GATA-6 Transcriptional Regulation of 15-Lipoxygenase-1 during NSAID-induced Apoptosis in Colorectal Cancer Cells[1]

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ABSTRACT

The mechanisms by which nonsteroidal anti-inflammatory drugs (NSAIDs) induce apoptosis in colorectal cancer cells are undergoing intensive investigation. We found previously that NSAIDs induce apoptosis in these cells by restoring 15-lipoxygenase-1 (15-LOX-1) expression. The present study examined the NSAID mechanism for up-regulating 15-LOX-1 in two colorectal cancer cell lines (RKO and DLD-1). We found that NSAID effects on 15-LOX-1 occurred at the level of transcriptional regulation. We then studied NSAID effects on GATA-6, a transcription factor that suppresses 15-LOX-1 expression. Beginning within 4 h, NSAIDs progressively down-regulated GATA-6 expression. Ectopic GATA-6 expression blocked NSAID induction of 15-LOX-1 and apoptosis. NSAIDs down-regulate GATA-6 to transcriptionally up-regulate 15-LOX-1 and induce apoptosis in colorectal cancer cells.

INTRODUCTION

Apoptosis is important for regulating and maintaining homeostasis in normal colon epithelia (1). The loss of the propensity to undergo apoptosis is a critical event in the development of human colorectal (2–4) and other (5) cancers, and restoring apoptosis is thought to be an important cancer therapeutic and preventive mechanism (5, 6). We have shown that 15-LOX[3]-1 is down-regulated and 13-S-HODE, a 15-LOX-1 product of linoleic acid, restores apoptosis in colorectal cancer cells (7). We showed recently that the up-regulation of 15-LOX-1 expression and 13-S-HODE production in colorectal cancer cells plays an important independent role in the apoptotic effects of NSAIDs, a very promising class of agents for the chemoprevention of colorectal cancers (8, 9). Various levels of the regulation of 15-LOX-1 expression have been shown (10, 11), but the mechanisms for the up-regulation of 15-LOX-1 by NSAIDs have not been reported previously.

GATA is a family of six transcriptional regulation proteins that play important roles in regulating cell differentiation during vertebrate embryogenesis (12). GATA-6 expression increases during proliferation and decreases during differentiation of intestinal cells (13). GATA-6 is expressed in undifferentiated cancer cells, especially colon cancer cells (13–15), in which GATA-6 suppresses the transcription of 15-LOX-1 (11). There are no previous reports on the relationship between GATA-6 and NSAID effects on 15-LOX-1. We designed our current study to test the hypothesis that the mechanism of NSAID-induced apoptosis in colorectal cancer cells involves GATA-6 transcriptional regulation of 15-LOX-1 expression.

MATERIALS AND METHODS

Materials. We obtained DLD-1 (human colon carcinoma) cells from the American Type Culture Collection (Manassas, VA) and RKO (human rectal carcinoma) cells from Dr. Michael Brattain (The University of Texas, San Antonio, TX). We purchased sulindac from Sigma Chemical Co. (St. Louis, MO), sulindac sulfone from LKT Laboratories, Inc. (St. Paul, MN), NS-398 from Cayman Chemical, Inc. (Ann Arbor, MI), and antihuman GATA-6 and histone antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Human GATA-6 wild-type cDNA subcloned into pcDNA1/Amp vector and a control pcDNA1/Amp vector that carries human GATA-6 cDNA with a deletion mutation in the zinc finger domains were gifts from Dr. Kenneth Walsh (Tufts University, Boston, MA) (16). Rabbit polyclonal antiserum to human recombinant 15-LOX-1 and standards of recombinant 15-LOX-1 were gifts from Drs. Mary Mulkins and Elliot Sigal (Roche Bioscience, Palo Alto, CA). Other reagents, molecular grade solvents, and chemicals were obtained from regular commercial manufacturers or as specified below.

Cell Culture. DLD-1 and RKO cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Life Technologies, Inc., Grand Island, NY). Cells were treated with NSAIDs at 60–80% confluence, cultured, and harvested for each assay as described below. The selected NSAID concentrations to induce apoptosis [NS-398 (120 μM), sulindac sulfone (300 μM), or sulindac (150 μM)] were based on prior studies in colorectal cancer cells and apply to all of the experiments described below (8, 17). NSAIDs were dissolved in DMSO. DMSO final concentrations were ≤0.5% (Ref. 8; DMSO had no independent effect on cell growth (data not shown)].

Northern Blot Analysis of 15-LOX-1 and GATA-6 RNA Expressions. Total RNA was isolated by TRI reagent (Sigma Chemical Co.), and Northern blot analyses were performed as described previously (18). RNA was separated (20 μg/lane) on 0.7% denaturing agarose gels containing 1.8% (v/v) formaldehyde and transferred onto nylon membranes (Bio-Rad). The membranes were hybridized overnight at 42°C with a ^{32}P-labeled cDNA probe in a solution containing 50% (v/v) formamide, 5× SSC, 50 mM sodium phosphate buffer (pH 6.5), 250 μg/ml sheared salmon sperm DNA, 10× Denhart's solution, and 10% dextran sulfate. The radiolabeled (^{32}P) cDNA probe for human 15-LOX-1 was 952 bp that were generated by reverse transcription-PCR of human 15-LOX-1 with the primers 5′-GAGTTGACTTTGAGG-TTTCGC-3′ and 5′-GCCCGTCTGTCTTATAGTGG-3′. A 644-bp human GATA-6 probe was generated by the primers 5′-TTGGATTGTCCTGT-GCCAACT-3′ and 5′-GGTTCACCCTCGGCGTTT-3′. The human GAPDH probe, used as control, was a 600-bp PCR piece made with 5′-CCACCCAT-GGCAAATTCCATGGCA-3′ primer and 5′-TCTAGACGGCAGGTCAG-GTCCACCACC-3′ primer. Amplified cDNA was cloned using the pGEM-T vector (Promega). After hybridization and washes, the blots were autoradiographed by exposure to hyperfilm-MP films (Amersham Corp).

Nuclear Run-On Assay. DLD-1 and RKO cells were cultured, treated with either sulindac, NS-398 or sulindac sulfone, and harvested 48 h later (19). Nuclei (5×10^7 cell/sample) were isolated by using NP-40 lysis buffer and stored at −80°C in storage buffer. Nuclei were thawed and incubated in 100 μl of 2× reaction buffer and 100 μCi of [α-^{32}P]UTP at 30°C for 30 min. RNase-free DNase and CaCl$_2$ were added to remove template DNA after transcription, and the mixture was incubated at 26°C for 30 min. To digest protein, 25 μl of 10× SET, 2 μl of 100 mg/ml proteinase K, and 5 μl of tRNA (Roche Molecular Biochemicals, Indianapolis, IN) were added for 30-min
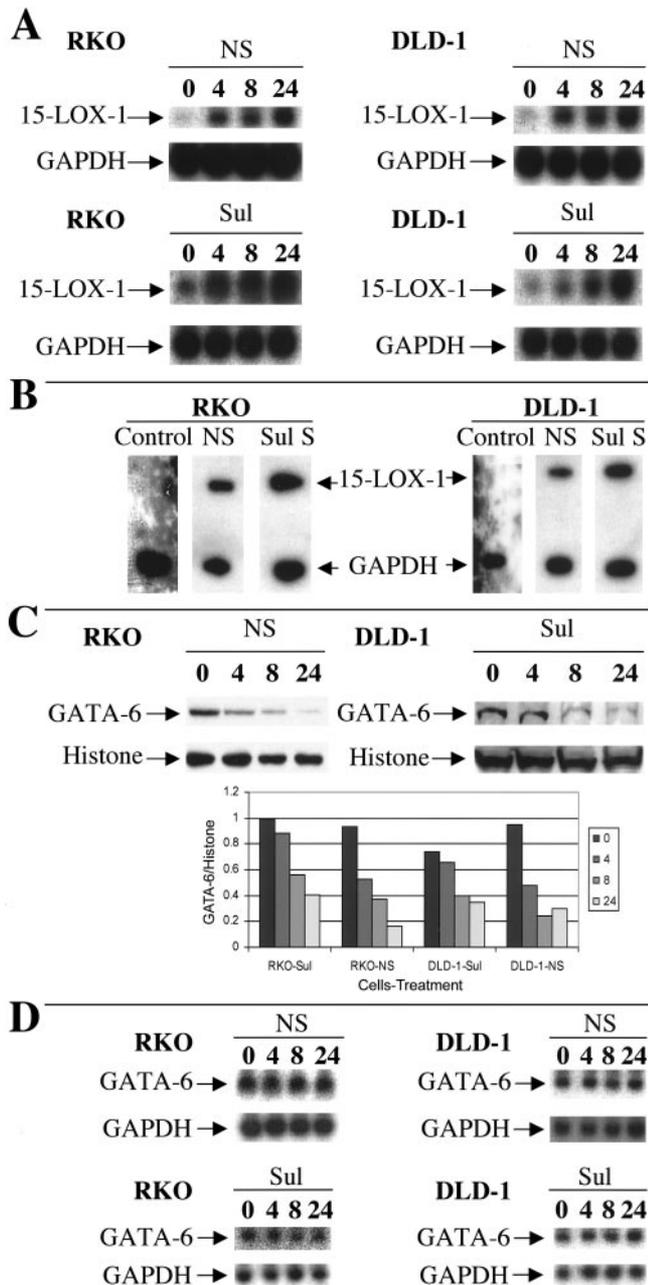
Fig. 1. NSAIDs increase 15-LOX-1 transcription and down-regulate GATA-6 in RKO and DLD-1 cells. *A,* cells were treated with NS-398 (*NS*) or sulindac (*Sul*) and processed for Northern blot analyses (see "Materials and Methods"). *B,* cells were treated with *NS* or sulindac sulfone (*Sul S*) and processed for run-on assays (see "Materials and Methods"). *C, NS* and *Sul* down-regulated GATA-6 protein expression in cells treated for the indicated time intervals and subjected to Western blot analyses using anti-GATA-6 antibody (see "Materials and Methods"). The bar graph indicates the values of the ratio of GATA-6:histone (protein expressions) for the time points assessed for these two cell lines and treatments. *D, NS* and *Sul* effects on GATA-6 RNA expression in RKO and DLD-1 cells treated for 0, 4, 8, or 24 h. RNA was extracted, blotted, and then probed with GATA-6- and GAPDH-radiolabeled probes. *A, B,* and *D,* GAPDH was used as an RNA loading control.

incubations at 37°C. The labeled nascent RNA transcripts were isolated with 0.75 ml of Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) plus 0.2 ml of chloroform and precipitated with isopropanol. The pellets were washed with ice-cold 70% ethanol and dissolved in 100 μl of 0.2% SDS. To detect newly synthesized RNA, 1 μg of 15-LOX-1 cDNA and 1 μg of GAPDH cDNA (as a control) were immobilized separately onto a nitrocellulose membrane, prehybridized at 65°C for 2 h in hybridization buffer (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), and hybridized at 65°C for 24 h with

10^6 cpm/ml labeled nascent RNA transcripts from each experiment. The membranes were then washed twice with 2× SSC at 65°C for 20 min and exposed to X-ray film for 24 h. The resulting bands were scanned electronically into images that were quantified by using the Integrated Density function in the NIH Image program (NIH Freeware). Results were expressed as the ratio of 15-LOX-1 mRNA to the content of GAPDH mRNA in each sample.

GATA-6 Western Blot Analyses. Cells were harvested in ice-cold PBS and centrifuged at 3000 rpm at 4°C for 5 min. The cell pellets were washed with PBS, lysed, and resuspended in a 0.2% NP40 lysis buffer. Nuclei were pelleted; nuclear protein was extracted; and the protein concentration was determined by the Bradford method (20). Equivalent amounts of protein (60 μg crude protein/sample) were subjected to SDS-PAGE, followed by electroblotting to nitrocellulose membrane. Blots were incubated with a rabbit antibody solution to human GATA-6 (0.1 μg/ml; Santa Cruz Biotechnology, Inc.) for 12 h at 4°C and then analyzed by the ECL method. HLA cell lysate was used as a positive control as specified by the manufacturer. Results were expressed as the ratio of GATA-6 protein expression to the content of histone protein expression in each sample.

Transient Transfection with GATA-6. RKO or DLD-1 cells were seeded in 100-mm dishes (100,000 cells/dish). Cells were transfected with either wild-type or mutated GATA-6 pcDNA1/amp vector at 40% confluence, using the calcium phosphate transfection method (16). After 24 h, the medium was changed, and cells were treated with either sulindac or NS-398. After another 72 h, cells were harvested, counted, assessed for viability using the trypan blue method (to assess growth inhibition), and processed for apoptosis assays as described below. In later experiments, transfected cells were grown after treatment with NS-398, sulindac, or nothing (control) and then harvested for determination of 15-LOX-1 protein expression as described below.

Immunocytochemistry Assessment for GATA-6 Transfection. To determine whether transient transfection with the GATA-6 vector was successful in increasing GATA-6 protein expression, transfected cells were analyzed by immunohistochemical staining. Cells (RKO and DLD-1) were cultured on coverslips, transfected with the GATA-6 vector, and grown for 48 h. The cells were fixed in 4% paraformaldehyde, washed in 0.1% NP40 PBS, and incubated with 6% BSA for 20 min at room temperature to block nonspecific binding of the antibodies. Fixed cells were incubated with a solution of rabbit antibody to human GATA-6 (1:100 dilution) for 24 h at 4°C. After washing, AlexaFlur 594 goat antirabbit IgG (1:200; Molecular Probes, Inc., Eugene, OR) was added as a secondary antibody for a 30-min incubation at room temperature. Coverslips were washed, placed on microscope slides, and examined by fluorescence microscopy. In negative control experiments, the primary antibody was replaced with nonimmune rabbit serum.

Western Blot Analysis of 15-LOX-1 Protein. Cells harvested for 15-LOX-1 expression experiments were lysed, sonicated, and kept frozen at −70°C until analyzed (8). An equal amount of protein from each sample was subjected to electrophoresis on an 8% SDS-polyacrylamide gel under reducing conditions. After transfer, blots were probed with a solution of rabbit antibody to human 15-LOX-1 and analyzed by the ECL method, as described previously (8).

Assessments of Apoptosis. Apoptosis was evaluated by several methods: DNA gel electrophoresis; microscopic examination to identify morphological changes associated with apoptosis; floating-cell ratio; staining with DAPI; flow cytometric cell cycle distribution analysis to determine sub-G_1 fractions; and TUNEL assay. Inverse-light (phase-contrast) microscopy was used to assess gross evidence of apoptosis and to determine floating cell ratio (8, 21). For DNA gel electrophoresis, cells were harvested 72 h after treatment and lysed. DNA was extracted from an equal number of cells, precipitated, subjected to electrophoresis on 1% agarose gels, and visualized by ethidium bromide staining, as described previously (8). Freshly harvested DLD-1 cells (~50,000/sample) were transferred to glass slides using a cytospin. Cells were fixed in acetic acid:ethanol (1:9, v/v), washed, stained with DAPI solution (1 μM; Molecular Probes, Inc.), and examined and photographed using a fluorescence microscope. The flow cytometric, cell cycle distribution analyses to determine subdiploid DNA (sub-G_1) peaks as a measure of apoptosis involved staining with propidium iodide, as described previously (8). Cells also were harvested and processed for TUNEL assay, as described previously (8).

Statistical Analysis. We used one-way ANOVA to compare various quantifiable outcome measures (*e.g.,* viable cell counts or the percentage of TUNEL-positive cells) in different experimental conditions (*e.g.,* GATA-6-transfected and nontransfected cells). Data were analyzed using SAS software
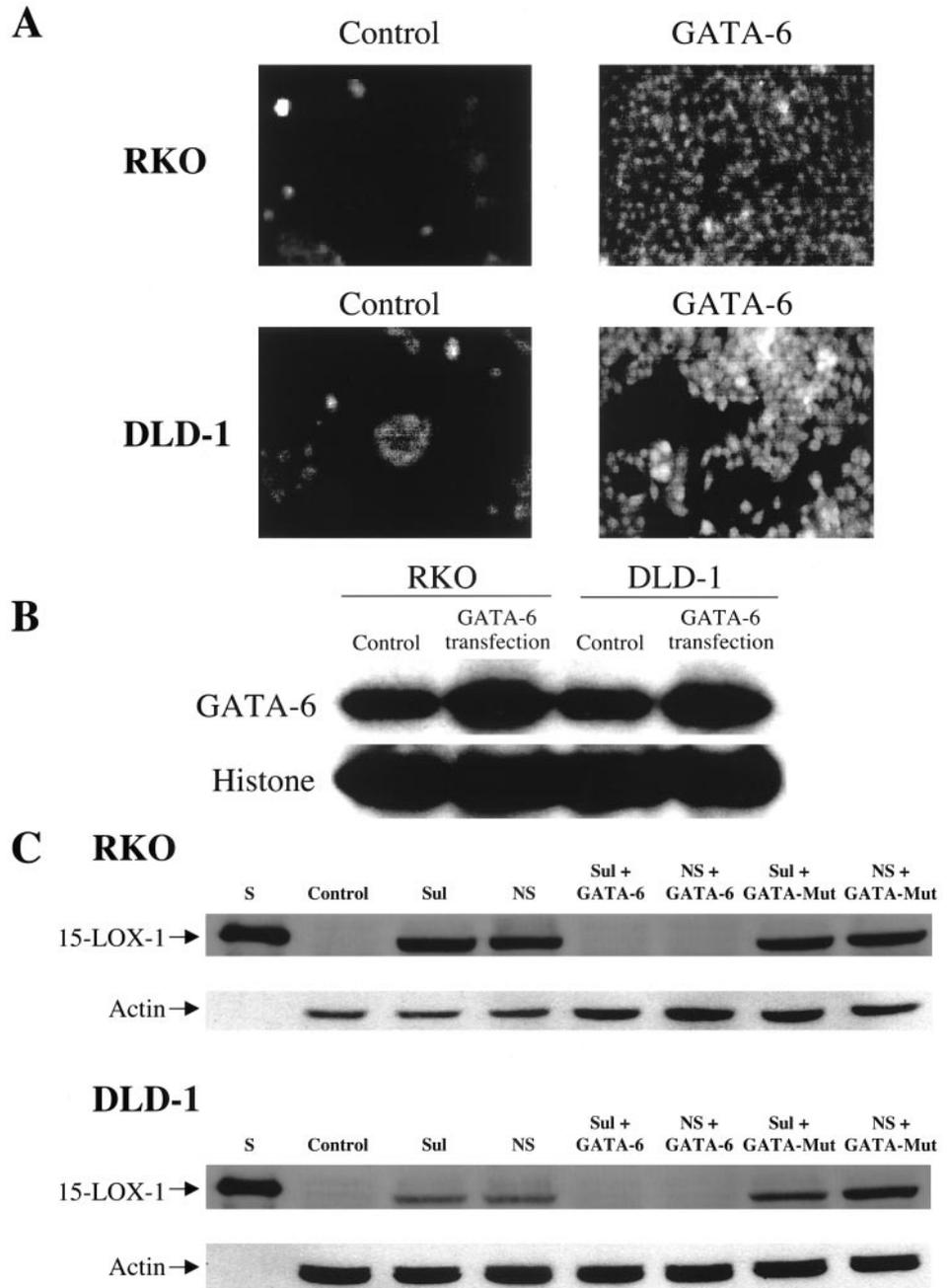
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Fig. 2. Transient transfection with a GATA-6 vector increased GATA-6 expression in human colorectal cancer cells (RKO and DLD-1) and blocked NSAID induction of 15-LOX-1 expression. Cells were transfected with pcDNA1/Amp carrying wild-type human GATA-6, which induced marked increases in GATA-6 expression (compared with nontransfected controls), as shown by immunocytochemistry ($\times$200; *A*) and Western blot analyses (*B*). *C*, cells were transfected with either human wild-type GATA-6 cDNA (GATA-6) or mutant GATA-6 cDNA (with a deletion mutation in the zinc finger; GATA-Mut), treated with either NS-398 (*NS*) or sulindac (*Sul*), cultured for 48 h, and harvested. 15-LOX-1 protein expression was analyzed by Western blot using an anti-15-LOX-1 antibody. Both *NS* and *Sul* induced 15-LOX-1 protein expression in both cell lines. GATA-6 transfection blocked sulindac and NS-398 from inducing 15-LOX-1 expression. GATA-Mut transfection failed to block NSAID induction of 15-LOX-1 expression. The lanes for the COX-2-expressing RKO cells (*top of panel*) and non-COX-2-expressing DLD-1 cells (*bottom of panel*) depict results for the following conditions: *Lane 1,* positive standard control (*S*) of recombinant human 15-LOX-1 protein; *Lane 2,* control experiment in cells without NSAID treatment; *Lanes 3* and *4, Sul-* or *NS*-treated cells; *Lanes 5* and *6,* cells transfected with GATA-6 and then treated with either *Sul* or *NS*; *Lanes 7* and *8,* cells transfected with GATA-Mut and then treated with either *Sul* or *NS*.

(SAS Institute, Cary, NC). The mean values and standard errors for the outcome variables are shown in bar charts. All reported *P*s are two-sided and were considered to be statistically significant at the 0.05 level. *P*s for comparing the difference between groups (*i.e.,* contrasts) are given after the Bonferroni adjustment for multiple comparisons.

RESULTS

NSAID Effects on Transcription of 15-LOX-1. 15-LOX-1 mRNA formation increased within 4 h of treatment with NS-398 or sulindac in RKO and DLD-1 cells (Fig. 1*A*). NS-398 and sulindac sulfone increased the initiation of 15-LOX-1 transcription (Fig. 1*B*). We observed the following NSAID-induced increases in newly synthesized 15-LOX-1 mRNA normalized with GAPDH (compared with no treatment). NS-398-induced increases of 9.6-fold in RKO cells and 5-fold in DLD-1 cells, and sulindac sulfone induced increases of 9.8-fold in RKO cells and 6.5-fold in DLD-1 cells.

NSAID Effects on GATA-6 Expression. GATA-6 protein expression decreased progressively for a period of at least 24 h, beginning within 4 h after sulindac or NS-398 treatment (Fig. 1*C*). At 24 h, the reductions in GATA-6 expression (normalized to histone expression) by sulindac and NS-398 were 59 and 83%, respectively, in RKO cells and 53 and 69%, respectively, in DLD-1 cells. In contrast to GATA-6 protein expression, GATA-6 RNA expression remained unchanged by either sulindac or NS-398 in RKO and DLD-1 cells (Fig. 1*D*).

Effects of Ectopic GATA-6 Expression on 15-LOX-1 Up-Regulation by NSAIDs. The ectopic expression of GATA-6 through vector transfection increased GATA-6 protein levels, as detected by immunocytochemical staining for GATA-6 in both RKO and DLD-1 cells (Fig. 2*A*). A control experiment in which the GATA-6 antibody was replaced with nonimmune rabbit serum showed no staining. Western blot analyses showed that the transfection increased GATA-6 expression (normalized to histone expression) by 197 and 200% in

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RKO or DLD-1 cells, respectively (Fig. 2*B*). The ectopic expression of GATA-6 suppressed the induction of 15-LOX-1 expression by sulindac and NS-398 in both RKO and DLD-1 cells (Fig. 2*C*). In contrast, sulindac or NS-398 induced 15-LOX-1 expression in RKO and DLD-1 cells transfected with a mutant GATA-6 vector (Fig. 2*C*).

Effects of Ectopic GATA-6 Expression on NSAID-induced Growth Inhibition and Apoptosis. The ectopic expression of wild-type GATA-6 suppressed the ability of both NS-398 and sulindac to inhibit cell growth in RKO cells (Fig. 3*A*; $P < 0.001$). In contrast,
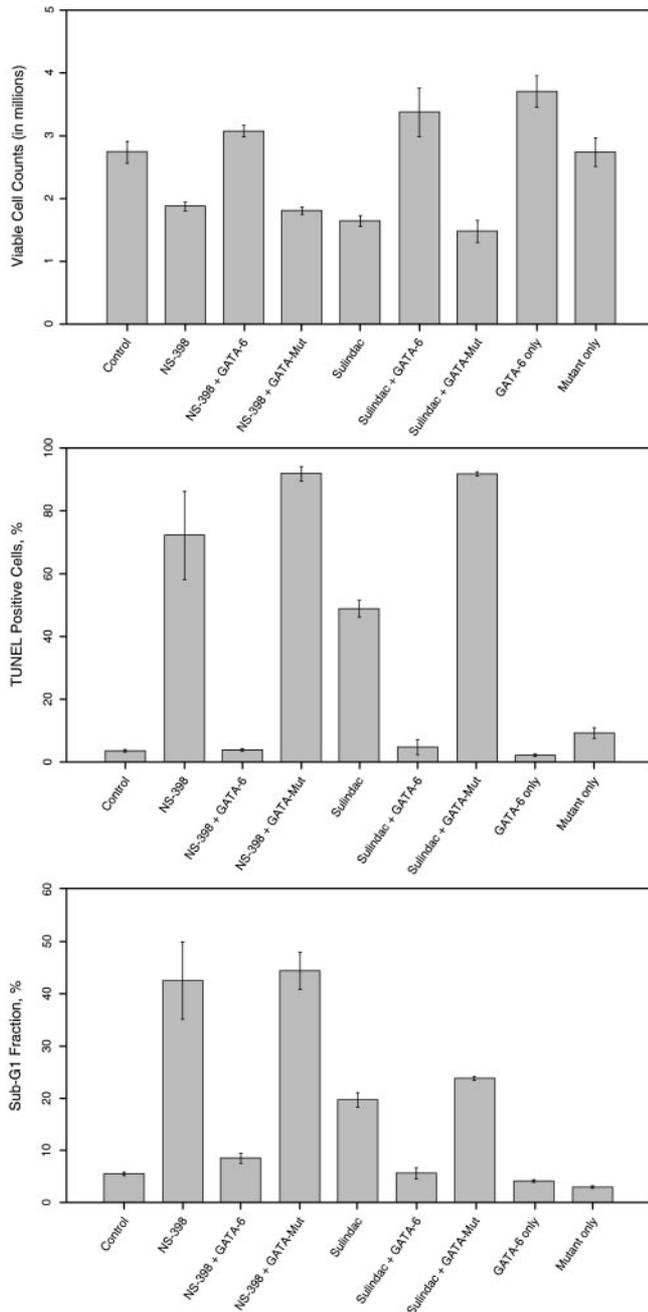

Fig. 3. GATA-6 ectopic expression blocked growth inhibition and apoptosis induction by NSAIDs in RKO cells. *Top*, cells were transfected with pcDNA1/Amp carrying either wild-type human GATA-6 or mutant human GATA-6 (*GATA-Mut*). Transfected cells were treated with either NS-398 or sulindac, cultured for 72 h, and harvested. Trypan blue staining was used to assess the number of viable cells. *Middle*, cells underwent TUNEL assay to measure apoptosis. *Bottom*, cells were stained with propidium iodide and assessed for sub-G_1 fractions by flow cytometry. All panels: the control GATA-Mut failed to block NSAID-induced growth inhibition or apoptosis. All values shown are the means of triplicate experiments; *bars*, SE.

transfection with a GATA-6 mutant vector failed to inhibit NSAID suppression of the growth of RKO cells (Fig. 3*A*). The suppressive effects of NS-398 *versus* NS-398 plus mutant GATA-6 vector and of sulindac *versus* sulindac plus mutant GATA-6 vector were similar on RKO cell growth ($P = 1$). Viable cell numbers were not reduced by either wild-type or mutant GATA-6 vector transfection in cells not treated with NSAIDs (Fig. 3*A*). The TUNEL assay indicated the following apoptosis results in RKO cells (Fig. 3*B*): NS-398 and sulindac induced apoptosis ($P < 0.001$); the ectopic expression of wild-type GATA-6 blocked the induction of apoptosis ($P < 0.001$); and, in contrast to ectopic wild-type GATA-6, the transfection of mutant GATA-6 vector increased apoptosis induction by either NS-398 ($P = 0.036$) or sulindac ($P < 0.001$; Fig. 3*B*). Similar apoptosis results in RKO cells were detected by sub-G_1 fraction analyses (Fig. 3*C*) or floating cell ratio measurements (data not shown). GATA-6 ectopic transfection blocked NS-398 and sulindac from increasing sub-G_1 fraction ($P < 0.001$), whereas mutant GATA-6 expression failed to block apoptosis induction by either NS-398 ($P = 1$) or sulindac ($P = 0.951$). This pattern of ectopic GATA-6 effects on NSAID-induced apoptosis also occurred in DLD-1 cells. Ectopic wild-type, but not mutant, GATA-6 expression blocked apoptosis induction in DLD-1 cells by either NS-398 or sulindac (Fig. 4).

DISCUSSION

The two major new findings of the present study are that the tested NSAIDs modulate 15-LOX-1 at the level of transcriptional regulation, and this modulation involves down-regulating the transcription factor GATA-6 expression. Whether using selective COX-2 inhibitors (NS-398), nonselective COX inhibitors (sulindac), or non-COX inhibitors (sulindac sulfone), the tested NSAIDs increased 15-LOX-1 transcription in COX-2-expressing (RKO) or COX-2-non-expressing (DLD-1) colorectal cancer cells. These transcriptional regulation results were produced in Northern blot analyses and run-on assays (19) and are consistent with other findings (10). Furthermore, our run-on assay results indicated that these NSAIDs promoted the initiation of 15-LOX-1 transcription (19).

We then studied NSAID effects on GATA-6, a transcription factor that suppresses 15-LOX-1 expression (11). The NSAIDs sulindac and NS-398 down-regulated GATA-6 protein expression in a time-dependent fashion in RKO and DLD-1 cells. The NSAID effects of down-regulated GATA-6 protein expression (detected in the present study within 4 h after treatment and persisting for at least 24 h) preceded 15-LOX-1 up-regulation [shown in our previous study to start 24 h after treatment (8, 9)] in colorectal cancer cells. In contrast with this marked reduction in GATA-6 protein expression, NSAIDs had no effect on GATA-6 RNA expression levels. These findings indicate that NSAIDs down-regulate GATA-6 through posttranscriptional effects.

This temporal connection led to our mechanistic examination of GATA-6 and 15-LOX-1 during NSAID-induced apoptosis in the *in vitro* model of RKO and DLD-1 cells transfected with GATA-6 to induce ectopic GATA-6 expression. The ectopic expression of GATA-6 inhibited the ability of NSAIDs to up-regulate 15-LOX-1 expression or induce growth inhibition or apoptosis. These findings established the important mechanistic link between 15-LOX-1 up-regulation and the down-regulation of GATA-6 expression during NSAID-induced apoptosis. Control experiments involving colorectal cancer cells transfected with mutant GATA-6 protein (that cannot bind DNA) failed to suppress NSAID-induced 15-LOX-1 expression and apoptosis, indicating that our results were not caused by nonspecific effects of transient transfection.

The results of our previous (8, 9, 22) and current studies can be
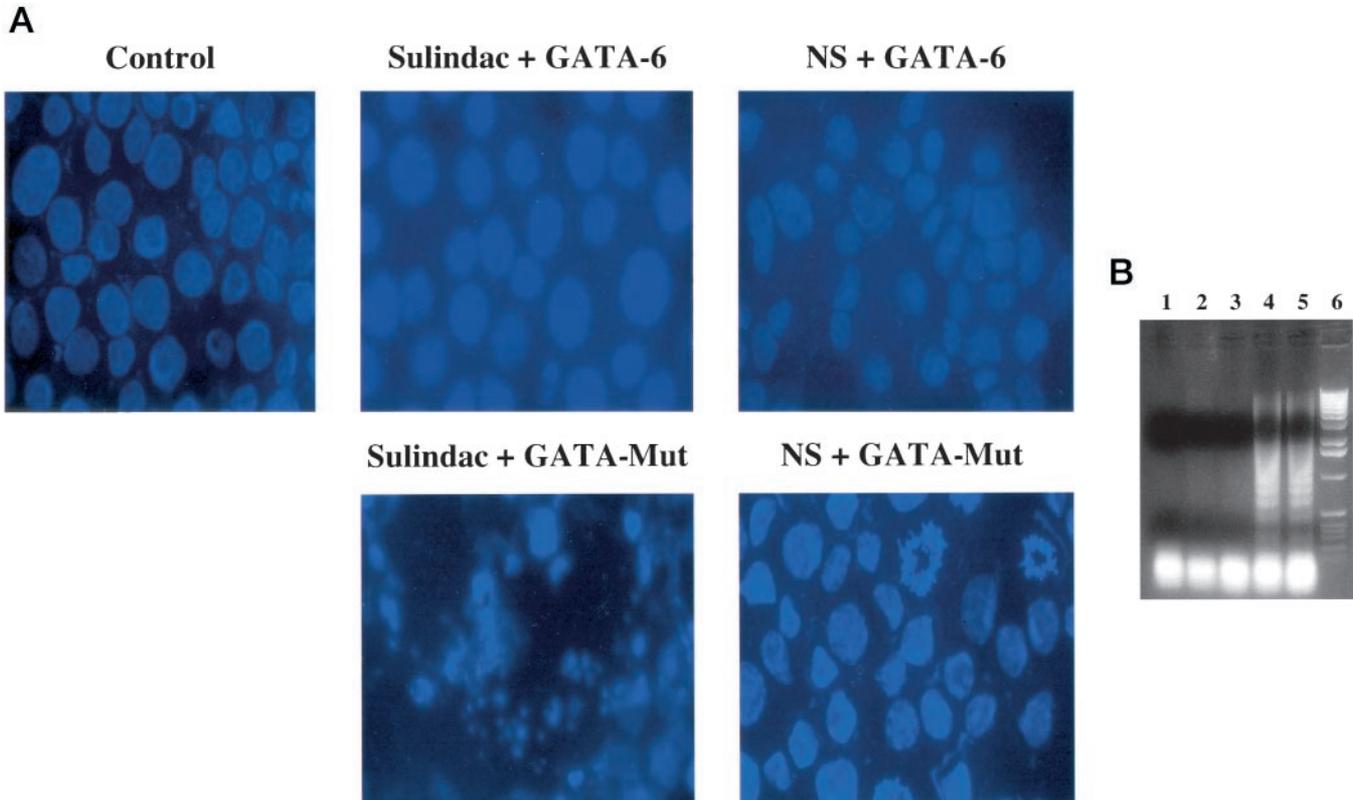
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Fig. 4. GATA-6 ectopic expression blocked apoptosis induction by NSAIDs in DLD-1 cells. Cells were transiently transfected with either wild-type human GATA-6 or mutant human GATA-6 (*GATA-Mut*), cultured, and treated with sulindac or NS-398 (*NS*). *A*, floating cells were stained with DAPI and examined by a fluorescence microscope (×400). GATA-6 transfection inhibited the typical morphological changes associated with apoptosis induction by NSAIDs, including nuclear shrinkage and fragmentation. The control cells were not transfected or treated. *B*, DNA was extracted and analyzed by agarose gel electrophoresis. *Lane 1,* control cells (untreated and nontransfected); *Lane 2,* sulindac + GATA-6; *Lane 3,* NS-398 + GATA-6; *Lane 4,* sulindac + GATA-Mut; *Lane 5,* NS-398 + GATA-Mut; *Lane 6,* a 1-kb standard DNA ladder. *A* and *B*, control transfections with GATA-Mut failed to block apoptosis induction by NSAIDs, as evidenced by the nuclear fragmentation and condensation (*A*) or typical ladder pattern (*B*).

summarized in (and suggest) a new molecular model including GATA-6 as a novel target of NSAID effects in cancer cells. NSAIDs down-regulate GATA-6, which permits the transcription of 15-LOX-1. The up-regulated 15-LOX-1 increases 13-*S*-HODE, which in turn triggers apoptosis. This model suggests that GATA-6-mediated down-regulation of 15-LOX-1 contributes to the ability of colorectal cancer cells to escape apoptosis. Findings by others support this concept (11, 13). The level of GATA-6 expression has been shown to be higher in the proliferating region of the intestinal crypt than in the intestinal villus tips, where cells undergo differentiation and apoptosis (13). Furthermore, GATA-6 in colon cancer cells is expressed during proliferation and down-regulated during differentiation induction (11, 13).

The present findings involving NSAID effects on GATA-6 and 15-LOX-1 provide important new insights into the signal transduction pathway governing NSAID-induced apoptosis in colorectal cancer cells. Further study of NSAID mechanisms of action, especially those involved in down-regulating GATA-6, should lead to new molecular targets for chemopreventive drug development (23).

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