Chemoprevention of Familial Adenomatous Polyposis by Low Doses of Atorvastatin and Celecoxib Given Individually and in Combination to APC^{Min} Mice

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Abstract

Preclinical and clinical studies have established evidence that cyclooxygenase-2 (COX-2) inhibitors and statins [hydroxy-3-methylglutaryl CoA reductase (HMGR) inhibitors] inhibit colon carcinogenesis. Chronic use of high doses of COX-2 inhibitors may induce side effects, and combining the low doses of agents may be an effective way to increase their efficacy and minimize the side effects. We assessed the chemopreventive efficacy of atorvastatin (Lipitor) and celecoxib individually or in combination in an animal model of familial adenomatous polyposis. Six-week-old male C57BL/6J-*APC*$^{min/+}$ mice were either fed diets containing 0 or 100 ppm atorvastatin or 300 ppm celecoxib, or a combination of both for ~80 days. Mice were sacrificed, and their intestines were scored for tumors. Normal-seeming mucosa and intestinal tumors were harvested and assayed for apoptosis (terminal deoxynucleotidyl transferase–mediated nick-end labeling) and HMGR and COX-2 protein expression and activity. We observed that 100 ppm atorvastatin significantly ($P < 0.002$) suppressed intestinal polyp formation. As anticipated, 300 ppm celecoxib decreased the rate of formation of intestinal polyps by ~70% ($P < 0.0001$). Importantly, the combination of 100 ppm atorvastatin and 300 ppm celecoxib in the diet suppressed the colon polyps completely and small intestinal polyps by >86% ($P < 0.0001$) compared with the control group. The inhibition of tumor formation by the atorvastatin and celecoxib combination was significant ($P < 0.005$) when compared with tumor inhibition by celecoxib alone. In addition, increased rates of apoptosis in intestinal tumors ($P < 0.01$-0.0001) were observed in animals fed with atorvastatin and celecoxib and more so with the combinations. Tumors of animals fed atorvastatin showed a significant decrease in HMGR-R activity. Similarly, tumors of mice exposed to celecoxib showed significantly lower levels of COX-2 activity. These observations show that atorvastatin inhibits intestinal tumorigenesis and that, importantly, when given together with low doses of celecoxib, it significantly increases the chemopreventive efficacy in an APC^{min} mice. (Cancer Res 2006; 66(14): 7370-7)

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doi:10.1158/0008-5472.CAN-05-4619

Introduction

Large bowel cancer is one of the leading causes of cancer deaths in both men and women in Western countries and in the United States. About 145,290 new cases of colorectal cancer and 56,290 related deaths are expected for the year 2005 (1). Epidemiologic and experimental studies indicate that the risk of developing colon cancer may be attributable to genetic and environmental factors, including promoting agents that occur endogenously (2). The progression from normal epithelium to colon cancer is a multistep process involving accumulation of multiple genetic alterations (3). The adenomatous polyposis coli (*apc*) gene, a tumor suppressor, is considered to be a gatekeeper in colon tumorigenesis (4), and its inactivation leads to the development of adenomatous polyps. Adenomatous polyps are the primary precursor lesion for the development of most colon carcinomas in humans, and as such, they represent an early clinical target for the prevention/intervention of colorectal cancer (5, 6). In most cases of colonic neoplasia, both inherited and somatic, the *APC* gene is either mutationally altered by the introduction of premature stop codons and/or deleted by loss of heterozygosity (6). The current mouse model has been used to unravel the basic mechanisms of intestinal tumor formation, including downstream targets and testing cancer chemopreventive agents (5–7).

Chemoprevention is likely to play a major role in the management of colorectal cancer (8–10). Recent developments in the cellular and molecular pathogenesis of colon cancer provide new insights for developing selective agents with potential chemopreventive properties against colon carcinogenesis. For example, understanding the role of nonsteroidal anti-inflammatory drugs (NSAID), particularly selective cyclooxygenase-2 (COX-2), in the development of colorectal cancer provided the basis for the application of selective COX-2 inhibitors (11–13). Studies by us and others support the hypothesis that the selective COX-2 inhibitor celecoxib (Fig. 1) suppresses colon cancer development more efficiently than the nonselective COX-2 inhibitors and with fewer side effects (14–17). Several clinical trials also support the use of celecoxib in familial adenomatous polyposis (FAP) patients (18); however, the apparent need for a high dose and the moderate degree of suppression of polyp formation (~30%) suggests that further strategies are required to improve the clinical efficacy of celecoxib. In this regard, recent adenoma prevention with celecoxib (APC) trials suggest that use of high doses of celecoxib may produce unwanted side effects, such as increased cardiovascular risks (19). The wide array of information on NSAIDs in cancer prevention, and a growing knowledge in the area of chemoprevention in general, has led to innovative

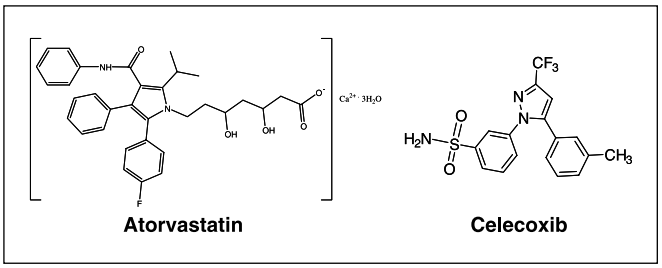

Figure 1. Chemical structures of atorvastatin (Lipitor) and celecoxib (Celebrex).

approaches using combinations of NSAIDs with other agents at low-dose levels. The combination of single agents with different modes of action represent a practical approach for improving the chemopreventive efficacy with little or no unwanted side effects.

It is known that hydroxy-3-methylglutaryl CoA reductase (HMGR) activities are up-regulated severalfold in colon tumors and possibly not regulated by feedback inhibition from cholesterol compared with normal mucosa. Recent studies suggest that HMGR inhibitors suppress chemically induced colon carcinogenesis in animal models (20, 21). Furthermore, clinical observations show an inverse relationship between the use of HMGR inhibitors and the reduction of colon cancers by as much as 51% (22). In two large clinical trials involving patients with coronary artery disease, use of the HMGR inhibitors pravastatin and simvastatin led to a 43% (23) and 19% (24) reduction, respectively, in the number of newly diagnosed cases of colon cancer during a 5-year follow-up period. In the same study, 83% of patients in both the pravastatin group and placebo group were given a daily dose of aspirin (for coronary artery disease prophylaxis; ref. 23). Despite aspirin ingestion, only patients taking pravastatin showed a reduction in the incidence of new cases of colon cancer, suggesting a possible synergistic effect of HMGR inhibitors with NSAIDs in colon cancer reduction. In support of this, we have previously shown that a combination of low doses of lovastatin and sulindac suppressed azoxymethane-induced colonic ACF formation in rats more effectively than each compound alone (25). Furthermore, we also showed that a combination of HMGR inhibitor lovastatin with COX-2 inhibitor celecoxib enhanced apoptosis in HT-29 colon cancer cells (26). Recently, Demierre et al. (27) reviewed extensively on the role of statins in cancer prevention.

The role of apoptosis in colon carcinogenesis has been extensively studied, suggesting that resistance to apoptosis in premalignant colonic epithelial cells will lead to development of colon tumors (28–30). Thus, identifying the mechanisms and approaches that up-regulate the apoptotic processes will provide useful strategies for regulating colon tumor growth. This hypothesis has been supported by several chemopreventive agents that have been shown to inhibit colon tumor growth by enhancing apoptosis (31–33). This information emphasizes the importance of understanding complementary pathways that lead to enhanced apoptosis and decreased proliferation, leading to synergistic chemopreventive efficacies. In the current study, we examined the chemopreventive efficacy of atorvastatin and celecoxib, either individually or in combination in an animal model of FAP. We also determined the activities and expressions of HMGR, COX-2, and caspase-3 and levels of apoptosis in intestinal tumors of $APC^{min/+}$ mice.

Materials and Methods

Animals, diets, HMGR, and COX-2 inhibitors. Heterozygous male Min (C57BL/6J-$APC^{min/+}$) and wild-type C57BL/6J male mice were obtained at 5 weeks of age from The Jackson Laboratory (Bar Harbor, ME). All ingredients for the semipurified diets were purchased from Dyets, Inc. (Bethlehem, PA) and stored at 4°C before preparation of the diets. Diets were based on the modified American Institute of Nutrition-76A (AIN-76A) diet with high fat (34). The composition of the high-fat semipurified diet is casein (21.3%), corn starch (43.5%), dextrose (12%), corn oil (12%), alphacel (5%), AIN mineral mix (3.5%), AIN-revised vitamin mix (1.2%), D,L-methionine (0.3%), and choline bitartrate (0.2%). The reason to increase fat content from 5% to 12% is to increase colon tumor burden in APC^{min}mice (33). The test agents (atorvastatin or celecoxib or a combination of both agents) were blended into the diet using a *V*-blender (Patterson-Kelley Co., East Stroudsburg, PA) after first being premixed with a small quantity of diet in a food mixer. The control diets and experimental diets containing agents were prepared weekly and stored in a cold room. Celecoxib and atorvastatin were kindly provided by the National Cancer Institute chemopreventive drug repository (Rockville, MD). The agent (atorvastatin and celecoxib) contents in the experimental diets were determined periodically in multiple samples taken from the top, middle, and bottom portions of individual diet preparations to verify uniform distribution. In this study, we used low doses of 100 ppm atorvastatin or 300 ppm celecoxib or 100 ppm atorvastatin + 300 ppm celecoxib in the control diets. The rationale for the selection of 100 ppm atorvastatin and 300 ppm of celecoxib is based on maximum tolerated dose (MTD) studies yielding <25% MTD of atorvastatin and <7% MTD of celecoxib.

Experimental procedure. The experimental protocol is summarized in Fig. 2. Following 5 days of quarantine, all mice were distributed so that average body weights in each group were equal (10 $APC^{min/+}$ mice in each group and 6 wild-type mice as parallel treatment groups to compare food intake, growth rate, and tumor formation in $APC^{min/+}$). The animals were then transferred to a holding room where they were housed individually in plastic cages with filter tops. Laboratory conditions were controlled to maintain a 12-hour light/dark cycle at 50% relative humidity and at 21 ± 1°C. The mice were fed the control or experimental diets until termination of the study (i.e., for ~80 days). The animals were weighed twice weekly and monitored daily for signs of weight loss or lethargy that might indicate intestinal obstruction or anemia. At 19 weeks of age, all mice were killed by CO_2 asphyxiation. This point in time was chosen to minimize the risk of intercurrent mortality caused by severe progressive anemia, rectal prolapse, or intestinal obstruction, which usually occurs among *Min* mice at ~19 weeks of age (7). After necropsy, the intestinal tracts were dissected from the esophagus to the distal rectum, spread onto filter paper, opened longitudinally with fine scissors, and cleaned with sterile saline. They were examined under a dissection microscope with ×5 magnification for tumor counts. This procedure was completed by two individuals who were blinded to the experimental group and the genetic status of the mice. Colonic and other small intestinal tumors that required further histopathologic evaluation to identify adenoma, adenocarcinoma, and enlarged lymph

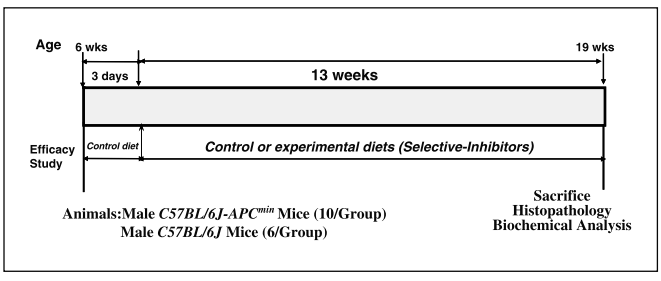

Figure 2. Experimental protocol for the evaluation of chemopreventive activity of atorvastatin and celecoxib in APC^{min} mice model. Groups of mice were fed control diet (AIN-76A), or diets containing 100 ppm atorvastatin, 300 ppm celecoxib, and 100 ppm atorvastatin + 300 ppm celecoxib until termination of the study. (Detailed information has been given in Materials and Methods).

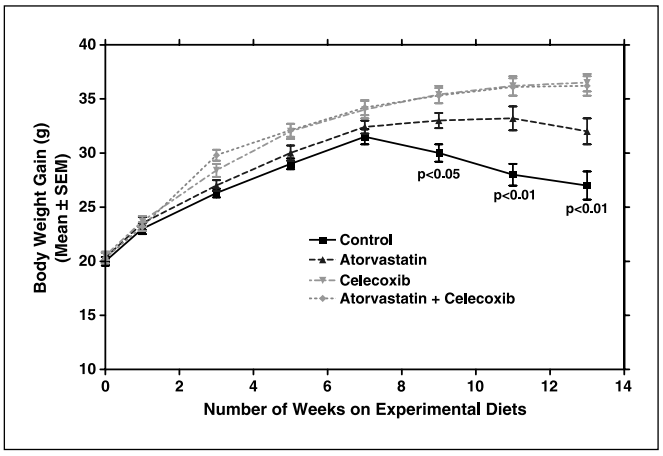

Figure 3. Body weights of *APC*^{min} mice given control and experimental diets containing atorvastatin and Celebrex individually and in combination. Body weight and diet composition were monitored during the experiment period. Body weights are plotted as a function of weeks of experimental diet feeding.

nodes were fixed in 10% neutral-buffered formalin, embedded in paraffin blocks, and processed by routine H&E staining. In addition, multiple samples of tumors from the small intestines and colons and normal-seeming colonic mucosa were harvested and stored in liquid nitrogen for analysis of HMGR, COX-2, and caspase-3 activities and expression levels.

Apoptosis. Intestinal tissues (normal mucosa and tumors) were fixed in 10% formalin for 24 hours and then embedded in paraffin. Sections, about 6 μm, were cut and mounted on slides, rehydrated, and stained using the terminal deoxynucleotidyl transferase (TdT)–mediated nick-end labeling (TUNEL) method for the detection of apoptosis (33). Briefly, slides were incubated with 3% H_2O_2 in PBS for 5 minutes, rinsed, and then incubated in TdT buffer [140 mmol/L cacodylate (pH 7.2), 30 mmol/L Tris-HCl, and 1 mmol/L $CoCl_2$] for 15 minutes at room temperature. TdT reaction mixture [0.2 unit/μL TdT, 2 nmol/L biotin-11-dUTP, 100 mmol/L cacohydrate, 2.5 mmol/L $CoCl_2$, 0.1 mmol/L DTT, and 0.05 mg/mL bovine serum albumin (BSA)] was added, and the slides were incubated for an additional 30 minutes at 37°C. After blocking with 2% BSA and incubation with avidin-biotin peroxidase complexes, the TUNEL reaction was visualized by chromogenic staining with 3,3′-diaminobenzidine, and slides were counter-stained by hematoxylin. Stained apoptotic epithelial cells (a minimum of 10 microscopic fields per section) were counted manually in a single-blend fashion.

HMGR activity. In colonic tumor tissues, HMGR was analyzed according to Brown et al. (35) by using [^{14}C]HMG-CoA as a substrate. Briefly, mucosa and tumor tissue were homogenized with a phosphate buffer (pH 7.2) containing 100 mmol/L sucrose, 50 mmol/L KCl, 2 mmol/L EDTA, 100 mmol/L potassium phosphate, and 3 mmol/L DTT. Microsomes were prepared by centrifugation at 100,000 × *g* for 1 hour at 4°C. The assay was carried out in total reaction mixture of 200 μL containing 20 μL of 35 nmol DL-3-hydroxy-3-methyl-[^{14}C]glutaryl-CoA (60 mCi/mmol; Amersham, Arlington Heights, IL), 20 μL of mercaptoethanol, 60 μmol/L of 150 μg of protein, and 100 μL of NAPDH-generating system (20 μL of 200 mmol/L glucose-6-phosphate, 30 μL of 1 mol/L phosphate buffer). The reaction was continued for 30 minutes at 37°C and then terminated with 15 μL of 6 N HCl. After centrifugation, the supernatant was extracted twice with 500 μL of ethyl acetate, evaporated to dryness, and then dissolved in acetone before being subjected to a silica gel TLC. The chromatogram was developed by 1:1 (v/v) of toluene/acetone solvent system, and the resulting radiolabeled separations were analyzed by a BioScan System 200 image scanning counter (BioScan, Inc., Washington, DC) equipped with B detector.

COX-2 activity. Activities in intestinal tumor samples were assayed using a slight modification of a previously published method (36). Briefly, 150 μL of the reaction mixture containing 12 μmol/L [^{14}C]arachidonic acid (AA; 420,000 dpm), 1 mmol/L epinephrine, 1 mmol/L glutathione in 50 mmol/L phosphate buffer (pH 7.4), and 30 μg of microsomal protein from intestinal polyps were used for each assay. To determine COX-2 activity, the reaction mixtures were preincubated with 150 μmol/L aspirin to block COX-1 activity and to modify COX-2 activity to 15-(*R*)-hydroxyeicosatetraenoic acid (HETE; refs. 37, 38). After incubation at 37°C for 20 minutes, the reaction was terminated by adding 40 μL of 0.2 mol/L HCl. The COX-mediated metabolites of AA were extracted with ethyl acetate (3 × 0.5 mL). The combined extracts were evaporated to dryness under N_2, dissolved in chloroform, and subjected to TLC on precoated plastic plates (silica G60, 20 × 20 cm, layer thickness = 150 μm). The TLC plates were developed with a solvent system containing chloroform/methanol/acetic acid/water (100:15:1.25:1, v/v/v/v). They were then exposed in an iodide chamber for 5 minutes to visualize the standards. The metabolites of [^{14}C]AA, corresponding to prostaglandin (PGE_2, $PGF_{2\alpha}$, PGD_2, 6-keto-and $PGF_{1\alpha}$) and thromboxane B_2 for COX-1 activity and 15-(*R*)-HETE for COX-2 activity were detected by their comigration (R_f values) with authentic standards. The area of each metabolite was determined in a Bioscan System 200 image scanning counter (Bioscan) equipped with a β-detector.

Caspase-3 activity. Colonic mucosal and tumor samples were homogenized in a buffer containing 50 mmol/L Tris (pH 7.4), 50 mmol/L β-glycerol phosphate, 15 mmol/L $MgCl_2$, 15 mmol/L EDTA, 100 μmol/L phenylmethylsulfonyl fluoride, 1 mmol/L 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; dichlorodiphenyltrichloroethane, and 100 μg digitonin. The homogenate was centrifuged at 12,500 × *g* for 20 minutes at 4°C. Caspase activities in the supernatant fractions were determined by fluorogenic assay–specific substrate of caspase-3 (39). Briefly, 10 μg of sample protein was incubated with 200 μmol/L fluorogenic peptide substrates (AC-DEVD-MCA, caspase-3) in 50 μL of a protease assay buffer containing 100 mmol/L HEPES (pH 7.5), 100% sucrose, 10 mmol/L DTT, and 0.1% 3-[(3-cholamidopropyl) dimethylaminol]-1-propanesulfomate. Following incubation at 37°C for 2 hours, the release of AMC was measured with excitation at 360 nm and emission at 460 nm using a fluorescence spectrophotometer. Unit enzyme activity of the caspase is defined as the release of 1 pmol of AMC per minute.

Western blot analysis of HMGR, COX-2, caspase-3, and poly(ADP-ribose) polymerase. Intestinal polyps isolated from individual mice were combined to obtain sufficient tissue (6-8 samples per group). Normal-seeming intestinal mucosal samples were homogenized in 1:3 volume of 100 mmol/L Tris-HCl buffer (pH 7.2) with 2 mmol/L $CaCl_2$. After centrifugation at 100,000 × *g* for 1 hour at 4°C, the resulting separations were subjected to 8% SDS-PAGE for HMGR, COX-2, and poly(ADP-ribose) polymerase (PARP)

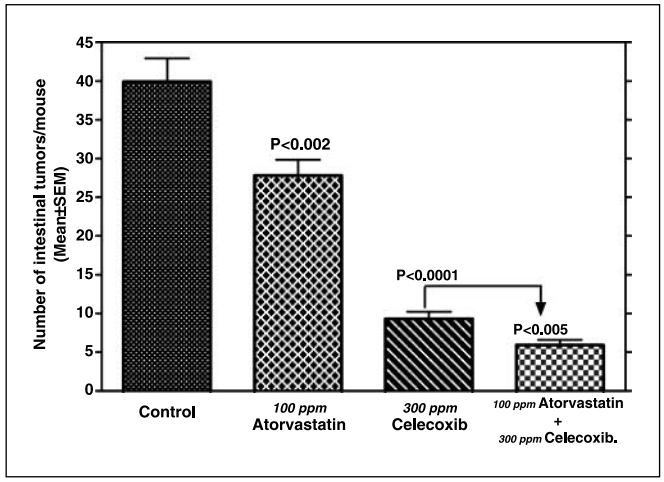

Figure 4. Effect of atorvastatin and celecoxib on intestinal tumor (small intestinal plus colon) formation in *APC*^{min} mice. *Columns,* mean number of intestinal tumors (n = 10) in *APC*^{min} mice given control and experimental diets containing 100 ppm atorvastatin and 300 ppm celecoxib individually and in combination; *bars,* SE.

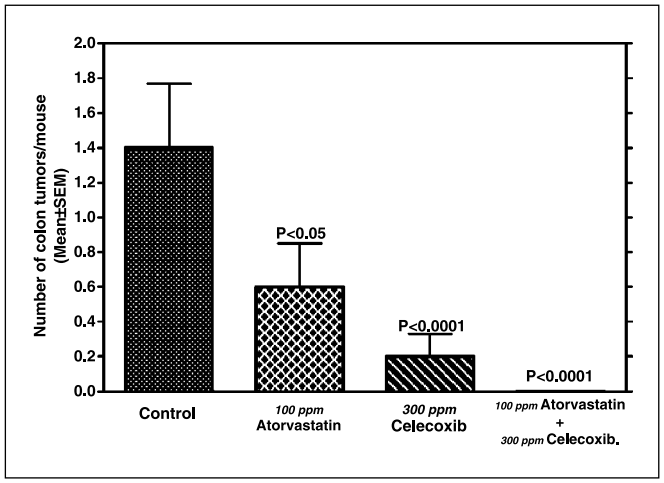

Figure 5. Effect of atorvastatin and celecoxib on colon tumor formation in APC^{min} mice. *Columns,* mean number of colon tumors ($n = 10$) in APC^{min} mice administered control and experimental diets; *bars,* SE. Significance between control and treatment groups were analyzed by *t* test.

and 12% for caspase-3. The proteins were electroplated onto polyvinylidene difluoride nitrocellulose membranes as described previously (8). These membranes were blocked for 1 hour at room temperature with 5% skim milk powder and probed with primary antibodies for 1 hour. The primary antibodies COX-2 (Cayman Chemicals, Ann Arbor, MI), HMGR (Upstate, Charlottesville, VA), caspase-3, and PARP (Santa Cruz Biotechnology, Santa Cruz, CA) were used 1:500 dilutions. Blots were washed thrice and incubated with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) at 1:2,500 dilutions for 1 hour. The membranes were washed thrice and incubated with Super-Signal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL) for 5 minutes and exposed to Kodak XAR5 photographic film and developed to detect proteins. Intensities of each band were scanned by a computing densitometer. α-Tubulin (Ab-1) mouse monoclonal antibody (Oncogene, San Diego, CA) was used at 1:1,000 dilution as the internal standard for all Western blots.

Statistical analyses. Differences in the body weights and tumor multiplicities (number of tumors per animal) were compared among the groups based on one-way ANOVA followed by Dunnett's multiple comparisons procedure in which several treatment groups are compared with a control. COX-2 and HMGR expression was compared between the mice fed the control diet and either celecoxib or Lipitor or the combined diets. All results are expressed as means ± SE and were analyzed by Student's *t* test. Differences were considered significant at the $P < 0.05$ level.

Results

General observations. Body weights of the wild-type mice fed the control and experimental diets containing test agents were comparable throughout the period of study (data not shown). $APC^{min/+}$ mice fed the control diet showed significantly lower body weight gain than the $APC^{min/+}$ mice fed the experimental diets containing either celecoxib or a combination atorvastatin and celecoxib (Fig. 3). The body weight loss in control group mice is mainly due to the increased small intestinal tumor burden and impairment of food absorption and anemia. Mice fed atorvastatin showed slightly lower bodyweight gains compared with celecoxib or combination due to the tumor burden. None of the animals fed with experimental diets produced any observable toxicity or any gross changes attributable to liver, kidney, or lung toxicity.

Intestinal tumor data (incidence and multiplicity). $APC^{min/+}$ mice developed intestinal tumors (polyps), and most of these tumors (>95%) occurred in the small intestine. On average, these mice developed between 18 to 62 tumors per mouse in the small intestine but only between 0 to 4 tumors per mouse in the colon. All of the histopathologically classified tumors in the small intestine and those in the colon were adenomas (adenomatous polyps), with no evidence of local invasion of the lamina propria. Figure 4 summarizes the chemopreventive effect of atorvastatin and celecoxib given individually and in combination according to tumor multiplicity of $APC^{min/+}$ mice. Dietary administration of atorvastatin at 100 ppm significantly ($P < 0.002$) suppressed intestinal polyp formation by about 30% (26.8 tumors per mouse) when compared with the control diet group (39.9 tumors per mouse). Dietary administration of celecoxib at 300 ppm significantly ($P < 0.0001$) decreased the formation of intestinal tumors by about 70% (9.5 tumors per mouse). Administration of these two agents in combination suppressed the tumor formation by about 86% compared with animals fed the control diet. Importantly, the inhibition of intestinal tumor formation in mice given the combination of atorvastatin at (100 ppm) and celecoxib (300 ppm) is highly significant ($P < 0.005$) when compared with tumor inhibition achieved by celecoxib alone. When data were analyzed for colon alone, we observed the following mean number of tumors per mouse: control, 1.4; 100 ppm atorvastatin, 0.6; 300 ppm celecoxib, 0.2; and 100 ppm atorvastatin plus 300 ppm celecoxib, 0 (Fig. 5). Colon tumor incidences were 80%, 40%, 10%, and 0% for control, atorvastatin, celecoxib, and combination of both the agents fed groups, respectively.

Apoptosis. Apoptotic rates in intestinal tumors by atorvastatin and celecoxib given individually and in combination in APC^{min} mice are summarized in Fig. 6. Induction of apoptosis was observed in mice fed with diets containing atorvastatin and celecoxib compared with control diet. Administration of atorvastatin induced a 3.5-fold increase in apoptosis compared with mice fed the control diet. Similarly, a 2.5-fold increase in induction of apoptosis was observed in intestinal tumors of mice fed with 300 ppm celecoxib. Atorvastatin induced greater apoptotic index than celecoxib, but it is not statistically not significant ($P > 0.08$). Interestingly, an additive induction (~5-fold) of apoptosis was observed in intestinal tumors in animals fed a combination of these two agents.

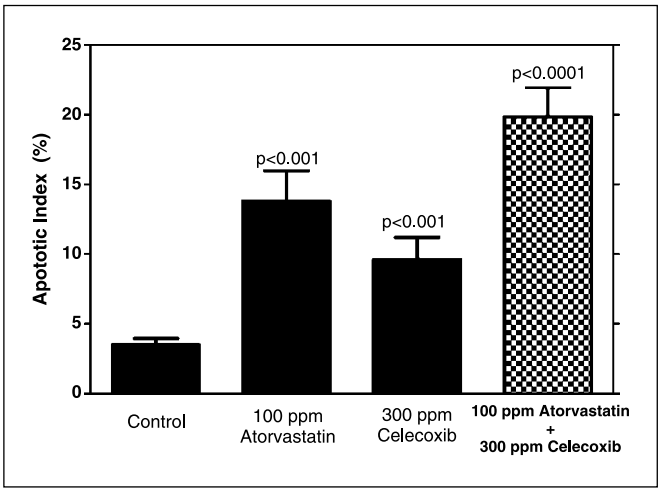

Figure 6. Effect of atorvastatin and celecoxib given individually and in combination in APC^{min} mice on intestinal apoptotic index by TUNEL method as detailed in Materials and Methods.

Modulation of HMGR activities. The effects of atorvastatin and celecoxib given individually and in combination on HMGR activity and expression levels in intestinal polyps of APC^{min} mice are shown in Fig. 7 and Fig. 10*A*. Administration of atorvastatin at 100 ppm levels significantly ($P < 0.005$) decreased the HMGR activity, and expression level was not significantly affected in the intestinal polyps. Diets containing 300 ppm celecoxib had no significant effect on HMGR activity and expression levels. However, a significant ($P < 0.0001$) decrease was observed in HMGR activity levels and to some extent in expression levels in intestinal polyps of APC^{min} mice fed with combinations of 100 ppm atorvastatin plus 300 ppm celecoxib.

Modulation of COX-2 activities. We studied the effect of atorvastatin and celecoxib given either individually or in combination on COX-2 activity (Fig. 8) and expression levels (Fig. 10*B*) in intestinal polyps of APC^{min} mice. Atorvastatin administration had no significant effect on COX-2 activity and expression levels. As anticipated, tumors of mice fed the celecoxib diet showed significantly ($P < 0.0001$) reduced levels of COX-2 activity and to some extent at COX-2 expression levels. Importantly, an additive inhibition in COX-2 activity and expression levels was observed in the mice fed with combinations of atorvastatin and celecoxib.

Modulation of caspase-3 activities. Caspase-3 seems to play a major role in the induction of apoptosis. Therefore, we next determined whether atorvastatin and celecoxib given individually and in combination could modulate the activity and expression levels of caspase-3 by inducing the apoptosis in intestinal polyps of APC^{min} mice. Figures 9 and 10 summarize the caspase-3 activity and expression levels in intestinal polyps of APC^{min} mice. Atorvastatin stimulated the intestinal tumor caspase-3 activity by 3-fold compared with the control diet. Similarly, a 2-fold increase in induction of caspase-3 activity was observed with 300 ppm celecoxib diet. Furthermore, an additive induction (~5-fold) in caspase-3 activity was observed in the animals fed with a combination of atorvastatin and celecoxib. A representative immunoblot analysis of caspase-3 expression in intestinal polyps of APC^{min} mice fed with atorvastatin and celecoxib individually and in combination is shown in Fig. 10*C*.

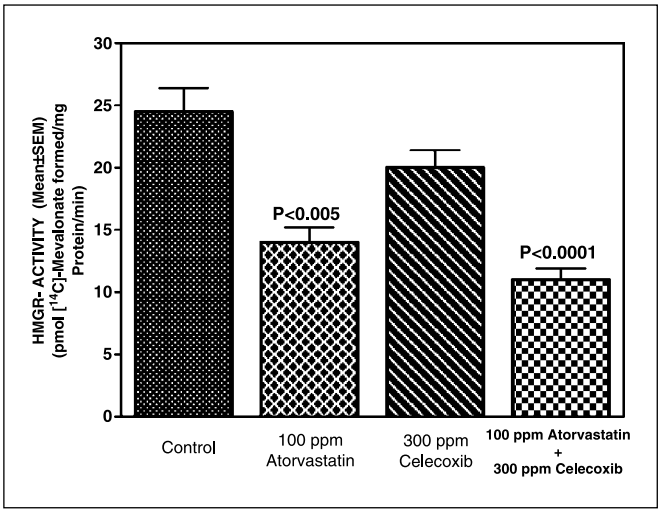

Figure 7. Effect of atorvastatin and celecoxib on intestinal tumor HMGR activity in APC^{min} mice. Tumor tissues obtained from different treatment groups were analyzed for HMGR activity (according to method described in Materials and Methods). *Columns,* mean; *bars,* SE. Significance was analyzed by *t* test.

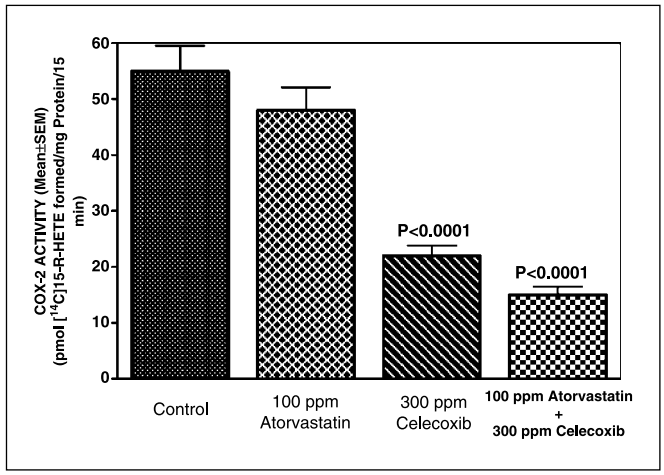

Figure 8. Effect of atorvastatin and celecoxib on intestinal tumor COX-2 activity in APC^{min} mice. Tumor tissues obtained from different treatment groups were analyzed for COX-2 activity (according to method described in Materials and Methods). *Columns,* mean; *bars,* SE. Significance was analyzed by *t* test.

PARP cleavage. We sought to determine whether increased activities of caspase-3 are associated with activation and cleavage of PARP in intestinal polyps of APC^{min} mice. Figure 10*D* summarizes the proteolytic cleavage of PARP by atorvastatin and celecoxib given individually and in combination in APC^{min} mice. PARP cleavage was clearly observed in atorvastatin and celecoxib given in intestinal polyps of APC^{min} mice compared with the control diet. The cleaved caspase-3 and PARP are the characteristic hallmarks of apoptosis that are observed in this present study.

Discussion

The specific objective of the present study was to develop novel strategies for the prevention of colorectal cancer in both preclinical and clinical settings. A key factor in testing the effectiveness of a combination of atorvastatin and celecoxib is to increase their efficacy while minimizing the side effects associated with long-term administration of the individual agents. Here, we examined the effects of atorvastatin and celecoxib, each possessing a different mode of action, on the inhibition of tumor formation and the modulation of HMGR and COX-2 expression and activities, including the levels of apoptosis in intestinal tumors of $APC^{min/+}$ mice. Our results suggest that atorvastatin and celecoxib given individually and/or in combination suppressed genetically predisposed neoplastic lesions associated with the *apc* gene in the gastrointestinal tract. Importantly, however, combining atorvastatin and celecoxib provides higher chemopreventive efficacy compared with the agent alone. Although, compared with human, the clinical usefulness of the $APC^{min/+}$ mice is moderated by the different geography of tumors occurring within the gastrointestinal tract, its clinical relevance for prevention studies is defined by its genetic etiology that closely mimics the mechanism of *APC* gene inactivation observed in FAP patients and most sporadic human colon adenomas (3, 40, 41).

Several studies have shown that chemopreventive agents that differ in their modes of action when administrated in combination are more efficacious even at low-dose levels. For example, combination of epidermal growth factor receptor (EGFR) inhibitor (EKB-569) and COX inhibitor (sulindac) suppressed intestinal polyp

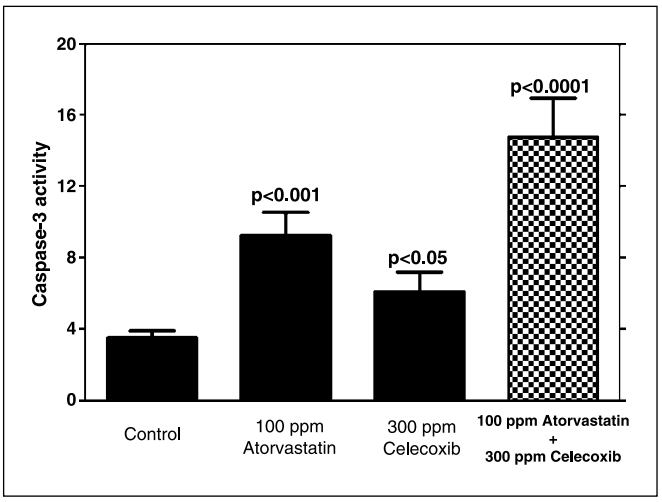

Figure 9. Effect of atorvastatin and celecoxib on intestinal tumor caspase-3 activity. Tissue lysates from different treatments groups were prepared, and activities of caspase-3 were measured using specific substrate as detailed in Materials and Methods. *Columns,* mean; *bars,* SE. Significance was analyzed by *t* test.

formation >95% more effectively in *Min* mice than each agents alone (42, 43). However, some of the toxicities associated with EKB-569 and possibly with EGFR inhibition limited the application of this combination in a clinical setting. Previously, we have shown that administration of low-dose combination of sulindac and lovastatin synergistically suppressed azoxymethane-induced colonic ACF formation in F344 rats (25). Furthermore, our most recent study has shown that low doses of Lipitor and Celebrex or aspirin given in combination synergistically suppressed both invasive and noninvasive adenocarcinomas of azoxymethane-induced colon carcinogenesis in the male F344 rat model (44). A recent clinical study also suggests that the use of statins was associated with a 47% relative reduction in risk of colorectal cancer after adjustment for other known risk factors (45). However, some of the recent clinical trial studies did not confirm the beneficial effect of statins against the colon cancer (46). In that study, the authors cannot rule out a reduction in risk of an effect associated with only specific types or doses of statins (46); moreover, they have not included any data from patients taking atorvastatin. Based on evidence from molecular mechanistic studies, preclinical and clinical studies further support development of statins for colon cancer prevention. Thus, our present results are consistent with the notion that agents with different modes of action can complement one another to inhibit colon tumorigenesis more effectively. This information emphasizes the importance of complementary pathways in enhancing chemopreventive efficacy of specific agents' combinations. The results of the present study show for the first time that a combination of 100 ppm atorvastatin and 300 ppm celecoxib suppresses the growth of colon tumors completely and small intestinal tumor formation by 86% and enhances apoptosis 2- to 3-fold compared with the actions of individual agents. Our present study further supports our previous observation that combining HMGR inhibitor lovastatin with celecoxib synergistically stimulates apoptosis in colon cancer cell lines (26). Several recent studies suggest that resistance to apoptosis in premalignant colonic epithelial cells may lead to the development of colon tumors (28–30). Our results suggest that atorvastatin produced a greater effect than celecoxib on apoptotic index and caspase-3 and PARP cleavage, but its ability to prevent tumors is lesser than celecoxib. Thus, in the present study, we have not observed any correlation between ability to prevent tumors to increase apoptosis and caspase-3 and PARP cleavage. It is possible that celecoxib ability to prevent tumors may predominately dependent on inhibition of cell proliferation when this agent is applied in low doses. Although, in the present study, we have not measured proliferative index, our previous *in vitro* results suggest that celecoxib at low concentrations significantly suppresses the cell proliferation and had limited effect on apoptosis (26, 47). COX inhibitors and other agents are known to induce apoptosis in various cell lines when applied in high concentrations (25, 48, 49). However, the results of this study show, for the first time, that low doses of atorvastatin in combination with celecoxib stimulate apoptosis in intestinal polyps to a far greater extent than what is seen with the individual agents. Exact mechanisms involved in the induction of apoptosis by atorvastatin and celecoxib are not yet fully understood; the data shown here would suggest the induction of caspase-3 expression in the tumors of atorvastatin- and Celebrex-treated APC^{min} mice might be implicated. Caspase-3, an effector molecule of apoptosis, plays an important role of downstreaming the apoptotic signaling pathway by cleaving and activating PARP and apoptotic processes (50, 51).

Mechanisms by which atorvastatin and celecoxib synergistically inhibit the development of adenomatous polyps is not fully known; it is likely due to the different modes of action of these agents when given in combination. At present, we do not know of any particular cellular target(s) that would be modulated by the combinational approach but not by individual agents. Discovery of cellular target(s) that modulated by low doses of efficient chemopreventive agent combinations is pivotal in understanding synergistic effects. It is well established that COX-2 plays a pivotal role in colon adenoma development in preclinical and clinical observations (10–15). Thus, celecoxib may block the activity of COX-2 that leads

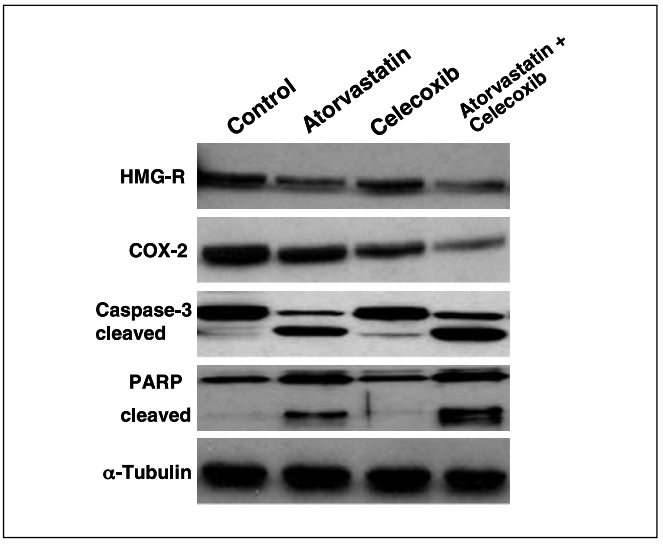

Figure 10. Expression levels of HMGR, COX-2, caspase-3, and PARP protein from tumor samples of mice administered with atorvastatin and celecoxib individually and in combination. Tissue lysates were homogenized in lysis buffer and were subjected to SDS-PAGE followed by Western blotting as described in Materials and Methods. Membranes were probed for HMGR, COX-2, caspase-3, and PARP specific primary antibodies and then peroxidase-conjugated appropriate secondary antibodies. Proteins were visualized with enhanced chemiluminescence detection system.

to reduced proliferation and enhanced apoptosis in colon tumor cells (12, 16, 17). In our recent studies, we have shown that celecoxib inhibits PGE_2 and its electrophilic metabolites in a dose-dependent manner in colon cancer cell lines (47, 52). This information suggests that one mechanism of COX-2-induced cell proliferation and lack of apoptosis may involve the impairment of p53 activity by electrophilic prostaglandins. Similar to NSAIDs, it has been shown that HMGR inhibitors, such as atorvastatin, modulate several molecular pathways, leading to induction of apoptosis and inhibition of tumor cell growth (53, 54). Precisely how the induction of apoptosis by atorvastatin occurs is not known. However, it is possible that inhibition of cholesterol formation by atorvastatin may alter the integrity of membranes (cell/mitochondrial) of tumor cells, thereby leading to activation of proapoptotic effector molecules, including caspase-3, as observed in the present investigation (55).

In summary, the present study shows for the first time that a dietary administration of atorvastatin and celecoxib in combination suppresses the tumorigenic effects of a germ line *APC* mutation in the *Min* mouse with increased efficacy compared with individual agents given alone. We have also shown an enhanced expression of apoptosis in mice given a combination of atorvastatin and celecoxib. Furthermore, our results suggest that stimulation of apoptosis induced by atorvastatin and celecoxib may be responsible, in part, due to the inhibition of HMGR and COX-2 activities, leading to the activation of caspase-3 and PARP. Although our understanding of the mechanisms of the chemopreventive action of atorvastatin and celecoxib individually and in combination is not yet fully appreciated, the development of effective combinations will serve as a practical approach toward the design of chemopreventive trials in humans.

Acknowledgments

Received 1/2/2006; revised 3/29/2006; accepted 5/17/2006.

Grant support: National Cancer Institute grants NCI-CA-94962 and NO1-CN-25114.

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We thank the technical expertise of the Research Animal Facility Biologists at the American Health Foundation, Valhalla, NY; Dr. Wade Williams for editing this article; Julie DeVore for assisting in the preparation of this article; and the AACR for the Travel Award to present part of this work at the 95th Annual AACR Meeting, 2004 in Orlando, FL.

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