexpressing LoVo cells.
effects of 5FU (Fig. 6b). Therefore, p53 function, which is not necessary for c-myc sensitization to low serum-induced apoptosis, is essential for the increased apoptosis induced by 5FU in cells overexpressing c-myc.

Patients with Optimal Response to 5FU-based Adjuvant Therapy in a Phase III Multi-Institutional Clinical Trial Had Tumors with Both an Amplified c-myc Gene and Wild-type p53. We have reported previously that long-term survival for patients given 5FU as adjuvant therapy was significantly increased from 40% for patients in the non-c-myc-amplified group to 70% in the c-myc-amplified group (8). Despite this marked difference, the above in vitro data predicted that further discrimination among patient subsets with different response to 5FU therapy could be achieved by investigating the interaction of both c-myc and p53 status with treatment. Therefore, we determined the mutation status of exons 5–8 of the p53 gene in these patients. In agreement with the predictions from the in vitro data, Fig. 7a illustrates that long-term (15-year) overall survival approached 90% for 5FU-treated patients with c-myc-amplified and p53 wild-type tumors versus only 40% for patients who did not receive adjuvant therapy (P = 0.05). Similar results were observed for disease-free survival (data not shown). In contrast, patients with amplified c-myc/p53 mutant (Fig. 7b), unamplified c-myc/wild-type p53 (Fig. 7c), or unamplified c-myc/mutant p53 tumors (Fig. 7c) did not benefit from the 5FU treatment (P = 0.23, 0.87, and 0.52, respectively), and only 40–50% of these patients survived to the 15-year follow-up of the study. These patients might be spared the toxicity and side effects associated with this treatment and are good candidates for alternative chemotherapeutic regimens, such as irinotecan and/or oxaliplatin, or other types of therapy, including gene therapy. In this regard, it has been suggested that the overexpression of c-myc in colon tumors may be a prime target for new therapeutic strategies (30). However, it is critical to note that the interactions of c-myc status with treatment response in vitro and in vivo clearly demonstrate that caution would have to be exercised in combining such novel strategies with standard 5FU-based therapy. An alternative approach would be to take advantage of the therapeutic opportunity afforded by c-myc amplification. In this respect, restoration of p53 function in the sensitization to 5FU was confirmed using antisense data, Fig. 7a illustrates that long-term survival probability of colon cancer patients.

In summary, our results show that high c-myc levels resulting from defective Wnt signaling sensitize colon carcinoma cells to low serum-induced apoptosis. Moreover, this is a p53-independent process, and further increasing c-myc levels and transactivation activity above the already high values in parental cells did not affect the apoptosis-promoting effects of serum starvation. However, in marked contrast, sensitization of the cells to 5FU required higher levels of c-myc achieved through the introduction of a c-myc expression vector as well as function of a wild-type p53 gene. Wild-type p53 was associated with the ability of the higher levels of c-myc to result in increased c-myc transactivation activity, and the role of both c-myc and p53 function in the sensitization to 5FU was confirmed using antisense c-myc and PFT-α, a specific p53 inhibitor, respectively. As predicted by the in vitro data, only patients whose primary colorectal tumor had an amplified c-myc gene and wild-type p53 showed benefit from the
standard 5FU-based adjuvant therapy. Analysis of the c-myc and p53 status accurately predicted the response to 5FU therapy, an observation with important clinical implications.

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