Inhibition of Extracellular-signal Regulated Kinases 1/2 Is Required for Apoptosis of Human Colon Cancer Cells In vitro by Sulindac Metabolites

Pamela L. Rice,1,2,3 K. Scott Beard,1 Linda J. Driggers,1 and Dennis J. Ahnen1,2,3

1Veterans Administration Medical Center, 2Department of Medicine, University of Colorado Health Sciences Center, and 3University of Colorado Comprehensive Cancer Center, Denver, Colorado

Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) including sulindac have shown potent chemopreventive and tumor regressive effects against colorectal cancer, the second leading cause of cancer death in the United States. However, the mechanisms by which sulindac inhibits tumor cell growth are not completely understood. We previously reported that sulindac metabolites inhibit the mitogen-activated protein/extracellular signal-regulated kinase extracellular signal-regulated kinase signaling cascade in colorectal cancer cells lines at doses that induce apoptosis, and inhibition of MEK/ERK activity with U0126 is sufficient to induce apoptotic cell death. To determine whether inhibition of MEK/ERK activity is necessary for sulindac-induced apoptosis of human colon cancer cells, stable transfectants were created that express an activated MEK1 gene in HT29 cells. HT29-MEK1(R4F) clones displayed a 10- to 20-fold increase in MEK1 activity compared with control HT29-pCEP4 clones. When compared with control HT29-pCEP4 clones, HT29-MEK1(R4F) clones were resistant to both apoptosis and inhibition of ERK1/2 phosphorylation induced by sulindac metabolites. These results suggest that inhibition of MEK/ERK signaling is necessary for the induction of apoptosis by sulindac metabolites.

Introduction

Colorectal cancer is the second leading cause of cancer death in the United States (1). Regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) including sulindac and aspirin is associated with a reduced incidence of colon adenomas and adenocarcinomas in humans as well as several animal models of colorectal cancer (2). NSAIDs are classically defined by their ability to catalytically inhibit the cyclooxygenase (COX) enzymes, COX-1 and COX-2, and COX metabolites of sulindac inhibiting colon tumor cell proliferation in vitro and in vivo; for example, the sulfone metabolite of sulindac inhibits colon tumor cell proliferation in vitro and in vivo (3), although it does not inhibit COX activity. Several COX-independent biochemical targets have been described for sulindac, including extracellular-signal-regulated kinases 1/2 (ERK1/2), cyclic guanosine 3′,5′-monophosphate–dependent phosphodiesterases 2/5 (PDE2/5; ref. 4), c-Jun NH2-terminal kinase-1 (JNK1; ref. 5), nuclear factor κB (NFκB) (6), and 15-lipoxygenase-1 (7). Induction of apoptotic cell death is thought to be a major mechanism of the growth inhibitory effects of sulindac on colon cancer cells. Prior work from our laborator identified ERK1/2 inhibition as an apoptotic mechanism of sulindac metabolites (8, 9). Sulindac metabolites inhibit ERK1/2 phosphorylation and activity at times and doses consistent with apoptosis, and inhibition of ERK1/2 with the selective MEK1/2 inhibitor, U0126, is sufficient to induce apoptosis of human colorectal cancer cells (8). In this article, we extend these initial observations to show that stable expression of a gene encoding a constitutively active form of MEK1 blocks both ERK1/2 inhibition and apoptosis induced by sulindac metabolites in HT29 cells. These results indicate that inhibition of ERK1/2 signaling is required for sulindac metabolite-induced apoptotic cell death in cultured human colon cancer cells in vitro.

Materials and Methods

Transfection. HT29 cells were stably transfected with a modified pCEP4 (Invitrogen, Carlsbad, CA) plasmid containing the constitutively active, hemagglutinin-tagged MEK1(R4F) vector under control of the cytomegalovirus promoter (kindly provided by Dr. Natalie Ahn, University of Colorado at Boulder, Boulder, CO) with LipofectAMINE (Life Technologies, Inc., Carlsbad, CA), according to the manufacturer’s instructions. The MEK1(R4F) construct was generated by site-directed mutagenesis of S218 and S222 residues to glutamate and aspartate, respectively, in addition to an amino-terminal deletion of amino acid residues 32 to 51 (10). Together these mutations result in enhanced MEK1 activity up to 410 times that of the wild-type enzyme (10). Stable empty vector control clones were transfected with the empty pCEP4 plasmid (Invitrogen). Individual transfected clones were selected by resistance to 600 μg/mL hygromycin, a concentration that caused 100% cell death of nontransfected HT29 cells within 2 weeks.

Cell Culture. Cells were maintained as described previously (9). All of the experiments were done in the presence of 600 μg/mL hygromycin to maintain selection of the stable clones, and expression of the MEK1(R4F) protein was confirmed in each experiment by Western blotting for hemagglutinin. For epidermal growth factor (EGF) experiments, cells were switched to serum-free medium 24 hours before EGF treatment.

Cell Lysates and Western Blotting. At time of harvest, cell lysates were prepared and Western blotting done as described previously (8), with primary antibodies raised against hemagglutinin (Covance, Berkeley, CA), Tyr 204 phosphorylated ERK1/2, total ERK1/2, (Santa Cruz Biotechnology, Santa Cruz, CA), and cleaved caspase-3 (Cell Signaling Technology, Beverly, MA). MEK1 In Vitro Kinase Assay. MEK1 was immunoprecipitated from 200 μg of total protein from each cell lysate with an agarose-conjugated anti-MEK1 primary antibody (Santa Cruz Biotechnology). Immunoprecipitates were collected by centrifugation, washed, and incubated with kinase inactive ERK2-glutathione S-transferase fusion protein and ATP in kinase buffer (Cell Signaling Technology). MEK1 kinase activity was determined after separating samples by SDS-PAGE and probing with phospho-specific ERK1/2 antibody.

Apoptosis Detection. Apoptosis was determined by examining nuclear morphology after staining cells with acridine orange and ethidium bromide as described previously (8). Results obtained by morphology were confirmed with Western blotting for cleaved caspase-3 as described above.

Statistical Analysis. Graphs represent means and SDs for three independently treated samples. Data were analyzed by the Student’s t test for paired samples, and statistical significance was accepted at P < 0.05.

Received 4/30/04; revised 6/21/04; accepted 7/1/2004.

Grant support: The Cancer Research Foundation of America and Veterans Affairs Merit Review Programs.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Pamela L. Rice, Department of Medicine, A009-151, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262. Phone: (303) 399-8020, extension 3655; Fax: (303) 393-5145; E-mail: pamela.rice@uchsc.edu.

©2004 American Association for Cancer Research.

8148

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2004 American Association for Cancer Research.
Results

Stable Expression of MEK1(R4F) Constitutively Activates MEK1 Activity in HT29 Colon Cancer Cells. Stable clones of HT29 that express MEK1(R4F) were originally identified by Western blotting for expression of hemagglutinin-tagged MEK1(R4F). Four hemagglutinin-expressing clones were identified and additionally characterized by a MEK1 in vitro kinase assay. The four HT29-MEK1(R4F) clones (clones-2, -7, -11, and -15) displayed 10- to 20-fold increased MEK1 kinase activity compared with three different vector control HT29-pCEP4 clones (-1, -11, and -12; Fig. 1A). Similar results were obtained with an in vitro kinase assay for hemagglutinin (data not shown), indicating that the increased MEK1 activity in the HT29-MEK1(R4F) clones was caused by expression of hemagglutinin-tagged MEK1(R4F).

Exogenous EGF normally activates MEK1/2 and ERK1/2 in the parental HT29 cell line (9). Treatment of control HT29-pCEP4 clones with 10 ng/mL EGF increased MEK1 activity 7- to 22-fold, as measured by in vitro kinase activity against ERK2 substrate (Fig. 1B). In contrast, EGF treatment did not increase the activity of MEK1 in HT29-MEK1(R4F) clones, indicating that MEK1 was constitutively activated.

Expression of MEK1(R4F) Blocks Apoptosis by Sulindac Metabolites. Control HT29-pCEP4 and HT29-MEK1(R4F) clones were grown to confluency then treated with sulindac sulfide or sulindac sulfone, and apoptotic cell death was measured 3 days after drug treatment. Both parental HT29 cells (data not shown) and HT29-pCEP4 vector control cells displayed a dose-dependent increase in apoptosis after treatment with either sulindac sulfone (Fig. 2A) or sulindac sulfide (Fig. 3A). In contrast, HT29-MEK1(R4F) clones failed to undergo apoptosis at the same concentrations of sulindac metabolites. Similar results were obtained in duplicate experiments with each of the four HT29-MEK1(R4F) clones and three individual HT29-pCEP4 control clones, indicating that the resistance of HT29-MEK1(R4F) clones to sulindac was not because of random insertion of the MEK1(R4F) genetic construct.

Expression of MEK1(R4F) Blocks Inhibition of ERK1/2 Phosphorylation by Sulindac Metabolites. We examined cell lysates from both control HT29-pCEP4 and constitutively active HT29-MEK1(R4F) clones treated with concentrations of sulindac metabolites that induced apoptosis in parental HT29 and vector control HT29-pCEP4 clones. Apoptotic concentrations of sulindac sulfone (Fig. 2B) and sulindac sulfide (Fig. 3B) inhibited the phosphorylation, but not total protein expression, of ERK1/2 in control HT29-pCEP4 and three different control pCEP4 clones. Numbers beneath blots represent densitometric analysis of bands, with vehicle-treated control samples being set at 1.0, and are an average of duplicate experiments of the same clone. Bars, ±SD. ■, pCEP; □, R4F.

Discussion

We have previously shown that sulindac metabolites inhibit both basal and EGF-induced MEK1/2 and ERK1/2 phosphorylation in human colon cancer cells at times and doses at which these drugs
induce apoptosis (8, 9). In addition, treatment with the selective MEK1/2 inhibitor, U0126, induced apoptosis at doses that inhibited ERK1/2 phosphorylation for 24 to 72 hours, indicating that down-regulation of MEK/ERK signaling is sufficient to induce apoptotic cell death. Furthermore, U0126 potentiated apoptosis induced by sulindac metabolites (8). In the current article, these observations are extended to show that expression of the constitutively active MEK1(R4F) gene increases MEK1 enzymatic activity in HT29 colon cancer cells. Expression of MEK1(R4F) in these cells blocks the ability of sulindac metabolites to induce apoptosis and inhibit ERK1/2 phosphorylation. Inhibition of ERK1/2 therefore seems both necessary and sufficient for the ability of sulindac to induce apoptosis in HT29 cells.

Although we have consistently seen down-regulation of ERK1/2 by a variety of nonselective NSAIDs (sulindac sulfide, indomethacin, and resveratrol) and related compounds in several different colon cancer cell lines, including HT29, HCT116, SW480, and HCT15, not all of the investigators have found down-regulation of ERK1/2 by all of the NSAIDs. Elder et al. (11) reported that the selective COX-2 inhibitor NS-398 induced a sustained increase in ERK1/2 activity in HT29 cells, and induction of ERK1/2 by NSAIDs has been reported in colon (12) and pancreatic cancer cell lines (13). The reasons for these differing results is not clear but could be because of structural and functional differences between sulindac and other NSAIDs, the doses used, or the exact cell culture conditions and time points. It is clear, however, that the ability of some NSAIDs and related compounds to down-regulate ERK1/2 is not unique to colon cancer cells. For instance, the sulindac derivative (Z)-5-Fluoro-2-methyl-1-(2-furanyl)-indene-3-acetic acid inhibited ERK1/2 phosphorylation in Ras-transformed Madin-Darby canine kidney cells from 4 to 24 hours after drug treatment (14). Similarly, the NSAIDs indomethacin and NS-398 have been shown to suppress ERK1/2 activation in A549 human lung cancer cells (15) and inhibit ERK2 activity in rat primary endothelial cells and human dermal microvascular endothelial cells (16). This article is the first to show that ERK1/2 inhibition is required for the apoptotic effects of sulindac metabolites. Inhibition of ERK1/2 may be an important mechanism for the chemopreventive effects of specific NSAIDs. ERK1/2 inhibition represents an attractive target for treatment and prevention of colorectal cancer and other cancers.

Prior reports have indicated a requirement in sulindac-induced apoptosis for protein kinase G–mediated activation of JNK1 (5), for down-regulation of β-catenin (17), and for the presence of Bax (17, 18). The results presented in this article add an unambiguous role for the necessity of down-regulation of ERK1/2 for the apoptotic effects of both sulindac sulfide and sulfone. The alternative targets for sulindac are currently being examined in HT29-MEK1(R4F) transfectants, to determine whether these different pathways are related or independent apoptotic mechanisms of sulindac. Although activation of the MEK/ERK signaling cascade can protect several cell types from apoptosis by various agents, the downstream mechanisms are poorly understood. In some cell types, activation of ERK1/2 results in transcriptional and posttranslational modulation of several Bcl-2 family proteins, including antiapoptotic (Bcl-2 and Bcl-XL; refs. 19, 20) and proapoptotic (Bad and Bim; refs. 9, 21, 22). MEK/ERK signaling may regulate mitochondrial events that lead to activation of caspases. If true, the HT29-MEK1(R4F) clones may be generally resistant to apoptosis induced by any agent that requires the mitochondrial apoptotic pathway and not specific to apoptosis by sulindac metabolites. Although the mechanism by which MEK1 activation protects HT29 cells from cleavage of caspase-3 and apoptosis by sulindac metabolites is unknown, we are currently investigating the Bcl-2 family of proteins and mitochondrial changes that lead to caspase-3 activation by the apotosome.

In conclusion, our results suggest that sulindac metabolites induce apoptosis of HT29 colon cancer cells by inhibiting phosphorylation of ERK1/2, and that inhibitory effect is both necessary and sufficient for the apoptotic effect of these drugs on colon cancer cells. Because ERK1/2 is activated in many colon tumors in vivo (23), inhibition of MEK/ERK signaling provides an attractive target for colorectal cancer prevention and treatment. This concept is supported by data indicating that treatment with PD184352, an in vivo MEK1/2 inhibitor, suppressed growth of colon 26 and HT29 xenografts in vivo (24), and that MEK1/2 inhibitors can sensitize cancer cells to other anticancer agents in vitro (8, 13). Although we have focused on the role of ERK1/2 for the growth inhibitory effects of sulindac, other biochemical targets have been identified for sulindac (4–7, 18). Sulindac may mediate its chemopreventive effects by multiple mechanisms and represents a pharmacological tool to identify potential biochemical targets against cancer cell survival. Using combination therapy with agents that specifically modulate relevant biochemical targets of sulindac, such as combining MEK1/2 and PDE2/5 inhibitors, may take advantage of synergistic growth inhibitory effects against cancer cells and could reduce toxicity associated with chronic intake of NSAIDs like sulindac. Use of such targeted therapies would be useful for both prevention and treatment of colorectal cancer.
References

Inhibition of Extracellular-signal Regulated Kinases 1/2 Is Required for Apoptosis of Human Colon Cancer Cells In vitro by Sulindac Metabolites


Cancer Res 2004;64:8148-8151.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/22/8148

Cited articles
This article cites 24 articles, 15 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/22/8148.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/64/22/8148.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.