Prevention of Azoxymethane-Induced Colon Cancer by Combination of Low Doses of Atorvastatin, Aspirin, and Celecoxib in F 344 Rats

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Abstract

Preclinical and clinical studies have provided evidence that aspirin, celecoxib, (cyclooxygenase-2 inhibitor), and statins (3-hydroxy-3-methylglutaryl CoA reductase inhibitors) inhibit colon carcinogenesis. Chronic use of high doses of these agents may induce side effects in ostensibly normal individuals. Combining low doses of agents may be an effective way to increase their efficacy and minimize toxicity. We assessed the efficacy of atorvastatin (lipitor), celecoxib, and aspirin, given individually at high dose levels and in combination at lower doses against azoxymethane-induced colon carcinogenesis, in male F 344 rats. One day after the last azoxymethane treatment (15 mg/kg body weight, s.c., once weekly for 2 weeks), groups of male F 344 rats were fed the AIN-76A diet or AIN-76A diet containing 150 ppm atorvastatin, 600 ppm celecoxib, and 400 ppm aspirin, 100 ppm atorvastatin + 300 ppm celecoxib, and 100 ppm atorvastatin + 200 ppm aspirin. Rats were killed 42 weeks later, and colon tumors were processed histopathologically and analyzed for cell proliferation and apoptosis immunohistochemically. Administration of these agents individually and in combination significantly suppressed the incidence and multiplicity of colon adenocarcinomas. Low doses of these agents in combination inhibited colon carcinogenesis more effectively than when they were given individually at higher doses. Inhibition of colon carcinogenesis by these agents is associated with the inhibition of cell proliferation and increase in apoptosis in colon tumors. These observations are of clinical significance because this can pave the way for the use of combinations of these agents in small doses against colon cancer. (Cancer Res 2006; 66(8): 4542-6)

Introduction

Colorectal cancer is one of the leading causes of cancer mortality in both men and women in the United States (1). Chemoprevention seems to play a major role in reducing the risk of death from colorectal cancer (2). Epidemiologic, clinical, and preclinical studies point to an inverse relationship between the use of nonsteroidal anti-inflammatory drugs (NSAID), including aspirin, and colorectal cancer development (3–5). Several studies indicate that cyclooxygenase-2 (COX-2)–specific inhibitors, including celecoxib, inhibit the intestinal adenomas in the mouse models of familial adenomatous polyposis (FAP) and in chemically-induced colon carcinogenesis in rats (6–15). FAP patients treated twice daily with 400 and 200 mg celecoxib had 31% and 12% reduction, respectively, in polyp number (16). However, long-term use of high doses of NSAIDs, including COX-2 inhibitors in humans, may result in gastrointestinal, cardiovascular, and renal effects, raising several concerns about clinical application.

Statins are a class of agents that inhibit 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), a rate-limiting enzyme in mevalonate synthesis, leading to inhibition of cholesterol biosynthesis. These drugs have also been used extensively to reduce serum cholesterol in patients with hypercholesterolemia. In this regard, statins have been shown to interfere with isoprenylation and subsequent membrane localization of G-proteins, including Ras and Ras-related proteins (17, 18). Randomized controlled clinical trials for preventing cardiovascular disease provided evidence that statins can prevent colorectal cancer (19). This excellent review by Demierre et al. (19) summarized the results of observational, preclinical, and randomized clinical studies, which show promise for cancer prevention in several organ sites by statins. Clinical trials in patients with coronary heart disease with pravastatin and simvastatin showed a lower number of newly diagnosed cases of colon cancer during a 5-year follow-up period (20). Other studies also indicated that statins are protective against colorectal cancer (21). *In vitro* studies using colon cancer cell lines indicate that statins inhibit cell growth and induce apoptosis (22–24). In human colon cancer cell lines, statins have been shown to induce apoptosis (22). Because HMG-CoA reductase, the target of statins, is overexpressed in colon cancer cell lines (23, 25), its inhibition is thought to contribute to this effect. Narisawa et al. (26, 27) were the first to report that lovastatin and pravastatin inhibit chemically induced colon carcinogenesis in F 344 rats. Farnesol and lanosterol, which are feedback inhibitors of HMG-CoA reductase, have been found to inhibit azoxymethane-induced colonic aberrant crypt foci (ACF) in the rat colon cancer model (28).

There is increasing interest to use a combination of low doses of chemopreventive agents that differ in their modes of action to increase their efficacy and minimize toxicity. Studies conducted in our laboratory have provided evidence that the combination approach provides greater efficacy than when the chemopreventive agents were given alone (7, 28–30). In this regard, Agarwal et al. (22) showed that combination of lovastatin and sulindac were more effective in inhibiting azoxymethane-induced colonic ACF in male F 344 rats than when these agents were given singularly. We also showed that treatment with combinations of low doses of celecoxib and lovastatin synergistically suppressed the growth of

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HT-29 human cancer cells as well as induced apoptosis (29). Here, we assessed the efficacy of celecoxib, aspirin, and atorvastatin given, individually at higher doses and in combination at lower doses against azoxymethane-induced colon adenocarcinomas, in male F 344 rats. In addition to their chemopreventive efficacy, we determined the effects of these agents on the modulation of cell proliferate and apoptotic indices in colon tumors.

Materials and Methods

Materials. Atorvastatin and celecoxib were provided by the National Cancer Institute's Repository. Azoxymethane was purchased from Midwestern Research Institute (Kansas City, MO).

Animals and diets. Weanling male inbred F 344 rats were obtained from Charles River Breeding Laboratories (Kingston, NY). All ingredients of the experimental diets were purchased from the Dytes, Inc. (Bethlehem, PA) and stored at 4°C before preparation of diets.

Male F 344 rats received at weaning were quarantined for 7 days and had access to AIN-76A diet. Following quarantine, all animals were randomly distributed by weight into various experimental groups and transferred to an animal holding room. They were housed in plastic cages with filter tops, three per cage, under controlled conditions of a 12-hour light/12- hour dark cycle, 50% humidity, and 21°C temperature. All experimental and control diets were prepared weekly in our laboratory and stored in a cold room. Animals had access to food and water at all times. Food cups were replenished with fresh diet twice weekly.

Efficacy studies. Following quarantine, all animals were distributed by body weight into control and experimental groups. At 7 weeks of age, groups of male F 344 rats (30 per group) intended for carcinogen treatment received s.c. injections of azoxymethane (15 mg/kg body weight, once weekly for 2 weeks) and vehicle controls (12 per group) received equal volume of normal saline. One day later, they were maintained on AIN-76A diet containing 0, 600 ppm celecoxib, 400 ppm aspirin, 150 ppm atorvastatin, 300 ppm celecoxib + 100 ppm atorvastatin, or 200 ppm aspirin + 100 ppm atorvastatin and continued for 42 weeks. The high doses of celecoxib and aspirin were selected based on our previous studies, which indicate that these doses were found to be nontoxic (7, 14, 15). The dose selection for atorvastatin was based on our unpublished data, indicating that 250 ppm atorvastatin was nontoxic.

Rats in each group were weighed once weekly until they reached 16 weeks of age and then once every 4 weeks until termination of the study at 42 weeks after the second azoxymethane treatment. As scheduled, all rats in each group were killed by CO_2 asphyxiation. The entire gastrointestinal tract was resected, opened, cleaned with normal saline, and examined for intestinal tumors under the dissection microscope. Colon tumors were noted grossly for their location and number. For histopathologic evaluation, colon tumors were fixed in 10% buffered formalin, embedded in paraffin blocks, and processed by routine histologic procedures with H&E staining. The stained sections were examined by a pathologist for tumor types (14). The end points were colon tumor incidence (% animals with colon adenocarcinomas) and multiplicity (number of adenocarcinomas per rat).

Cell proliferation and apoptosis in colon tumors. We investigated whether the inhibition of colon tumorigenesis by the chemopreventive agents given individually and in combination is associated with the modulation of cell proliferation and apoptosis in colon adenocarcinomas. These variables were measured in formalin-fixed, paraffin-embedded blocks of colon tumors. The number of colon tumors that were evaluated in each group are as follows: control group, n = 7; atorvastatin, 150 ppm, n = 10; celecoxib, 600 ppm, n = 11; aspirin, 400 ppm, n = 11; atorvastatin, 100 ppm + celecoxib, 300 ppm, n = 8; and atorvastatin, 150 ppm + aspirin 200 ppm, n = 8. Tumor samples were randomly picked for cell proliferation and apoptosis. Four-micrometer-thick sections were cut from the paraffin-embedded blocks and mounted on glass slides coated with 3-aminopropylmethoxysilane.

Apoptosis and proliferation. The apoptotic cells were detected using an ApopTag *In situ* Apoptosis Detection kit (Chemicon, Temecula, CA). The assay was done according to the manufacturer's manual. After deparaffinization, the sections were incubated in proteinase K for 15 minutes at room temperature. The sections were then incubated with terminal deoxynucleotidyl transferase enzyme at 37°C for 1 hour, washed in three changes of PBS, and incubated with anti-digoxignenin conjugate in a humidified chamber at room temperature for 30 minutes. The color was developed by incubating the sections with peroxidase substrate and then counterstained with hematoxylin for 30 seconds. For detection of proliferative cells, proliferating cell nuclear antigen (PCNA) antibody (1:50; DAKO, Carpinteria, CA) was used. The assay was done following the manufacturer's protocols. The scoring of apoptotic and proliferative cells was done at ×400, and at least 1,000 cells were counted at each section. A positive control slide of rat mammary glands provided by the manufacturer was used as positive control for the *in situ* apoptosis detection assay. For PCNA staining, colonic crypt cells were used as an internal positive control.

Evaluation of staining. For PCNA staining, cells with a blue nucleus were considered unlabeled/negative, whereas cells with a brown nucleus were considered labeled/positive. The apoptotic and proliferative indices were calculated as number of positive cells in the lesions divided by the total cell number counted multiplied by 100.

Statistical analysis. Differences in tumor incidence (% animals with colon adenocarcinomas) were analyzed by Fisher's exact probability test, and the tumor multiplicity (number of colon adenocarcinomas/rat), cell proliferation, and apoptosis indices were analyzed by Student's t test. All results were expressed as mean ± SE. Differences were considered significant at $P < 0.05$.

Results

General observations. Body weights of animals treated with vehicle (saline) or azoxymethane and given chemopreventive agents alone or in combination were comparable with those fed control diet throughout the study (data not shown). Animals fed chemopreventive agents alone or in combination showed no evidence of gastrointestinal ulcers and bleeding or other signs of toxicity nor any gross changes indicative of toxicity at the selected doses. This was true whether the rats were treated with azoxymethane or saline. Saline-treated rats fed the control or experimental diets containing chemopreventive agents showed no evidence of colon tumors.

Efficacy of atorvastatin, celecoxib, or aspirin given individually on colon tumor incidence and multiplicity. Results summarized in Fig. 1 show that the incidence of colon adenocarcinomas was 70% in animals fed the control diet. Administration of 150 ppm atorvastatin, 600 ppm celecoxib, or 400 ppm aspirin alone significantly inhibited incidence of colonic adenocarcinomas by about 34% ($P < 0.05$), 61% ($P < 0.001$), and 29% ($P < 0.05$), respectively, compared with those fed the control diet. The percentage inhibition of colon adenocarcinomas by atorvastatin, celecoxib, and aspirin was 34%, 61%, and 29%, respectively, compared with those fed the control diet. The degree of inhibition of incidence of colon tumor is more pronounced with 600 ppm celecoxib compared with 150 ppm atorvastatin or 400 ppm aspirin. Figure 2 summarizes the results on colon adenocarcinoma multiplicity. Administration of atorvastatin at 150 ppm and celecoxib at 600 ppm alone significantly suppressed the multiplicity of adenocarcinomas of the colon (number of adenocarcinomas per rat; $P < 0.05$) and ($P < 0.001$), respectively, compared with those animals fed the control diet. However, 400 ppm aspirin, slightly albeit not significantly inhibited multiplicity of the adenocarcinomas of the colon ($P > 0.05$). The percentage of inhibition of colon tumor multiplicity by atorvastatin, celecoxib,

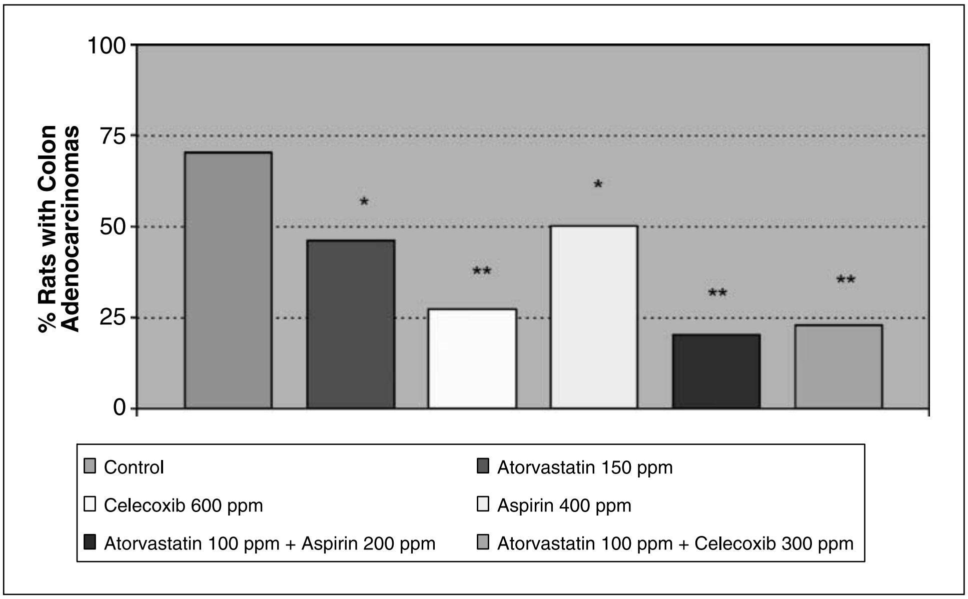

Figure 1. Effects of 150 ppm atorvastatin, 600 ppm celecoxib, and 400 ppm aspirin administered individually and 100 ppm atorvastatin + 300 ppm celecoxib and 100 ppm atorvastatin + 200 ppm aspirin on azoxymethane-induced colon adenocarcinoma incidence (% animals with tumors). *, $P < 0.05$, significantly different from the control diet group by Fisher's exact probability test; **, $P < 0.001$, significantly different from the control diet group.

and aspirin compared with those fed the control diet was by about 37%, 76%, and 17%, respectively.

Efficacy of low doses of atorvastatin, celecoxib, or aspirin given in combination on colon tumor inhibition. Administration of 100 ppm atorvastatin + 300 ppm celecoxib resulted in the inhibition of incidence of adenocarcinomas to about 71% ($P < 0.001$; Fig. 1) and multiplicity to about 90% ($P < 0.0001$) compared with those fed the control diet (Fig. 2). In addition, dietary administration of atorvastatin at 100 ppm together with aspirin at 200 ppm significantly suppressed the incidence of adenocarcinomas of the colon by about 67% ($P < 0.001$) and the multiplicity by about 64% ($P < 0.01$) compared with rats fed control diet (Figs. 1 and 2). These results suggest that low doses of these agents in combination inhibit colon carcinogenesis better than when they are given individually at high doses.

Effect of atorvastatin, celecoxib, or aspirin given individually and in combination on cell proliferation and apoptosis in colon tumor. The results summarized in Table 1 indicate that administration of 150 ppm atorvastatin, 600 ppm celecoxib, and 400 ppm aspirin alone and 100 ppm atorvastatin + 300 ppm celecoxib and 100 ppm atorvastatin + 200 ppm aspirin in combination significantly suppressed the proliferative index and

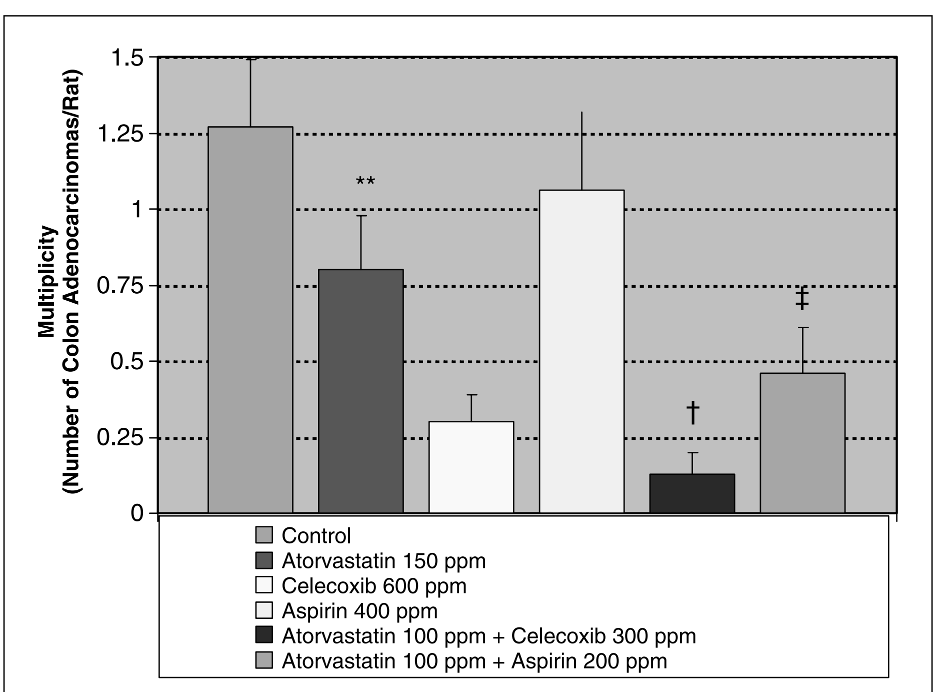

Figure 2. Effect of 150 ppm atorvastatin, 600 ppm celecoxib, and 400 ppm aspirin administered individually and 100 ppm atorvastatin + 300 ppm celecoxib and 100 ppm atorvastatin + 200 ppm aspirin on multiplicity of azoxymethane-induced colon adenocarcinomas (tumors/rat). *Columns,* means; *bars,* SE. *, $P < 0.05$, significantly different from the control group by Student's *t* test; ‡, $P < 0.01$, significantly different from the control diet group; **, $P < 0.001$, significantly different from the control diet group; †, $P < 0.0001$, significantly different from the control diet group.

Table 1. Effect of atorvastatin, celecoxib, and aspirin given individually and in combination on apoptotic and proliferatic indices in azoxymethane-induced colonic adenocarcinomas

Group no.	Treatment	Apoptotic index (%)	*P*	Proliferative index (%)	*P*
1	Control	4.15 ± 0.85*	—	54.44 ± 5.78	—
2	Atorvastatin, 150 ppm	8.23 ± 1.43 (98%)†	<0.05	37.28 ± 4.29 (32%)‡	<0.010
3	Celecoxib, 600 ppm	11.00 ± 2.30 (165%)†	<0.02	33.81 ± 3.30 (38%)‡	<0.002
4	Aspirin, 400 ppm	13.83 ± 0.31 (233%)†	<0.001	37.26 ± 4.79 (32%)‡	<0.01
5	Atorvastatin, 100 ppm + celecoxib, 300 ppm	12.97 ± 1.62 (212%)†	<0.003	41.61 ± 1.52 (24%)‡	<0.02
6	Atorvastatin, 100 ppm + aspirin, 200 ppm	10.06 ± 1.80 (142%)†	<0.01	41.90 ± 3.26 (24%)‡	<0.05

*Mean ± SE.
†% Increase compared with those fed the control diet.
‡% Decrease compared with those fed the control diet.

significantly increased the apoptotic index compared with those fed the control diet. These findings suggest that inhibition of colon carcinogenesis by these agents are due to alterations in cell proliferation and apoptosis.

Discussion

The major goal of this study is to develop novel strategies for colon cancer prevention by means of combining low doses of potential chemopreventive agents to increase their chemopreventive efficacy and to minimize toxic side effects associated with long-term administration of such agents to otherwise healthy individuals.

The present study shows for the first time that the administration of atorvastatin alone at 150 ppm in the diet significantly suppressed azoxymethane-induced colon tumorigenesis. Previous studies showed that pravastatin, another HMG-CoA reductase inhibitor, suppressed methylnitrosourea- and 1,2-dimethylhydrazine-induced colon carcinogenesis, and lovastatin inhibited azoxymethane-induced colonic ACF in F 344 rats (22, 26, 27). Our studies on atorvastatin and those of others on pravastatin and lovastatin are further evidence for the potential of these agents as chemopreventives against colon carcinogenesis. The role of apoptosis in colon carcinogenesis has been extensively studied, suggesting that resistance to apoptosis in premalignant colonic epithelial cells will lead to the development of colon tumors (29–31). HMG-CoA reductase inhibitors and COX-2 inhibitors are known to induce apoptosis in various cell lines when applied in high concentrations (22, 29, 31–35). In the present study, administration of atorvastatin or celecoxib individually or in combinations significantly induced the colon tumor cell apoptosis and inhibited cell proliferation. These results are consistent with our earlier observations that HMG-CoA reductase inhibitors and COX-2 inhibitors induce apoptosis (22, 29). The mechanisms by which HMG-CoA reductase and COX-inhibitors induce tumor cell apoptosis and reduce proliferation are yet to be fully established.

Our results provide compelling evidence that when compared with individual high doses of atorvastatin and celecoxib, combinations of these agents at low doses induce greater inhibition of colon carcinogenesis. Our results of this study indicate that celecoxib, atorvastatin, and/or aspirin given individually at high doses and in combination at low doses showed no evidence of gastrointestinal ulceration and bleeding, suggesting absence of any side effects. The results of this study are in line with previous studies indicating that combination of low doses of piroxicam, an NSAID together with difluoromethylornithine (an ornithine decarboxylase inhibitor; ref. 30), lovastatin, and sulindac (an NSAID), were more effective in inhibiting the colon carcinogenesis than the agents given alone (22). Human clinical trials using NSAIDs and statins should consider relevant data regarding the efficacy and safety of these agents at very high dose levels, because it is imperative that chemopreventive agents be well tolerated and safe for long-term use.

Statins are most commonly used in the elderly population to treat coronary heart disease and hypercholesterolemia. Therefore, the potential antitumor effects of statins shown here are particularly relevant (19). Additional cohorts at risk of cancer are also likely to benefit by taking statins (19). The rationale for combinations with statins is based on the knowledge of biosynthetic mechanisms, where in addition to inhibiting HMG-CoA reductase, they also inhibit the isoprenylation and subsequent membrane localization of the ras family of oncogenes as well as potentially reducing the flow and production of estrogen and testosterone in at risk individuals. Together, our data indicate that atorvastatin as a single agent or combined with celecoxib and aspirin should be evaluated against colorectal cancer. In a broader sense, our present observations are of clinical significance because this can pave the way for the use of combination of these agents in small doses whose added chemopreventive effect would be significant with minimal side effects. Significantly, individuals currently treated with statins are also given NSAIDs and COX-2 inhibitors at low doses of combinations of value in the area of public health.

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