Atorvastatin and Celecoxib Inhibit Prostate PC-3 Tumors in Immunodeficient Mice

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Abstract

Purpose: To investigate the effects and mechanisms of atorvastatin and celecoxib administered individually or in combination on human prostate cancer PC-3 cells cultured in vitro or grown as xenograft tumors in immunodeficient mice.

Experimental Design: Human prostate cancer PC-3 cells in culture were treated with atorvastatin and celecoxib alone or in combination. Severe combined immunodeficient (SCID) mice were injected s.c. with PC-3 cells. The mice received daily i.p. injections starting 2 days before tumor cell inoculation and continuing during the course of treatment with atorvastatin (10 µg/g body weight/d), celecoxib (10 µg/g/d), a combination of atorvastatin (10 µg/g/d) and celecoxib (10 µg/g/d), or a combination of atorvastatin (5 µg/g/d) and celecoxib (5 µg/g/d).

Results: Atorvastatin in combination with celecoxib had stronger effects on growth inhibition and apoptosis of PC-3 cells than either agent used individually. Atorvastatin and celecoxib in combination also had a stronger inhibitory effect on activation of nuclear factor-κB and extracellular signal-regulated kinase 1/2 in PC-3 cells than either agent alone. Treatment of SCID mice with combinations of atorvastatin and celecoxib more effectively inhibited the formation and growth of PC-3 tumors in the mice than either agent administered alone.

Conclusions: A combination of atorvastatin and celecoxib had a more potent inhibitory effect on the growth of PC-3 cells cultured in vitro or grown in SCID mice than either agent alone. A combination of atorvastatin and celecoxib may be an effective strategy for the prevention of prostate cancer.

Early studies have shown the presence of prostate cancer cells in a large number of men, but in many of these individuals, the cells remain dormant and do not form clinically relevant tumors that kill the individual (1). Why these cells multiply and form lethal metastatic tumors in some individuals but remain dormant in others is an important question. Autopsies of elderly men in Asia and the United States indicated that most elderly men without clinically diagnosed prostate cancer in both Japan and the United States had a high incidence of histologically diagnosed prostate cancer. However, prostate cancer deaths in the United States were considerably higher than in Japan (2–4). These observations indicate that environmental/lifestyle factors influence the progression of dormant prostate cancer cells to lethal cancers. Prostate cancer cells often invade the bone marrow. These cells remain dormant, and the time between dissemination to evidence of clinically important metastatic disease may be prolonged for more than 10 years (discussed in ref. 5). The mechanism for the activation and growth of dormant prostate cancer cells is unknown. A recent study suggested that a low ratio of phosphorylated extracellular signal-regulated kinase (phospho-Erk)/p38 is associated with dormancy for several cancer cell lines including the PC-3 androgen-independent prostate cancer cell line and that increased phospho-Erk is associated with increased proliferation (6). An important approach to cancer prevention is initiating changes in the diet or lifestyle or the administration of drugs that either prevent the formation of cancer cells or prevent the formation of lethal cancer from otherwise dormant cancer cells. In the present study, we investigated the effects of administration of atorvastatin (Lipitor) and celecoxib (Celebrex) alone and in combination on the formation and growth of tumors after s.c. injecting androgen-independent PC-3 human prostate cancer cells into immunodeficient mice.
Atorvastatin (Lipitor) is a synthetic statin (7). The statin family of drugs inhibits 3-hydroxy-3-methylglutaryl CoA reductase and is used clinically as a safe and effective approach for the control of hypercholesterolemia (7). Recent studies have indicated that in addition to the cholesterol-lowering effect, statins have pleiotropic activities that modulate other biological processes, such as cell proliferation and apoptosis (8). Although epidemiologic studies investigating statin use and total cancer risk as the primary outcome have yielded conflicting results (9–11), a recent case-control study indicates that statins may reduce the risk of total prostate cancer as well as more aggressive prostate cancer (12). In another clinical study, Moyad et al. (13) reported that a significantly lower prostate-specific antigen level, a lower percentage of positive biopsy score and an earlier clinical stage were found in the statin use group. Although atorvastatin and other statin drugs have been found to induce apoptosis in prostate cancer cells (14, 15), the effects of atorvastatin alone or in combination with other agents on the formation and growth of prostate xenograft tumors in immunodeficient mice are not known. Celecoxib (Celebrex) is a selective cyclooxygenase-2 inhibitor. Recent studies showed that celecoxib suppressed prostate carcinogenesis and caused regression of prostatic intraepithelial neoplasia in the transgenic adenocarcinoma of mouse prostate model (16, 17). In a pilot study, prostate cancer patients who had relapsed after radiation therapy or radical prostatectomy were treated with celecoxib 200 mg twice daily (18). Follow-up prostate-specific antigen levels were obtained at 3, 6, and 12 months after initiating treatment. Decreased serum prostate-specific antigen levels and increased prostate-specific antigen doubling time were found in some of the patients, suggesting that celecoxib may help prevent or delay disease progression in these prostate cancer patients (18). In a recent study, we found that celecoxib in combination with atorvastatin significantly suppressed the incidence and multiplicity of colon adenocarcinoma in the azoxymethane-induced rat colon carcinogenesis model (19). It is of great interest to investigate the effects of celecoxib in combination with atorvastatin on the development and growth of prostate cancer in animal models.

Combinations of low doses of anticancer agents that have different mechanisms of action may have significantly improved efficacy for inhibiting tumor formation and tumor growth when compared with individual anticancer substances alone. In the present study, we assessed the effects of atorvastatin and celecoxib alone or in combination on growth and apoptosis in cultured human prostate cancer PC-3 cells. We also assessed the effects of administration of these two agents alone or in combination on the formation and growth of PC-3 xenograft tumors in immunodeficient mice. We found that combinations of atorvastatin and celecoxib synergistically inhibited the growth and induced apoptosis in cultured PC-3 cells. Administration of a combination of these two agents also had a stronger inhibitory effect than either agent alone on the formation and growth of PC-3 xenograft tumors in immunodeficient mice.

Materials and Methods

Cell culture and reagents. PC-3 cells were obtained from the American Type Culture Collection. Atorvastatin and celecoxib were provided by the National Cancer Institute's Repository. Propylene glycol, polysorbate 80, benzyl alcohol, ethanol, and DMSO were purchased from Sigma. Matrigel was obtained from BD Biosciences. RPMI 1640 tissue culture medium, penicillin-streptomycin, t-glutamine, and fetal bovine serum were from Life Technologies. PC-3 cells were maintained in RPMI 1640 tissue culture medium containing 10% fetal bovine serum that was supplemented with penicillin (100 units/mL)-streptomycin (100 μg/mL) and t-glutamine (300 μg/mL). Cultured cells were grown at 37 °C in a humidified atmosphere of 5% CO2 and were passaged twice weekly. PC-3 cells were initially seeded at a density of 0.2 × 10^5 cells/mL in 35-mm tissue culture dishes (2 mL/dish) for assays of proliferation and apoptosis and seeded at a density of 1 × 10^4 cells/mL of medium in 100-mm tissue culture dishes (10 mL/dish) for the Western blot analysis. Atorvastatin and celecoxib were dissolved in DMSO and the final concentration of DMSO in all experiments was 0.2%.

Determination of the number of viable cells. The number of viable cells after each treatment was determined using a hemacytometer under a light microscope (Nikon Optiphot). Cell viability was determined by the trypan blue exclusion assay, which was done by mixing 80 μL of cell suspension and 20 μL of 0.4% trypan blue solution for 2 min. Blue cells were counted as dead cells and the cells that did not absorb dye were counted as live cells.

Morphologic assessment of apoptotic cells. Apoptosis was determined by morphologic assessment in cells stained with propidium iodide (20). Briefly, cytospin slides were prepared after each experiment and cells were fixed with acetone/methanol (1:1) for 10 min at room temperature, followed by 10 min with propidium iodide staining (1 μg/mL in PBS) and analyzed using a fluorescence microscope (Nikon Eclipse TE200). Apoptotic cells were identified by classic morphologic features, including nuclear condensation, cell shrinkage, and formation of apoptotic bodies (20). At least 200 cells were counted in each sample and the percentage of apoptotic cells was presented.

Western blotting. After treatment, PC-3 cells were washed with ice-cold PBS and lysed with 800 μL of lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 50 mmol/L sodium chloride, 30 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 100 μmol/L sodium orthovanadate, 2 mmol/L iodoacetic acid, 5 mmol/L ZnCl2, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.5% Triton X-100]. The homogenates were centrifuged at 12,000 × g for 15 min at 4 °C. The protein concentration of whole-cell lysates was determined with a Bio-Rad protein assay kit (Bio-Rad). Equal amounts (20 μg) of protein were then resolved on a 10% Criterion Precast Gel (Bio-Rad) and transferred to a polyvinylidene difluoride membrane using a semidy transfer system. The membrane was then probed with anti-β-actin/ERK1/2 primary antibody (Cell Signaling Technology). After hybridization with primary antibody, the membrane was washed with TBS thrice and then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and washed with TBS thrice. Final detection was done with enhanced chemiluminescent reagents. The extent of protein loading was determined by blotting for β-actin. The membrane was incubated in stripping buffer [100 mmol/L β-mercaptoethanol, 2% SDS, 62.5 mmol/L Tris-HCl (pH 6.7) at 50 °C for 30 min with occasional agitation before incubating in blocking buffer and reprobing using anti-β-actin (Santa Cruz Biotechnology).

Nuclear factor-κB–dependent reporter gene expression assay. Nuclear factor-κB (NF-κB) transcriptional activity was measured by the NF-κB-luciferase reporter gene expression assay (21). A NF-κB luciferase construct was stably transfected into PC-3 cells and a single stable clone, PC-3 C4 (21), was used in the present study. In brief, PC-3 C4 cells were treated with atorvastatin or celecoxib alone or in combination for 24 h, and the NF-κB-luciferase activities were measured using the luciferase assay kits from Promega. After treatments, the cells were washed with ice-cold PBS and harvested in 1× reporter lysis buffer. After centrifugation, 10 μL aliquots of the supernatants were measured for luciferase activity using a Luminometer from Turner Designs Instrument. The luciferase activity was normalized against known protein concentrations and expressed as percentage of luciferase.
activity in the control cells, which were treated with DMSO solvent. The protein level was determined by Bio-Rad protein assay kits according to the manufacturer’s instructions.

Caspase-3 (active form) immunostaining. An antibody that reacts with active form of caspase-3 was purchased from R&D Systems. Tumor sections used for the measurement of caspase-3 (active form) were stained by the horseradish peroxidase–conjugated avidin method with some modification. Briefly, sections were incubated with caspase-3 primary antibody (1:2,000 dilution) for 30 min at room temperature followed by incubation with a biotinylated antirabbit secondary antibody for 30 min and incubation with conjugated-avidin solution (ABC Elite kit purchased from Vector Laboratories) for 30 min. Color development was achieved by incubation with 0.02% 3,3′-diaminobenzidine tetrahydrochloride containing 0.02% hydrogen peroxide for 10 min at room temperature. The slides were then counterstained with hematoxylin and dehydrated, and coverslips were added for permanent mounting. A positive reaction was shown as a brown precipitate in the cytoplasm and/or perinuclei of the cells. The percentage of caspase-3–positive cells was determined in each tumor.

Formation and growth of PC-3 tumors in immunodeficient mice: effect of treatment with atorvastatin and celecoxib. Male severe combined immunodeficient (SCID) mice (6-7 weeks old) were obtained from Taconic Farms, Inc. The animals were housed in sterile filter-capped microisolator cages and provided with sterilized food and water. Prostate cancer PC-3 cells (2 × 10⁶ cells/0.1 mL/mouse) suspended in 50% Matrigel (Collaborative Research, Bedford, MA) in RPMI 1640 were injected s.c. into the right flank of the mice. The mice were injected i.p. with (a) vehicle (5 mg/g body weight), (b) atorvastatin (10 μg/g; 5 μL vehicle/g), (c) celecoxib (10 μg/g; 5 μL vehicle/g), (d) atorvastatin (10 μg/g) + celecoxib (10 μg/g), or (e) atorvastatin (5 μg/g) + celecoxib (5 μg/g) once daily starting 2 days before the injection of tumor cells and continuing for 42 days. In all experiments, animals in the different experimental groups received the same amount of vehicle (5 μL/g body weight), which consisted of propylene glycol, polysorbate 80, benzyl alcohol, ethanol, and water (40.05:5.1:10:48.5). Tumor size (length × width) and body weight were measured every 3rd day. At the end of the study, mice were sacrificed and tumors were excised, weighed, and placed in phosphate-buffered formalin at room temperature for 48 h and then placed in ethanol for 48 h before preparing paraffin sections as described previously (22). All animal experiments were carried out under an Institutional Animal Care and Use Committee–approved protocol.

Statistical analyses. Tumor-free distribution was analyzed using the Kaplan-Meier method (23). The log-rank test was used to determine the difference between tumor-free distribution. The analyses of increase in tumor size were based on mixed effect regression (repeated measurement) model (24). The treatment effects were assessed by comparing the rates of change over time between treatment groups (i.e., comparing the slopes between treatment groups). The ANOVA method with Newman-Keuls and Tukey-Kramer test (25) was used for the comparison of viable cells, apoptosis, tumor size, and body weight among different treatment groups at the end of the treatment.

Results

Effects of atorvastatin or celecoxib alone or in combination on the growth and apoptosis of human prostate cancer PC-3 cells. In initial studies, we assessed the effects of various concentrations of atorvastatin and celecoxib on the growth and death of PC-3 cells. In these experiments, PC-3 cells were treated with different concentrations of atorvastatin (2-20 μmol/L) or celecoxib (2-20 μmol/L) for 96 h. The number of viable and dead cells was determined by a trypan blue exclusion assay. We found that treatment of PC-3 cells with atorvastatin or celecoxib inhibited cell growth and caused cell death in a concentration-dependent manner (Fig. 1). Treatment of PC-3 cells with 2 to 20 μmol/L atorvastatin for 96 h resulted in a 6% to 54% decrease in the number of viable cells when compared with control cells treated only with DMSO solvent (Fig. 1). Celecoxib (2-20 μmol/L) caused 5% to 43% decrease in the number of viable PC-3 cells (Fig. 1). Statistical analysis using the Tukey-Kramer multiple comparison test showed that the difference for the number of viable cells between the control and 5 μmol/L atorvastatin-treated cells or between the control and 5 μmol/L celecoxib-treated cells was statistically significant (P < 0.05). The number of viable cells was significantly lower in cells treated with atorvastatin (P < 0.001 at both 10 and 20 μmol/L) or celecoxib (P < 0.001 at both 10 and 20 μmol/L) compared with the control (Fig. 1).

The effects of atorvastatin and celecoxib in combination on the growth and death of PC-3 cells were also studied. In these experiments, PC-3 cells were treated with atorvastatin (2, 5, or 10 μmol/L) alone or in combination with celecoxib (2, 5, or 10 μmol/L) for 96 h. The number of viable and dead cells was determined by a trypan blue exclusion assay. Treatment with atorvastatin (2 μmol/L) or celecoxib (2 μmol/L) alone had little or no effect on the growth of PC-3 cells, whereas their combination caused a 27% decrease in the number of viable cells compared with the control (Fig 2A). Treatment with atorvastatin (5 μmol/L) or celecoxib (5 μmol/L) alone caused a small decrease in the number of viable cells (Fig 2A). A combination of atorvastatin (5 μmol/L) and celecoxib (5 μmol/L) caused a 48% decrease in viable cells compared with the control (Fig 2A). At a higher concentration (10 μmol/L), atorvastatin and celecoxib in combination also caused a stronger growth inhibition compared with either agent alone (Fig 2A). In additional studies, we determined the effect of atorvastatin or celecoxib alone or in combination in prostate cancer LNCaP, CWR22Rv1, and Du145 cells. As shown in Fig 2B, treatment of the various lines of prostate cancer cells with a combination of atorvastatin (5 μmol/L) and celecoxib...
(5 μmol/L) resulted in a stronger growth inhibition than either agent alone at 5 μmol/L. Statistical analysis using the Tukey-Kramer multiple comparison test showed that the number of viable PC-3 cells after the treatment was significantly lower in the combination groups ($P < 0.01$ for 2 μmol/L atorvastatin + 2 μmol/L celecoxib; $P < 0.001$ for both 5 μmol/L atorvastatin + 5 μmol/L celecoxib and 10 μmol/L atorvastatin + 10 μmol/L celecoxib) compared with cells treated with individual compound at the same concentration (Fig. 2A). Differences for the number of viable LNCaP, CWR22Rv1, and Du145 cells between combination groups and single compound-treated groups were also statistically significant ($P < 0.01$ for CWR22Rv1 cells; $P < 0.001$ for LNCaP and Du145 cells; Fig. 2B).

We also determined apoptosis in PC-3 cells treated with atorvastatin and celecoxib alone or in combination by using a morphologic assessment of apoptotic cells (20). Apoptotic cells were identified by classic morphologic features, including nuclear condensation, cell shrinkage, and formation of apoptotic bodies. Morphologically distinct apoptotic cells from representative samples are shown in Fig. 3A to D. A combination of atorvastatin (10 μmol/L) and celecoxib (10 μmol/L) caused a stronger induction of apoptosis in PC-3 cells than either agent alone. As shown in Fig. 3E, treatment of PC-3 cells with atorvastatin (10 μmol/L) alone resulted in 20% apoptotic cells and treatment with celecoxib (10 μmol/L) alone resulted in 18% apoptotic cells. Treatment with a combination of atorvastatin (10 μmol/L) and celecoxib (10 μmol/L) resulted in 46% apoptotic cells. Statistical analysis using the Tukey-Kramer multiple comparison test showed that apoptosis was significantly increased in cells treated with atorvastatin (10 μmol/L) + celecoxib (10 μmol/L) compared with cells treated with atorvastatin (10 μmol/L) or celecoxib (10 μmol/L) alone ($P < 0.001$).

**Effects of atorvastatin and celecoxib alone or in combination on the level of activated Erk1/2 and NF-κB.** The level of activated Erk1/2 in PC-3 cells was evaluated by Western blot analysis using an anti–phospho-Erk1/2 antibody that detects active, phospho-Erk1/2. In these experiments, PC-3 cells were treated with atorvastatin (10 μmol/L) or celecoxib (10 μmol/L) alone or in combination for 24 h and analyzed by Western blotting. Treatment of PC-3 cells with atorvastatin (10 μmol/L) or celecoxib (10 μmol/L) alone resulted in a small but measurable decrease in the level of phospho-Erk1/2 at 24 h after the treatment, and the combination of atorvastatin (10 μmol/L) and celecoxib (10 μmol/L) caused a stronger decrease in the level of phospho-Erk1/2 than either agent alone (Fig. 4A). The extent of protein loading was determined by blotting for β-actin, and the levels of phospho-Erk1/2 in the Western blot was analyzed by absorbance measurement and normalized for actin. The level of phospho-Erk1 relative to control (1.00) was 0.93 in cells treated with atorvastatin, 0.71 in cells treated with celecoxib, and 0.62 in cells treated with the combination of atorvastatin and celecoxib. The relative level of phospho-Erk2 was 1.00 in control, 0.95 in cells treated with atorvastatin, 0.78 in cells treated with celecoxib, and 0.64 in cells treated with the combination of atorvastatin and celecoxib.

To investigate the effect of atorvastatin and celecoxib on the activation of NF-κB, we used a NF-κB-luciferase reporter gene expression assay in PC-3 C4 cells (21). PC-3 C4 is a cell line derived from stable transfection of PC-3 cells with a NF-κB luciferase construct (21). In these experiments, PC-3 C4 cells were treated with atorvastatin (10 μmol/L) and celecoxib (10 μmol/L) alone or in combination for 24 h. Treatment of PC-3 C4 cells with atorvastatin (10 μmol/L) or celecoxib (10 μmol/L) alone caused a modest decrease in luciferase activity, and the combination of atorvastatin (10 μmol/L) and celecoxib (10 μmol/L) had a stronger effect than either agent alone (Fig. 4B).

**Effects of i.p. injections of atorvastatin and celecoxib alone or in combination on the formation and growth of PC-3 xenograft tumors in immunodeficient mice.** Male SCID mice were injected i.p. with vehicle (5 μL/g body weight), atorvastatin (10 μg/g; 5 μL vehicle/g), celecoxib (10 μg/g; 5 μL vehicle/g), atorvastatin (10 μg/g) + celecoxib (10 μg/g), or atorvastatin (5 μg/g) + celecoxib (5 μg/g) once daily for 42 days starting 2 days before the s.c. inoculation of PC-3 cells (14 mice were in the vehicle-treated control group and 12 mice were in each of the other

![Fig. 2. Effect of atorvastatin and celecoxib alone or in combination on the growth of prostate cancer cells. PC-3 (0.2 × 10⁶ cells/mL) cells (A), or LNCaP (0.5 × 10⁶ cells/mL), CWR22Rv1 (0.5 × 10⁶ cells/mL), and Du145 (0.2 × 10⁶ cells/mL) cells (B) were seeded in 35-mm tissue culture dishes (2 mL/dish) and incubated for 24 h. The cells were then treated with atorvastatin alone or in combination with celecoxib for 96 h. The number of viable cells after each treatment was determined by a trypan blue exclusion assay. Columns, mean of three separate experiments; bars, SE. Differences for the number of viable cells between a combination group and a single agent-treated group were analyzed by the Tukey-Kramer multiple comparison test. **, $P < 0.01$; *** $P < 0.001$.](www.aacnjournals.org)
groups). Tumor size (length × width; square centimeter) and body weight were measured once every 3 days. As shown in Fig. 5, treatment with atorvastatin or celecoxib alone had little or no effect on the formation of tumors, and all mice had a tumor at 23 days after injection of the tumor cells. However, administration of a combination of atorvastatin and celecoxib inhibited the formation of measurable tumors. At 23 days after injection of the tumor cells (25 days of drug treatment), 67% of the mice had tumors with the low-dose combination and 58% of the mice had tumors after the high-dose combination. At 29 or 42 days after injection of the tumor cells (when the experiment was terminated), 8% of the animals had tumors with the low-dose combination and 58% of the animals on the high-dose combination remained tumor-free. The log-rank test showed that the difference in tumor formation between the control group and the atorvastatin (10 μg/g/d) + celecoxib (10 μg/g/d) group was statistically significant (P = 0.035), and the difference between the control group and the atorvastatin (10 μg/g/d) + celecoxib (10 μg/g/d) group was also statistically significant (P = 0.013).

The effects of the various treatments on tumor growth are described in Fig. 6. The rates of change in tumor size (Fig. 6) were significantly different between any two groups (P < 0.0001) except between the atorvastatin- and celecoxib-treated groups (P = 0.8858) and between the high-dose combination and low-dose combination group (P = 0.1255). The mean ± SE for average tumor size (square centimeter) after 42 days of treatment was 0.45 ± 0.02 for the control group, 0.36 ± 0.02 for the atorvastatin group, 0.34 ± 0.03 for the celecoxib group, 0.23 ± 0.03 for the atorvastatin (5 μg/g/d) + celecoxib (5 μg/g/d) group, and 0.21 ± 0.04 for the atorvastatin (10 μg/g/d) + celecoxib (10 μg/g/d) group. Statistical analysis using ANOVA with the Newman-Keuls multiple comparison test showed that the differences in the average tumor size between the control group and the atorvastatin group or between the control group and the celecoxib group were statistically significant (P < 0.05). The differences in the average tumor size between the control group and the atorvastatin (5 μg/g/d) + celecoxib (5 μg/g/d) group or between the control group and the atorvastatin (10 μg/g/d) + celecoxib (10 μg/g/d) group were also statistically significant (P < 0.001). The average tumor size in the

Fig. 3. Effect of atorvastatin and celecoxib on apoptosis of PC-3 cells. PC-3 cells were seeded at a density of 0.2 × 10^5 cells/mL in 35-mm tissue culture dishes and incubated for 24 h. The cells were then treated with atorvastatin (10 μmol/L) or celecoxib (10 μmol/L) alone or in combination for 96 h. The cells were fixed with methanol/acetone (1:1) for 10 min and stained with propidium iodide (1 μg/mL) for 10 min. Apoptotic cells were determined by morphologic assessment using a fluorescence microscope. Arrows, apoptotic cells. A, control PC-3 cells. B, PC-3 cells treated with 10 μmol/L atorvastatin. C, PC-3 cells treated with 10 μmol/L celecoxib. D, PC-3 cells treated with a combination of atorvastatin (10 μmol/L) and celecoxib (10 μmol/L). E, percentage of apoptotic cells. Columns, mean of three separate experiments; bars, SE. Differences for the number of apoptotic cells between combination group and single agent-treated group were analyzed by the Tukey-Kramer multiple comparison test. ***, P < 0.001.

Fig. 4. Effects of atorvastatin and celecoxib alone or in combination on the activation of Erk1/2 and NF-κB in PC-3 cells. A, PC-3 cells were seeded at a density of 1 × 10^5 cells/mL of medium in 100-mm culture dishes (10 mL/dish) and incubated for 24 h. The cells were then treated with atorvastatin (10 μmol/L) or celecoxib (10 μmol/L) alone or in combination for 24 h. Activated Erk was determined by using Western blot with an anti-phospho-Erk1/2 antibody. B, PC-3 C4 cells were seeded at a density of 0.2 × 10^5 cells/mL of medium in 60-mm culture dishes (10 mL/dish) and incubated for 24 h. The cells were then treated with atorvastatin (10 μmol/L) alone or in combination with celecoxib (10 μmol/L) for 24 h. The NF-κB transcriptional activity was measured by a luciferase activity assay. Columns, mean of three separate experiments; bars, SE.
atorvastatin (10 μg/g/d) + celecoxib (10 μg/g/d) group was significantly smaller than that in the atorvastatin (10 μg/g/d) or celecoxib (10 μg/g/d) group (P < 0.01). There were also statistically significant differences in the average tumor size between the atorvastatin (5 μg/g/d) + celecoxib (5 μg/g/d) group and the atorvastatin (10 μg/g/d) group (P < 0.05) and between the atorvastatin (5 μg/g/d) + celecoxib (5 μg/g/d) group and the celecoxib (10 μg/g/d) group (P < 0.05). The mean ± SE for the percentage of initial body weight was 106.6 ± 1.4% for the vehicle-treated control group, 101.9 ± 1.9% for the atorvastatin group, 104.4 ± 2.3% for the celecoxib group, 107.1 ± 3.0% for the atorvastatin (5 μg/g/d) + celecoxib (5 μg/g/d) group, and 103.5 ± 2.9% for the atorvastatin (10 μg/g/d) + celecoxib (10 μg/g/d) group. Statistical analysis using ANOVA with the Newman-Keuls multiple comparison test showed that the differences in the percentage of initial body weight between the control group and any of the treatment groups were not statistically significant (P > 0.05).

Effects of i.p. injections of atorvastatin and celecoxib alone or in combination on proliferation and apoptosis in PC-3 xenograft tumors in immunodeficient mice. The effects of daily i.p. injections of atorvastatin and celecoxib alone or in combination for 42 days after injection of PC-3 cells on proliferation and apoptosis in PC-3 tumors described in Figs. 5 and 6 were studied by determining mitotic cells and caspase-3 (active form)–positive cells in these tumors. The percentage of mitotic cells was decreased significantly in tumors from mice treated with atorvastatin + celecoxib when compared with the control group (Table 1). Apoptosis as measured by the percentage of caspase-3 (active form)–positive cells in tumors was increased significantly in the atorvastatin + celecoxib group (Table 1). The ratio of the percentage mitotic cells/percentage caspase-3 (active form)–positive cells, which is a potential index of the balance between cell proliferation and cell death, was also determined in the PC-3 tumors. We found that the ratio of the percentage mitotic cells/percentage caspase-3 (active form)–positive cells ± SE in tumors was 1.35 ± 0.09 for the vehicle-treated control group, 0.93 ± 0.09 for the atorvastatin group, 0.91 ± 0.05 for the celecoxib group, 0.70 ± 0.06 for the atorvastatin (5 μg/g) + celecoxib (5 μg/g) group, and 0.53 ± 0.03 for the atorvastatin (10 μg/g) + celecoxib (10 μg/g) group (Table 1).

Discussion

The present study shows for the first time that administration of atorvastatin in combination with celecoxib inhibited the formation and growth of prostate PC-3 xenograft tumors in immunodeficient mice. Atorvastatin or celecoxib alone had little or no effect in delaying the formation of PC-3 tumors (Fig. 5). Although celecoxib has been shown to inhibit prostate carcinogenesis in an animal model (16, 17), the effects of atorvastatin alone or in combination with celecoxib on the formation and growth of prostate tumors in animals injected with prostate cancer cells have not been studied previously. Our results provide evidence that combinations of atorvastatin and celecoxib had a stronger inhibitory effect on the formation and growth of prostate PC-3 xenograft tumors in immunodeficient mice than the individual compounds alone. Combinations of atorvastatin and celecoxib (2 + 2, 5 + 5, and 10 + 10 μmol/L) all had a stronger inhibitory effect on the growth of PC-3 cells than either agent alone at the same concentration (Fig. 2A). Moreover, a combination of atorvastatin and celecoxib at a...
low 5 μmol/L concentration had an even more potent inhibitory effect on the growth of PC-3 cells than either agent used individually at a higher 10 μmol/L concentration (Fig. 2A). Treatment of PC-3 cells with atorvastatin (10 μmol/L) or celecoxib (10 μmol/L) alone resulted in 19.7% and 18.2% apoptosis, respectively (Fig. 3E). A combination of atorvastatin (10 μmol/L) and celecoxib (10 μmol/L) caused 45.6% apoptosis (Fig. 3E). Our in vitro studies suggested that atorvastatin and celecoxib in combination produced a more-than-additive or possible synergistic effect on growth inhibition and apoptosis in PC-3 cells. In the in vivo experiment, we found that a combination of low-dose levels of atorvastatin (5 μg/g body weight) and celecoxib (5 μg/g body weight) had an even more potent inhibitory effect on the formation of PC-3 tumors in immunodeficient mice than either agent used alone at a higher dose (10 μg/g body weight; Fig. 5). This low-dose combination also had a stronger inhibitory effect on the growth of PC-3 tumors (53.3% decrease in tumor size at day 42) than a higher dose of atorvastatin (10 μg/g body weight; 20% decrease in tumor size at day 42) or celecoxib (10 μg/g body weight; 24.4% decrease in tumor size at day 42; Fig. 6). Our in vivo study suggested that administration of a combination of atorvastatin and celecoxib had a more-than-additive or possible synergistic effect for inhibiting the formation and growth of PC-3 tumors in immunodeficient mice. A combination of atorvastatin and celecoxib may be an effective strategy for the prevention of prostate cancer.

It is of considerable interest that daily i.p. injections of celecoxib or atorvastatin alone had little or no effect in delaying the formation of androgen-independent PC-3 prostate tumors that occurred after the s.c. injection of PC-3 cells, whereas administration of a combination of the two drugs delayed the formation of tumors and some mice treated with the combination of drugs were tumor-free at the end of the study (Fig. 5). At 23 days after the injection of tumor cells, all of the control animals or animals treated with celecoxib or atorvastatin alone had a tumor, whereas 33% of the animals treated with the low-dose combination and 42% of the animals treated with the high-dose combination remained tumor-free (Fig. 5). At 42 days after the injection of tumor cells (when the experiment was terminated), 8% of the animals on the low-dose combination remained tumor-free and 29% of the animals on the high-dose combination remained tumor-free (Fig. 5). Because no toxicity was observed in the present study, it is possible that increasing the dose or altering the dosing regimen would improve efficacy for the combination. Our results suggest that administration of a combination of atorvastatin and celecoxib enhanced the dormancy of PC-3 cells that were injected into immunodeficient mice.

The mechanisms by which atorvastatin inhibits the growth and induces apoptosis in prostate cancer cells are not clear. Atorvastatin is a 3-hydroxy-3-methylglutaryl CoA reductase inhibitor that reduces the synthesis of isoprenoids, geranylgeranyl pyrophosphate, and farnesylpyrophosphate and their precursor mevalonate (26). Notably, geranylgeranyl pyrophosphate and farnesylpyrophosphate are required for the function of Rho proteins and Ras, respectively (27). Because Ras and Rho are important signaling molecules in cell proliferation and survival, atorvastatin and other statin drugs may interfere with Ras/Rho activity and thus inhibit the growth and stimulate apoptosis in cancer cells. Ras activation is a component of the signaling pathways for many growth factor receptors that are up-regulated in advanced prostate cancer (28). One of the downstream effectors of Ras activation is Erk1/2. Constitutive activation of Erk1/2 has been observed in prostate cancer (29, 30). In the present study, we found that atorvastatin at a high concentration (20 μmol/L) inhibited the activation of Erk1/2 (data not shown) and induced cell death (Fig. 1). These results suggest that the decrease in Erk1/2 activation may be involved in atorvastatin-induced cell death in PC-3 cells. Future studies are needed to determine if the decrease in Erk1/2 activation induced by atorvastatin in PC-3 cells is mediated by inhibition of Ras function.

Celecoxib, a selective cyclooxygenase-2 inhibitor, was shown to inhibit prostate carcinogenesis in the transgenic adenocarcinoma of mouse prostate model (16, 17). Recent clinical studies showed that long-term use of a high dose of celecoxib was associated with an increased cardiovascular risk (31, 32). Toxic side effects caused by a high dose of celecoxib may limit the use of this drug for the prevention of prostate cancer. An effective strategy to reduce side effects is the use of suitable combinations of a low dose of celecoxib together with other preventive agents. Results of our in vitro and in vivo studies indicated that a combination of celecoxib and atorvastatin at a

<table>
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<th>Table 1. Effects of treatment with atorvastatin, celecoxib, or atorvastatin + celecoxib on the percentage of mitotic cells and the percentage of caspase-3 (active form)–positive cells in PC-3 tumors</th>
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<tbody>
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<td>Treatment</td>
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<tr>
<td>Control</td>
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<td>Atorvastatin (10 μg/g)</td>
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<tr>
<td>Celecoxib (10 μg/g)</td>
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<td>Atorvastatin (5 μg/g) + celecoxib (5 μg/g)</td>
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<td>Atorvastatin (10 μg/g) + celecoxib (10 μg/g)</td>
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NOTE: Each value represents the mean ± SE. Pairwise comparisons for percentage mitotic cells, percentage caspase-3–positive cells, and ratio of percentage mitotic cells/caspase-3–positive cells between each treatment group and control group were analyzed by the Tukey-Kramer multiple comparison test.

*P < 0.001.
†P < 0.01.
low dose has an even more potent effect than either agent used individually at a higher dose for inhibiting the growth and inducing apoptosis in cultured PC-3 cells and PC-3 tumors in immunodeficient mice (Figs. 1–3 and 6; Table 1). In addition, our studies showed that a combination of atorvastatin and celecoxib had a stronger inhibitory effect than either agent alone toward activated Erk1/2 and NF-κB in PC-3 cells (Fig. 4). These results identified Erk1/2 and NF-κB as potential targets for the design of synergistic combinations of chemopreventive agents for the prevention of prostate cancer.

The NF-κB family of transcription factors is constitutively activated in various human malignancies including prostate cancer (33). An inverse correlation between androgen receptor status and NF-κB activity was observed in prostate cancer cell lines. NF-κB may promote cell growth and proliferation in prostate cancer cells by regulating expression of genes, such as c-myc, cyclin D1, and IL-6. NF-κB may also inhibit apoptosis in prostate cancer cells through activation of expression of antiapoptotic genes, such as Bcl-2 (33). Using a luciferase reporter gene expression assay, we found that a combination of atorvastatin and celecoxib had a stronger inhibitory effect on the transcription activity of NF-κB than either agent alone. This observation, together with the finding that atorvastatin in combination with celecoxib potently inhibit the activation of Erk1/2, indicates that inhibition of NF-κB and Erk1/2 activation may contribute to the strong effects on growth inhibition and apoptosis induced by the combination of atorvastatin and celecoxib.

In summary, the present report indicates that although daily i.p. injections of atorvastatin or celecoxib alone does not inhibit the formation of tumors after the injection of human prostate cancer PC-3 cells into immunodeficient mice, administration of a combination of atorvastatin and celecoxib significantly inhibited tumor formation. Administration of atorvastatin or celecoxib alone inhibited tumor growth and decreased the ratio of mitotic cells/apoptotic cells but administration of a combination of these drugs was considerably more effective. Clinical trials with atorvastatin in combination with celecoxib in patients with prostate cancer may be warranted.

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