Influence of K-*ras* Activation on the Survival Responses of Caco-2 Cells to the Chemopreventive Agents Sulindac and Difluoromethylornithine[1]

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Abstract

The nonsteroidal anti-inflammatory drug sulindac and the ornithine decarboxylase inhibitor difluoromethylornithine (DFMO) are both potent inhibitors of colon carcinogenesis in experimental models of this disease. The combination of these two agents is undergoing evaluation as a strategy for colon cancer chemoprevention in humans with resected colon polyps. We evaluated the effects of the major sulfide and sulfone metabolites of sulindac and DFMO alone, or in combinations, on the growth and survival of Caco-2 colon cancer-derived cells and in clones of these cells transfected with an activated K-*ras* oncogene. Both the sulfide and sulfone metabolites of sulindac reduced cell viability, measured by colony-forming assays, primarily by inducing apoptosis. Expression of an activated K-*ras* oncogene caused cells treated with either sulindac sulfide or sulfone to undergo apoptosis earlier than nontransfected controls. However, clonogenic survival, measured 2 weeks after drug treatment, was the same in both Caco-2 and *ras*-transfected Caco-2 cells treated with sulindac metabolites. A 24-h treatment with DFMO caused a dose-dependent decrease in the colony-forming ability of cells expressing an activated K-*ras* but had no effect on the viability of the parental Caco-2 cells. The DFMO-dependent decrease in colony formation in K-*ras*-activated cells occurred in the absence of apoptosis. Assessment of cell survival by colony-forming assays indicated that these two agents acted in an additive manner when combined. These data indicate that K-*ras* can influence the kinetics of apoptosis induction by sulindac metabolites and cell survival in response to DFMO. However, cytotoxicity induced by these agents occurs via unique mechanisms. These studies suggest that the combination of DFMO and sulindac may be useful in human cancer prevention strategies.

Introduction

Mutational activation of the K-*ras* oncogene is an important genetic alteration in colorectal neoplasia. K-*ras* mutations have been detected in ~50% of sporadic human colorectal tumors (1, 2). K-*ras* mutations have been detected in aberrant crypt foci, as well as in adjacent regions of histologically normal mucosa (3). These findings suggest that the mutation of K-*ras* may be a relatively early event in the temporal development of colon cancer. K-*ras* is also mutated in chemically induced rodent tumors with a frequency similar to that of human cancers (4, 5). Although the role of K-*ras* in tumorigenesis is unclear, activation of this gene has been correlated with deficient apoptosis in human colorectal neoplasms (6).

The NSAIDs,[4] including piroxicam (7, 8), indomethacin (9), and sulindac (10, 11), effectively inhibit colon carcinogenesis in the AOM-treated rat model. NSAIDs also inhibit the development of tumors harboring an activated K-*ras* (12). Accumulating evidence suggests that NSAIDs inhibit carcinogenesis via the induction of apoptosis in tumor cells (10, 13–15). A number of studies suggest that the chemopreventive properties of the NSAIDs, including the induction of apoptosis, is a function of their ability to inhibit prostaglandin synthesis (14, 16, 17). Recent studies, however, indicate that NSAIDs may act through both prostaglandin-dependent and -independent mechanisms (18–21). Sulindac sulfone, a metabolite of the NSAID sulindac, lacks COX-inhibitory activity yet induces apoptosis in tumor cells (10, 15), possibly by a mechanism involving inhibition of a cyclic guanosine 3′,5′-monophosphate phosphodiesterase (22), and inhibits tumor development in several rodent models of carcinogenesis (10, 15, 20).

The polyamine biosynthesis inhibitor also decreases K-*ras* mutations and colon carcinogenesis in the AOM-treated rat. The combination of DFMO and the NSAID piroxicam has been shown to have a synergistic chemopreventive effect in the azoxymethane-treated rat model of colon carcinogenesis (7), although DFMO exerted a greater suppressive effect than piroxicam on K-*ras* mutation and tumorigenesis when each agent was administered separately (7, 23, 24). In one study, administration of DFMO or piroxicam to AOM-treated rats reduced the number of tumors harboring K-*ras* mutations from 90 to 36

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[4] The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; DFMO, difluoromethylornithine; AOM, azoxymethane; ODC, ornithine decarboxylase.

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and 25%, respectively (8). Both agents also reduced the amount of biochemically active p21 *ras* in existing tumors. (23).

The importance of K-*ras* activation in NSAID-mediated chemoprevention has not yet been determined. NSAIDs induce apoptosis in both colon tumor cell lines and animal tissues and appear to reduce K-*ras* tumor development; however, K-*ras* activation has not been investigated as a mechanism of NSAID-mediated cytotoxicity. It is also not known whether such cytotoxicity is dependent on the anti-inflammatory properties of the NSAIDs. The effect of polyamine depletion on NSAID-mediated chemoprevention has been studied only in piroxicam-treated rat models. The NSAID sulindac is metabolized to two different molecules that differ in their ability to inhibit COX, yet both are able to exert chemopreventive effects via the induction of apoptosis. Sulindac sulfone lacks COX-inhibitory activity and most likely facilitates the induction of apoptosis in a manner independent of blocking prostaglandin synthesis.

A number of clinical trials are under way that use a combination of DFMO and NSAIDs to potentiate chemoprevention. Although DFMO has been used successfully in combination with the NSAID piroxicam in animal models, the combination of DFMO and sulindac has not been investigated. Unlike piroxicam, sulindac is metabolized to two chemopreventive agents that most likely differ in their mechanism of action. Polyamines may play a role in the efficacy of one or both of these agents, because polyamines have been shown to mediate the induction of apoptosis in many rodent cell lines (25–28). Several studies have shown that the proto-oncogene c-*myc* is a transcriptional activator of ODC (29, 30) and that ODC is a mediator of c-*myc*-induced apoptosis in murine myeloid cells (31, 32). Polyamine depletion in c-*myc*-overexpressing murine myeloid cells prevented apoptosis upon growth factor withdrawal (31).

Materials and Methods

Cell Culture and Drug Treatments. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Caco-2 cells were maintained in MEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and a 1% penicillin (10,000 units/ml)-streptomycin (10,000 mg/ml) solution (Life Technologies, Inc.). Transfected Caco-2 cells resistant to neomycin were maintained in 400 μg/ml G418. Cells were maintained at 37°C in the presence of 5% CO_2 in air.

All cells were passaged into new medium every 2–3 days, just prior to confluence. Drug additions were performed at the time of cell seeding and in MEM for each cell line unless otherwise noted. DFMO was provided by Marion Merrel Dow Co. (Cincinnati, OH). The two metabolites of sulindac, sulindac sulfone and sulfide, were generous gifts from Dr. Gary Piazza (Cell Pathways, Inc., Horsham, PA).

Cell Number and Viability Determinations. Caco-2 cells were removed from the monolayer by treatment with trypsin (~1500 units/ml; Calbiochem, San Diego, CA)-EDTA (0.7 mM) and counted using a hemocytometer. A sample of the cell suspension was combined in a 1:1 volume ratio with trypan blue dye (Life Technologies, Inc.), and at least two independently prepared suspensions were counted on a hemocytometer, two counts each. For all cell types, viability was determined by the percentage of cells able to exclude the trypan blue dye.

Apoptosis Quantitation. Apoptosis was quantitated by morphological examination of cells on prepared slides. Each sample was prepared by pooling trypsinized cells with the aspirated culture medium and an additional saline wash of the tissue culture plate. Slides for light microscopy were prepared by placing 20,000 or 40,000 cells into a cytospin cup and pelleting the cells onto slides via centrifugation using a cytospin (Shandon Lipshaw, Pittsburgh, PA) at 600 rpm for 2 min. Cells were affixed to the slides by immersion of the slide into 100% methanol for at least 1 min. Slides were stained using a 1:10 (for Chinese hamster ovary cells) or a 1:20 (for Caco-2 cells) dilution of Modified Giemsa stain (Sigma Chemical Co., St. Louis, MO). Apoptotic cells were identified by characteristic chromatin condensation, cytoplasmic vacuole formation, cell shrinkage, and formation of apoptotic "bodies" (33). Apoptotic bodies that were not enclosed by membranes were not included in the cell scoring. At least 500 cells were scored for each treatment for each day, and frequencies were expressed as a percentage of the total cells counted.

Clonogenic Assays. Caco-2 cell survival was determined by colony-forming efficiency. Caco-2 cells or the K-*ras*-transfected clones were plated at a cell density of 2×10^5 cells per 60-mm dish in MEM, in the presence or absence of DFMO at varied concentrations. Cells were grown for 24 h, and then the DFMO/MEM media was removed. The plates were rinsed twice with saline, and MEM containing varied concentrations of sulindac sulfide or sulfone was added. Cells were grown for an additional 24 h and then replated at three serially diluted concentrations, with three plates seeded for each concentration. Approximately 21 days later, the plates were stained for colony formation.

Plates were removed from the incubator, and an equal volume of a 3:1 volume ratio of methanol and acetic acid was added to each plate for 5 min. The supernatant was aspirated, and the colonies were stained by the addition of ~1 ml of crystal violet stain (5 mg/ml in 100% ethanol; Sigma). The plates were then rinsed in distilled water and air dried. Colonies were defined as consisting of a minimum of 50 cells, with each plate containing 20 or more colonies for inclusion in the data set. Standard deviations were prepared from data in which all three plates contained a sufficient number of colonies to be included.

Establishment and Characterization of Clones. A cDNA encoding an activated K-*ras*^{Val12} was purchased from the American Type Culture Collection (Rockville, MD). This cDNA was ligated into the multiple cloning site of a pCDNA3 mammalian expression vector (Invitrogen Corp., Carlsbad, CA), and vectors containing the K-*ras* insert were isolated according to standard protocols (34). Large-scale amounts of plasmid (mg quantities) suitable for mammalian cell transfection were purified using the Nucleobond Plasmid kit (Clontech Laboratories, Inc., Palo Alto, CA).

The calcium phosphate transfection method was used to introduce the pCDNA3-K-*ras* plasmid into Caco-2 cells, according to established protocols (34).

One week after transfection, geneticin (Life Technologies, Inc. Germantown, MD) was added to the media at a concentration of 400 μg/ml. Stable clones were isolated with the use of trypsin-soaked filter discs placed over isolated colonies on a tissue culture plate and then transferred to the wells of a 48-well plate. Clones were maintained in 400 μg/ml of geneticin and screened via a RFLP method to confirm presence of the mutant K-*ras* (4) and by Western blot for K-*ras* expression.

Preparation of Whole-Cell Lysates of Caco-2 Cells. Whole-cell lysates of Caco-2 cells were prepared according to commercially available protocols (Santa Cruz Biotechnology, Santa Cruz, CA). Plates were kept on ice, and cells were scraped off the tissue culture plates in the presence of RIPA buffer (PBS,

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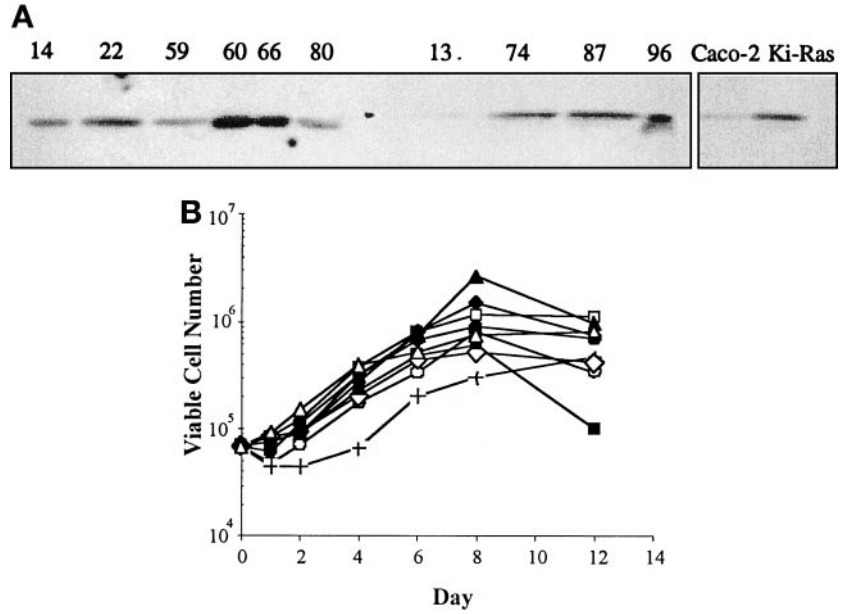

Fig. 1. K-*ras* transfection of Caco-2 human colon adenocarcinoma cells. Caco-2 cells were transfected with a plasmid directing the overexpression of an activated K-*ras* gene and subject to drug resistance selection and clone isolation as described in "Materials and Methods." *A*, detection of K-*ras* expression in various transfected clones by Western blot, using an antibody that detects both normal and activated $p21^{K\text{-}ras}$. *Numbered lanes*, clonal isolates of transfected cells; *Caco-2*, untransfected parental cells; *Ki-ras*, pooled clones. *B*, growth of parental (○) and transfected clone numbers 13 (+), 14(◇), 22 (●), 59 (■), 60 (▲), 66 (◆), and 96 (△). Cells were harvested at the indicated times and analyzed for viable cell number as determined by dye exclusion.

1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 30 μg/ml aprotinin, and 100 mM sodium orthovanadate). The cell suspension was then passed twice through a 22-gauge needle and was centrifuged at 10,000 × *g* for 20 min to clarify the lysate.

Western Blot. Characterization of K-*ras* expression in transfected Caco-2 clones was performed by Western blot, using a mouse monoclonal antibody directed against both normal and activated K-*ras* (Santa Cruz Biotechnology). Samples were electrophoresed onto a 12.5% acrylamide gel, and the proteins were then transferred onto a Hybond nitrocellulose membrane (Amersham Life Science, Inc., Arlington Heights, IL). Samples were run at 50 V for 16 h in a transfer apparatus (Bio-Rad Laboratories, Hercules, CA) in transblot buffer (30% methanol, 50 mM Tris, and 191 mM glycine). The nitrocellulose membrane was blocked for 1 h in Blotto (TBS, 5% nonfat dry milk, and 0.05% Tween 20; Sigma). A mouse monoclonal IgG directed against K-*ras* (Santa Cruz Biotechnology) was diluted 1:100 in Blotto and applied to the membrane for 2 h. Membranes were washed in Tween-TBS for three washes of 5 min each, and then a horse radish peroxidase-conjugated goat antimouse IgG secondary antibody was diluted in Blotto and applied to the membrane. The membrane was incubated in secondary antibody for 1 h, and then the membrane was washed again in Tween-TBS. Visualization was made with the ECL Detection system (Amersham Life Science, Inc., Arlington Heights, IL).

Protein Gel Electrophoresis. SDS-PAGE was performed according to the method of Laemmli (35), except that bisacrylamide was replaced with *N,N′*-diallyltartardiamide (Bio-Rad Laboratories). Samples were diluted in electrophoresis buffer (2% SDS, 5% β-mercaptoethanol, and 3% sucrose in 300 mM Tris-HCl, pH 7.0) and heated at 100°C for 5 min before loading on a 12.5% acrylamide gel.

Protein Quantitation. Protein measurements of the lysates used for Western blotting were performed with the DC protein assay kit (Bio-Rad Laboratories), using the modified method suitable for use with detergents. Protein concentrations for both assay types were estimated by comparing the absorbance of unknown samples to that of known BSA standard concentrations in the same assay.

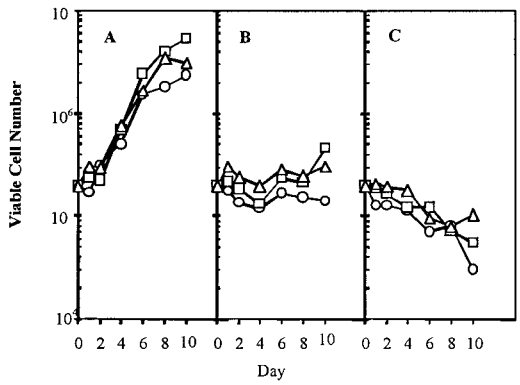

Fig. 2. Effect of sulindac sulfide and sulfone on the growth of K-*ras*-transfected cells. Caco-2 parental cells (○), K-*ras* clone 60 (□), and K-*ras* clone 66 (△) were seeded in the presence of vehicle (*A*), 120 μM sulindac sulfide (*B*), or 600 μM sulindac sulfone (*C*) and harvested at 2-day intervals. The viable cell number was determined by trypan blue dye exclusion.

Statistical Method. Analysis of covariance model was used in the study, where the dose was the covariant variable. The data were normalized by dividing each value by the mean value of colony-forming efficiency at 0 dose level.

Results

Isolation and Characterization of Caco-2-transfected Cells. Caco-2 human colon adenocarcinoma cells were used to determine the effects of sulindac and DFMO on cells containing an activated K-*ras*. Caco-2 cells are one of the few colon tumor lines that contain a normal K-*ras* gene (36, 37). These cells possess many of the characteristics of mature enterocytes, including spontaneous differentiation and hydrolase secretion (38).

Caco-2 cells were stably transfected with a plasmid directing the overexpression of an activated K-*ras*, and individual clones were subsequently isolated. Ten clonal populations were characterized by Western blot using an antibody that reacts with

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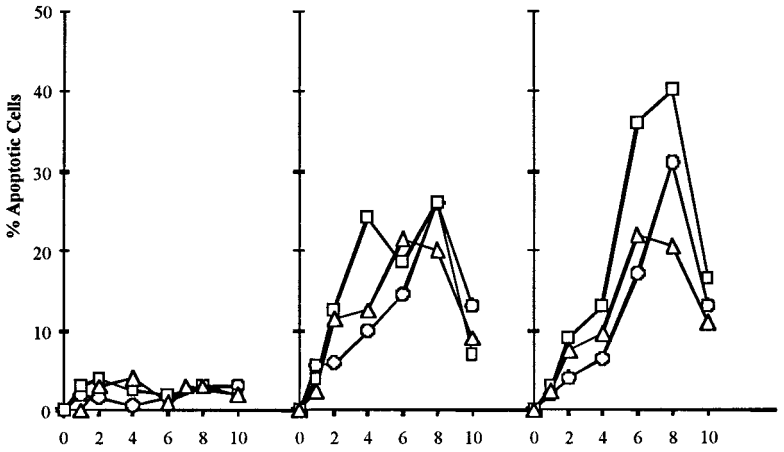

Fig. 3. Induction of apoptosis by sulindac sulfide and sulfone in K-*ras*-transfected cells. Caco-2 parental cells (○), K-*ras* clone 60 (□), and K-*ras* clone 66 (△) were seeded in the presence of vehicle (*A*), 120 μM sulindac sulfide (*B*), or 600 μM sulindac sulfone (*C*) and harvested at 2-day intervals. Apoptosis was determined by light microscopy.

both normal and mutated K-ras proteins (Fig. 1). K-ras protein was detectable in the parental cells and was present in increased amounts in most of the transfectants. One clone, 13, had levels of K-*ras* below that of the parental Caco-2 cells, whereas clones 14, 22, 59, 80, 74, and 87 were intermediate expressors, and contained levels of K-*ras* similar to the uncloned K-*ras*-transfected population. Clones 60, 66, and 96 showed the highest level of expression. Clones 60 and 66 were used for further study.

Effect of Sulindac Sulfone and Sulfide on Growth of Cells Expressing an Activated K-*ras*. Growth of parental Caco-2 cells as well as clones 60 and 66 were measured in the presence of vehicle, 120 μM sulindac sulfide, or 600 μM sulindac sulfone (Fig. 2). In the presence of vehicle only, the expression of activated K-*ras* did not confer a growth advantage to either transfected cell line. The number of viable cells in all sulfide-treated cultures remained relatively unchanged throughout the 10-day growth period, whereas treatment of cultures with the sulfone derivative resulted in a pronounced cell loss beginning 6 days after treatment. K-*ras* activation did not confer a selective toxicity in the presence of either sulindac metabolite.

Effect of K-*ras* on Apoptosis Induced by Sulindac Sulfide or Sulfone. Sulindac has been shown to cause apoptosis in the AOM-treated rodent model. In this model, sulindac also reduces the number of tumors harboring an activated K-*ras*, which suggests that apoptosis may occur by a K-*ras*-dependent mechanism. To test this hypothesis, Caco-2 parental cells and clones 60 and 66 were treated with vehicle, 120 μM sulindac sulfide, or 600 μM sulindac sulfone, and apoptosis of each culture was quantitated by light microscopy (Fig. 3). The percentage of cells undergoing apoptosis in response to vehicle alone were minimal, with a peak apoptosis index of 4%. Caco-2 parental cells exposed to sulfide displayed a gradual increase in apoptosis that peaked at 8 days in culture. Clone 60 cells, and to a lesser extent, clone 66, displayed an earlier onset of apoptosis. Clone 60 reached peak apoptosis levels at day 2 compared with day 8 of the parental cells, whereas clone 6 reached a peak level of apoptosis by day 6. In both the Caco-2 parental and clone 60 cells, induction of apoptosis by sulindac sulfone was more pronounced than with the sulfide. Upon exposure to the sulfone, clone 60 cells reached a peak level of apoptosis by day 4, compared with day 8 in the parental cells. K-*ras* appears to accelerate the onset of apoptosis in response to both sulindac sulfone and sulfide.

Effect of K-*ras* Activation on the Survival of Cells Exposed to Sulindac Sulfide or Sulfone. In an effort to better characterize the apoptosis response to the sulindac metabolites in cells with an activated K-*ras*, the colony-forming efficiencies of Caco-2 and clone 60 cells were measured after exposure to increasing concentrations of sulindac sulfide or sulfone (Fig. 4). Cells were treated for 24 h and then replated at diluted concentrations in fresh media. By this method, a 50% decrease in cell number occurred at approximately 225 μM sulindac sulfide and 800 μM sulindac sulfone. The expression of an activated K-*ras* did not affect the final measure of viability caused by either sulindac metabolite, because the transfected cells displayed the same toxicity as the parental cells. Activation of K-*ras* was unable to confer a selective cytotoxicity in the presence of either sulindac metabolite.

Effect of DFMO on the Colony-forming Efficiency of Sulindac Sulfone- and Sulfide-treated Caco-2 Cells. Caco-2 cells and K-*ras* transfectants exhibit a growth cessation in response to polyamine depletion, which is not accompanied by a loss of viability in short-term experiments. Polyamine depletion in these experiments appears to have no effect on sulindac-induced cytotoxicity. To further characterize the effects of polyamine depletion, both alone and in combination with sulindac, colony-forming efficiencies of Caco-2 and clone 60 cells were analyzed after exposure to 5 mM DFMO and increasing concentrations of sulindac sulfide or sulfone (Fig. 5). Exposure of the K-*ras*-transfected cells to 5 mM DFMO had a much more pronounced effect on cell survival than that exhibited by the parental Caco-2 cells. Treatment of the transfected cells with 5 mM DFMO alone resulted in a 60% decrease in cell survival compared with untreated transfectants, whereas in the parental cells, DFMO treatment alone had little effect. For both Caco-2 and clone 60 cell lines, the combination of DFMO and increasing concentrations of either sulindac metabolite resulted in an even further decrease in cell survival, which suggests that DFMO did not inhibit sulindac-induced cytotoxicity.

When the survival of DFMO-treated clone 60 or parental Caco-2 cells was normalized to like DFMO-treated controls, the survival rates of the DFMO-treated cells mirrored those of the non-DFMO-treated cells in response to increasing concentrations of sulindac sulfide (Fig. 6). The interaction of DFMO with either sulindac sulfide or sulindac sulfone was evaluated using the analysis of covariance model, where the dose was the covariant variable. This analysis was unable to detect any

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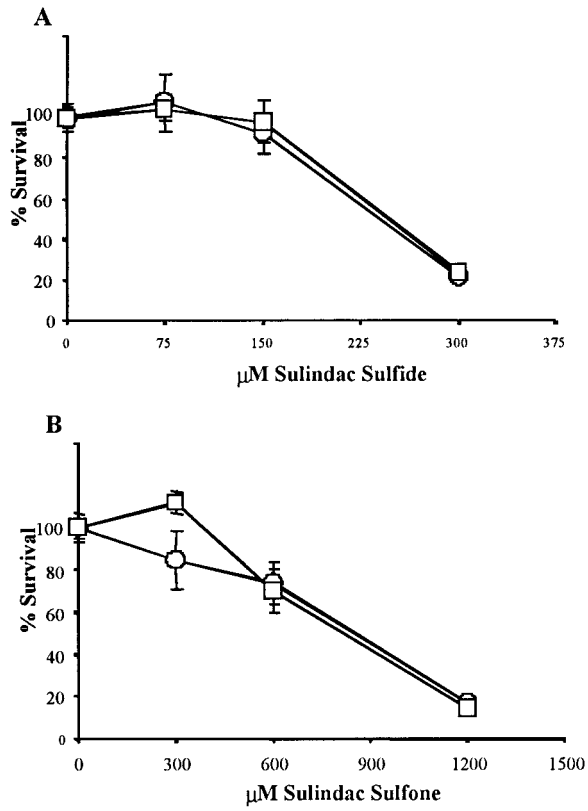

Fig. 4. Survival of Caco-2 parental or K-*ras* clone 60 cells in sulfide or sulfone. Caco-2 parental cells (○) and K-*ras* clone 60 (□) were treated for 24 h with increasing concentrations of sulindac sulfide (0, 75, 150, and 300 μM; *a*) or sulindac sulfone (0, 300, 600, and 1200 μM; *b*). Cells were replated at diluted concentrations in the absence of drug and grown for 21 days. Plates were stained with crystal violet, and the number of colonies on each plate was determined. Plating efficiency was determined by dividing the number of colonies by the total number of cells plated. These numbers were then normalized to the plating efficiency of non-drug-treated controls of the same line. Data points without error bars contain a SD too small to be represented; *bars*, SD.

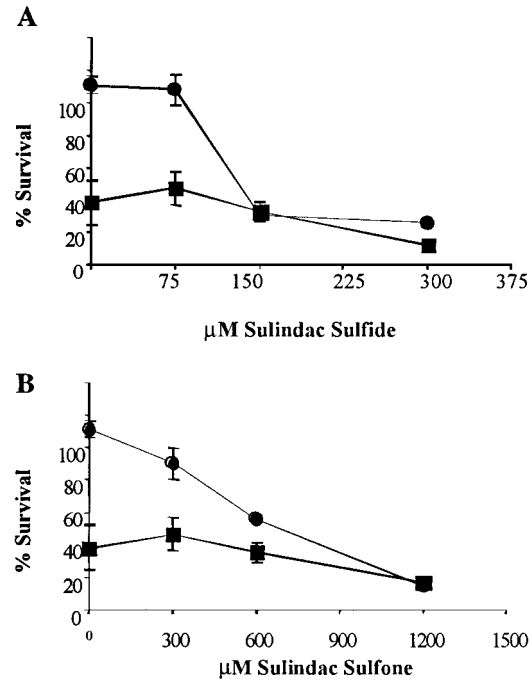

Fig. 5. Effect of DFMO on survival of Caco-2 parental or K-*ras* clone 60 cells in sulindac sulfide or sulfone. Caco-2 parental (●) or K-*ras* clone 60 (■) cells were treated for 24 h with 5 mM DFMO and then treated for an additional 24 h with increasing concentrations of sulindac sulfide (0, 75, 150, and 300 μM; *a*) or sulindac sulfone (0, 300, 600, and 1200 μM; *b*) in the absence of DFMO. Cells were replated at diluted concentrations and grown for 21 days. Plates were stained with crystal violet, and the number of colonies on each plate was determined. Plating efficiency was determined by dividing the number of colonies by the total number of cells plated. These numbers were then normalized to the plating efficiency of non-drug-treated controls of the same line. Data points without error bars contain a SD too small to be represented; *bars*, SD.

interaction between either DFMO and sulindac sulfide or DFMO and sulindac sulfone (Table 1).

Effect of Increasing Concentrations of DFMO on Survival of Cells with an Activated K-*ras*. The 60% decrease in survival of clone 60 cells treated with 5 mM DFMO suggests that polyamine depletion was selectively toxic to cells expressing an activated K-*ras*. To further examine this response, Caco-2 cells and clones 60 and 66 were treated with increasing concentrations of DFMO for 24 h and then replated for colony formation (Fig. 7). Caco-2 cells were refractory to DFMO treatment and maintained nearly 100% survival at concentrations up to 5 mM DFMO. In contrast, both clones 60 and 66 exhibited increasing toxicity with increasing concentrations of DFMO, with 50% cell survival occurring at only 0.5 mM DFMO. At 5 mM DFMO, cell survival for both transfectants was decreased to 20–40% of controls. In these experiments, polyamine depletion was selectively toxic to cells with an activated K-*ras* at concentrations as low as 50 μM. Cultures treated with DFMO for varying times and concentrations were evaluated for induction of apoptosis, using assessment of morphological criteria as described in "Materials and Methods." We found no evidence for induction of apoptosis in any DFMO-treated cultures. Thus, although DFMO killed cells expressing the activated *ras* gene, this process involved an apoptosis-independent mechanism.

Discussion

In the present study, the polyamine synthesis inhibitor DFMO was selectively toxic to cells containing an activated K-*ras*. K-*ras* activation did not affect the quantitative level of cytotoxicity, as measured by colony formation, exerted by either metabolite of the NSAID sulindac. However, the rate of killing, as measured by apoptosis induction, induced by the sulindac metabolites was increased by K-*ras* activation. These results suggest that the cytotoxic effects of DFMO and the NSAIDs occur through unique K-*ras*-dependent mechanisms.

The expression of an activated K-*ras* did not appear to change the growth rate of transfected Caco-2 cells, even in clones that expressed high levels of the protein. Thus, the role of activated K-*ras* expression is cell specific. Activated K-*ras* expression is associated with an increase in growth rates in DLD-1 and HCT116 human colon cell lines (39). Others have also failed to observe an effect of activated H-*ras* expression on growth of Caco-2 cells (40). However, both de Vries *et al.* (40) and we found that *ras* activation increases tumorigenicity of Caco-2 cells. Just as *ras* plays pivotal roles in proliferation, *ras* also participates in several mechanisms leading to apoptosis. Activated K-*ras* increases the rate of ceramide-induced apoptosis in colon cancer cells (41). Our findings indicating en-

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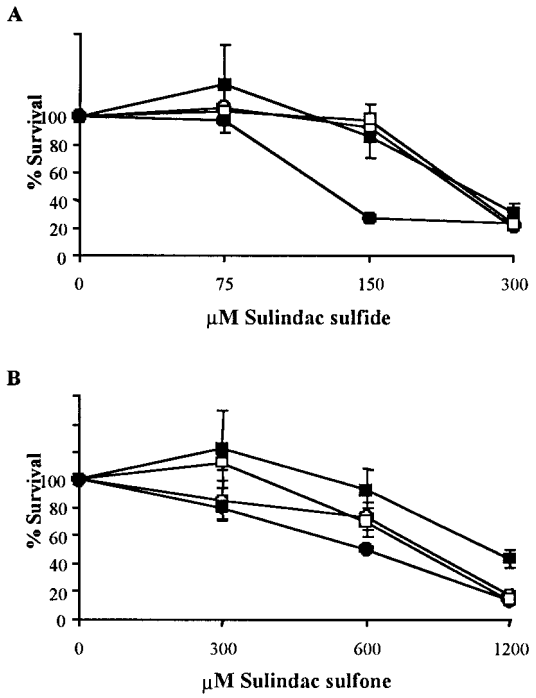

Fig. 6. Cell survival responses as shown in Fig. 5 but normalized to exclude effects attributable to DFMO alone. Symbols and error bars are as described in the legend to Fig. 5. Survival values resulting from combined treatments with DFMO and either sulindac sulfide or sulindac sulfone were normalized to cultures treated with DFMO alone for equivalent times and concentrations to detect possible interactions between these agents.

Table 1 Statistical significance of interaction between DFMO and sulindac metabolites

	Interaction between DFMO and dose effect of sulindac sulfide	Interaction between DFMO and dose effect of sulindac sulfone
Caco-2 parental	0.1269	0.5257
Caco-2 #60	0.6595	0.5999

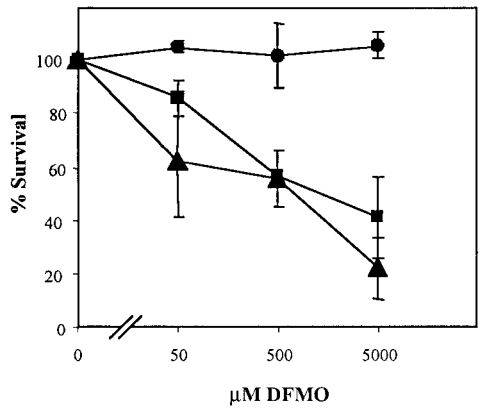

Fig. 7. Effect of DFMO on survival of Caco-2 and K-*ras*-transfected cells. Caco-2 (●), K-*ras* 60 (■), or K-*ras* 66 (▲) cells were treated with 0, 50, 500, and 5000 μM DFMO for 24 h prior to subsequent dilution and reseeding in drug-free media. Cells were grown for ~21 days and then analyzed for colony-forming efficiency. Plating efficiency was determined by dividing the number of colonies by the total number of cells plated. These values were then normalized to the plating efficiency of untreated control cells of like cell type. Data points without error bars contain a SD too small to be represented; *bars*, SD.

hanced rates of killing induced by sulindac derivatives may be related, because NSAIDs may induce apoptosis in part by a ceramide-dependent mechanism (42). The role of *ras* in regulating proliferation and apoptosis have been reviewed recently (43, 44).

A number of studies suggest that NSAID-mediated chemoprevention occurs through the induction of apoptosis (13, 15, 45, 46). However, the role of K-*ras* activation in this process has not been investigated extensively. One group has reported that K-*ras* activation in rat enterocytes confers resistance to apoptosis induced by sulindac sulfide, although not with sulfone (47). In the present study, we found that treatment of Caco-2 cells with sulindac sulfide and sulfone resulted in a K-*ras*-dependent induction of apoptosis. Apoptosis induction by both sulfide and sulfone occurred several days earlier in K-*ras*-transfected cells than in parental cells, although maximal levels of apoptosis in both transfectants and parental cells were similar. Our results are consistent with those of other groups who showed that *ras* activation induces the expression of the antiapoptotic protein COX-2 (48). Similarly, we found that K-*ras* activation in Caco-2 cells up-regulates COX-2 (49). The difference between our results and those of Arber *et al.* (47) is unknown but may relate to different patterns of gene expression induced by K-*ras* in rat enterocytes compared with human cells.

The K-*ras*-dependent acceleration of apoptosis seen with sulindac treatment appeared to be inconsequential with respect to overall cytotoxicity. Survival studies with increasing concentrations of sulindac sulfide or sulfone did not reveal any differences in colony-forming efficiency between K-*ras* transfectants and parental Caco-2 cells, although cytotoxicity in both cell types was correlated with increased drug concentrations. These data suggest that although K-*ras* activation may increase the rate at which cells die from apoptosis, the final level of cell survival influenced by sulindac metabolites is unaffected by an activating mutation in K-*ras*.

Treatment of Caco-2 or cells expressing either a normal or mutant K-*ras* with DFMO and either sulindac sulfide or sulfone resulted in additive effects on cell survival. Our results corroborate previous studies in animal models with DFMO and the NSAID piroxicam (7). In the present study, treatment of Caco-2 cells with DFMO did not increase the toxicity of the sulindac metabolites in long-term survival studies. Thus, DFMO and sulindac appear to use unique mechanisms to suppress cell viability. This possibility is further supported by the selective cytotoxicity of DFMO seen in cells with an activated K-*ras*. Treatment of both K-*ras* clones 60 and 66 with increasing concentrations of DFMO led to a dose-dependent decrease in colony-forming efficiency, whereas survival of parental cells was unaffected. Twenty-four h of treatment with 5 mM DFMO was sufficient to reduce colony-forming efficiency of the K-*ras* transfectants by 40%, although cells were subsequently incubated in normal media for an additional 24 h prior to replating. Concentrations as low as 50 μM DFMO were also moderately cytotoxic. In short-term growth experiments, where cells were exposed to 5 mM DFMO continuously over a 6-day time course, exclusion of trypan blue dye in either the K-*ras* cells or parentals was unaffected, and we failed to observe any apoptotic cells in DFMO-treated cultures (data not shown). The cells appeared to be metabolically active during this time period, yet the

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colony-forming efficiency data indicate they were unable to proliferate. The cytotoxicity of DFMO on cells with an activated K-*ras* is consistent with other studies showing potent chemopreventive effects of DFMO on AOM-induced tumorigenesis as well as the development of tumors with an activated K-*ras* (8, 24).

The mechanism of K-*ras*-dependent toxicity of DFMO is not yet established. In results to be presented elsewhere, we show that K-*ras* activation suppresses the expression of the spermidine spermine *N′*-acetyltransferase, the first enzyme in polyamine catabolism.[5] Treatment of cells expressing an activated K-*ras* with DFMO are thus unable to generate new polyamines either from synthesis or catabolism. This deficit may be sufficient to kill these cells.

Administration of both piroxicam and DFMO in the AOM-rat model decreased both the number of existing tumors as well as the number of tumors with an activated K-*ras* (7). The mechanism by which DFMO prevents K-*ras*-dependent tumorigenesis in the AOM-rat model is unknown. It has been proposed that NSAIDs and DFMO act through a common pathway involving the inhibition of prostaglandin synthesis (7), because ODC activity can be inhibited by agents that inhibit COXs (7, 50). The induction of apoptosis may occur through the inhibition of prostaglandin synthesis, or in the case of sulindac sulfone, a prostaglandin-independent mechanism that is also independent of K-*ras* activation. The activation of K-*ras* may lead to the down-regulation of genes that are not necessary for growth but are necessary for cell survival, such as spermidine spermine *N′*-acetyltransferase. This down-regulation may not cause toxicity unless the cell undergoes the additional stress of polyamine depletion, in which case the genes normally expressed during conditions of stress are not available to protect the cell from a cytotoxic response.

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Influence of K-*ras* Activation on the Survival Responses of Caco-2 Cells to the Chemopreventive Agents Sulindac and Difluoromethylornithine

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