The Effect of Statins in Colorectal Cancer Is Mediated Through the Bone Morphogenetic Protein Pathway

LIUDMILA L. KODACH,* SYLVIA A. BLEUMING,* MAIKEL P. PEPPELENBOSCH,‡ DANIEL W. HOMMES,§ GIJS R. VAN DEN BRINK,*,§ and JAMES C. H. HARDWICK*,§

*Center for Experimental and Molecular Medicine, Academic Medical Center, Amsterdam, The Netherlands; ‡Department of Cell Biology, University Medical Center Groningen, University of Groningen, The Netherlands; and §Department of Gastroenterology & Hepatology, Leiden University Medical Center, Leiden, The Netherlands

Background & Aims: **Epidemiological evidence suggests that statins prevent colorectal cancer (CRC), but the biological mechanism remains obscure. Statins induce bone morphogenetic protein (BMP) expression in bone cells. We have previously shown that BMPs act as tumor suppressors in CRC. We hypothesized that the action of statins in CRC involves the induction of BMPs.** ***Methods:*** **We investigated the effects of statins on CRC cell lines using immunoblotting, measurements of apoptosis and cell proliferation, and luciferase reporter assays. The effect of statins was confirmed in a xenograft mouse model.** ***Results:*** **CRC cell lines show widely differing sensitivities to statin treatment. Sensitive cell lines show induction of BMP2 protein levels and a BMP2 reporter construct, activation of the BMP pathway, and induction of the BMP target gene ID-2, whereas resistant cell lines do not. The addition of the specific inhibitor of BMPs, noggin, completely prevents lovastatin-induced apoptosis in sensitive cells. Sensitive cell lines express the central BMP pathway element SMAD4, whereas the resistant cell lines do not. Targeted knockout of SMAD4 leads to the loss of statin sensitivity and reconstitution with SMAD4, to the restoration of statin sensitivity. In a xenograft mouse model, tumors from sensitive and insensitive cell lines were treated with oral simvastatin. Significant inhibition of tumor growth using sensitive cells but increased tumor growth when using insensitive cells was observed.** ***Conclusions:*** **Statins induce apoptosis in CRC cells through induction of BMP2. Statin therapy may only be effective in SMAD4-expressing CRCs and may have adverse effects in SMAD4-negative tumors.**

BASIC–ALIMENTARY TRACT

A recent trial specifically designed to investigate the incidence of colorectal cancer (CRC) in statin users showed a highly significant 47% reduction.[1] Several investigators are now suggesting that the time is ripe for large-scale, prospective, clinical, or observational trials of statin therapy as a chemopreventative or adjuvant treatment in CRC,[2,3] but the epidemiological evidence for a beneficial effect is conflicting,[4] and the biological mechanism of action of statins in CRC remains obscure.

HMG-CoA reductase inhibitors are extensively used to reduce serum cholesterol and to decrease the incidence of cardiovascular and cerebrovascular events.[5,6] Statins prevent formation of mevalonate from HMG-CoA by inhibiting the enzyme HMG-CoA reductase and thereby inhibiting cholesterol synthesis.[7] As well as reducing cholesterol levels, statins inhibit the generation of other products of the mevalonate pathway, including mevalonate and the downstream isoprenoids (farnesyl pyrophosphate and geranylgeranylpyrophosphate). Posttranslational isoprenylation is important in determining the membrane localization and function of many cellular proteins including small GTPases such as Ras and Rho.[8] In vitro studies show that statins inhibit cellular proliferation and induce apoptosis in colon cancer cells.[9,10] Because Ras mutations are frequent in tumors,[11] and Rho proteins participate in growth-factor signaling,[12] the study of the action of statins in tumor cells has largely focused on their ability to inhibit these small GTPases.[13] However, there is evidence that this may not be the only mechanism by which statins inhibit proliferation and induce apoptosis.[2,14]

A screen of 30,000 compounds for their ability to activate a BMP-2 promoter construct in mouse osteoblast cell lines found that 2 statins (simvastatin and lovastatin) had the highest activity and went on to show the anabolic properties of statins on bone in vivo in mice.[15]

BMPs are part of the transforming growth factor-β superfamily. They signal by cooperatively binding type I and type II BMP receptors, which in turn phosphorylate the BMP-specific SMADs1, 5, and 8, which then complex with SMAD4. The complex translocates to the nucleus and modulates gene transcription of BMP target genes such as ID-2. The BMP pathway has recently been impli-

Abbreviations used in this paper: BMP, bone morphogenetic protein; CRC, colorectal cancer; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase.

0016-5085/07/$32.00
doi:10.1053/j.gastro.2007.08.021

cated in CRC with the identification of germline mutations in BMPR1a and SMAD4 in families with familial juvenile polyposis syndrome.[16] Affected individuals have a greatly increased risk of developing cancer.[17,18] We have shown that BMP2 acts to promote apoptosis in mature epithelial cells in the colon.[19]

In this article, we have investigated the effects of statins on CRC cell lines and xenografts in order to test the hypothesis that statins act through their actions on the BMP pathway. We show that statins induce BMP2 in CRC cells and that noggin specifically blocks statin-induced apoptosis in colon cancer cell lines. Our results suggest that loss of SMAD4 confers resistance to statins and leads to growth promotional effects. This implies that statin therapy may only be effective in a subgroup of SMAD4-expressing CRCs.

Materials and Methods

Cell Culture

DLD1, SW480, HT29, and HCT116 colon cancer cell lines were obtained from the ATCC and cultured in Dulbecco's Modified Eagle's Medium (Gibco, Paisley, Scotland) with 4.5g/L glucose and L-glutamine. This was supplemented with penicillin (50 U/mL) and streptomycin (50 μg/mL) and, where serum was used, with 10% fetal calf serum (Gibco). HCT116 SMAD4−/− cells were cultured in McCoy's 5A Medium (Sigma Aldrich, St. Louis, MO) supplemented with 0.4 mg/mL G418 and 0.1 mg/mL hygromycin B. Cells were grown in monolayers in a humidified atmosphere containing 5% CO_2. Simvastatin, lovastatin and pravastatin were all obtained from Sigma Aldrich.

Cell Count

Cells were trypsinized, and 20 μL of cell suspension was taken for counting using Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter B.V., Mijdrecht, The Netherlands) according to the manufacturer's instructions.

MTT Assay

Cells were trypsinized and taken up in Dulbecco's Modified Eagle's Medium with 0.5% fetal calf serum. 5×10^3 cells were seeded in flat-bottomed tissue-culture-treated 96-well plates (Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands) and allowed to adhere for 12 hours. Cells were then stimulated with different concentrations of statins at 24 and 48 hours. After treatment, MTT solution was added (final concentration 0.5 mg/mL, stock solution 5 mg/mL MTT in phosphated buffered saline) for 3 hours. The medium was discarded, and the cells were lysed in acidified 2-propanol. Absorbance was measured at 550 to 560 nm. Ten wells were used for each treatment condition (n = 10).

Immunoblotting

Cells at 60% to 80% confluence from 6-well plates (Greiner Bio-One B.V., Alphen a/d Rijn) were washed in ice-cold PBS and scraped into 200 μL of 2 × sample buffer (125 mmol/L Tris/HCl, pH 6.8; 4% sodium dodecyl sulfate (SDS); 2% β-mercaptoethanol; 20% glycerol, 1 mg bromophenol blue). Protein concentration was measured using the RC DC protein assay kit (Biorad, Hercules, CA) according to the manufacturer's instructions. The lysates were sonicated and then heated at 95° for 5 minutes. Fifty micrograms of protein from each sample was loaded onto SDS-PAGE and blotted onto PVDF membrane (Millipore, Bedford, MA). The blots were blocked in block buffer (2% low-fat milk powder in Tris-buffered saline with 1% Triton [TBST]) for 1 hour at room temperature and washed 3 × 10 minutes in TBST before overnight incubation at 4°C with primary antibody in primary antibody buffer (TBST with 0.2% low-fat milk powder). Primary antibodies to BMP2 (mouse monoclonal) were from R&D (Abingdon, UK). Goat polyclonal antibodies to pSMAD1, 5, and 8 were from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibodies to SMAD4, rabbit polyclonal antibodies to Id1, and rabbit polyclonal antibodies to β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Blots were then washed 3 × 10 minutes in TBST and incubated for 1 hour at room temperature in 1:2000 horseradish peroxidase-conjugated goat anti-rabbit, rabbit anti-goat, or goat anti-mouse secondary antibody (Dako, Glostrup, Denmark) in block buffer. After a final 3- × 10-minute wash in TBST, blots were incubated for 5 minutes in Lumilite Plus (Boehringer-Mannheim, Mannheim, Germany), and chemiluminescence was then detected using a Lumi-Imager (Boehringer-Mannheim).

Annexin V and 7-Amino-Actinomycin D Assays

Control and treated cells were collected and resuspended in 1 × binding buffer (0.01 mol/L Hepes/NaOH, pH 7.4, 0.14 mmol/L NaCl and 2.5 mmol/L $CaCl^2$) at a concentration of 2 x 10^7 cells/mL. Subsequently, 100 μL of cell suspension was transferred to a 5-mL tube, and Annexin V-APC (5 μL) and 7-amino-actinomycin D (7-AAD) (5 μL) was added. The cells were incubated at room temperature for 15 minutes, after which 400 μL of 1 × binding buffer was added, apoptosis was analyzed by flow cytometry (Becton Dickinson FACSCalibur, Rockville, MD), and data were analyzed using the software Cell Quest Pro (BD Biosciences Pharmingen, Breda, the Netherlands).

Luciferase Reporter Assay

Cells were transiently transfected either with BMP2-Luc vector or BRE-Luc vector reporter in combination with a cytomegalovirus promoter-driven Renilla luciferase vector (Promega, Madison, WI) using Lipofectamine Plus (Invitrogen, Breda, The Netherlands) accord-