ORIGINAL ARTICLE

Atorvastatin inhibits proliferation and promotes apoptosis of colon cancer cells via COX-2/PGE2/β-Catenin Pathway

Shuyan Cai, Zhigang Gao

Department of General surgery, Beijing Chaoyang Hospital, Capital Medical University, Beijing, China.

Summary

Purpose: To explore the effects of atorvastatin (ATST) on the proliferation and apoptosis of colon cancer cells through the cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2)/ β catenin pathway.

Methods: HCT116 cells were cultured and transfected, and they were treated with ATST at different concentrations for different time. The association between the expressions of COX-2 and PGE2 and the survival time of patients with colon cancer was analyzed via Kaplan-Meier survival analysis. Then the protein expressions of COX-2, β -catenin and apoptosis-related molecules in HCT116 cells were determined using Western blotting, and the proliferation of HCT116 cells was detected via cell counting kit-8 (CCK-8) assay.

Results: There was a significant difference in the survival rate between HCT116 cells treated with 30 μ M ATST and those treated with 0 μ M ATST. The survival time was obviously longer in patients with low expressions of COX-2 and PGE2 than that those with high expressions of COX-2 and

PGE2. Low expressions of COX-2 and PGE2 in colon cancer tissues indicate a longer survival time. Moreover, a positive correlation was found between HCT116 cell density and COX-2 level, HCT116 cell density and PGE2 level, and COX-2 and PGE2 levels. ATST could down-regulate COX-2 and β -catenin, and knocking down COX-2 could lower β -catenin. After treatment with ATST and ATST + anti-COX-2, the activity of cleaved caspase-9, caspase-3 and PARP was remarkably enhanced, suggesting that ATST and ATST + anti-COX-2 can promote apoptosis of HCT116 cells. It was found that ATST and ATST + anti-COX-2 could also inhibit the proliferation of HCT116 cells.

Conclusions: ATST inhibits the proliferation and promotes the apoptosis of colon cancer HCT116 cells through down-regulating the COX- $2/PGE2/\beta$ -catenin signaling pathway.

Key words: COX-2/PGE2/β-catenin pathway, atorvastatin, colon cancer, cell proliferation and apoptosis

Introduction

Colon cancer is one of the most common malignant tumors in the world, and its morbidity rate increases due to the unbalanced diet, such as eating red and processed meats [1,2]. Such a dietary habit will lead to the increased levels of cholesterol and low-density lipoprotein in the blood, ultimately causing hyperlipidemia. Studies have demonstrated that hyperlipidemia can raise the incidence of colon cancer [3,4], because the increased levels of cholesterol and low-density lipoprotein will enhance the proliferation and invasion of cancer cells [5,6].

Statins can reduce the content of both cholesterol and low-density lipoprotein, so they can be used to not only prevent cancer, but also reduce symptoms in patients with hyperlipidemia. Nielsen et al [7] reported that statins may reduce the cancer mortality in the Danish population. According to clinical studies, statins have certain value in the treatment of cancer, but its efficacy on colon cancer remains controversial [8,9]. Therefore, it is necessary to evaluate the anticancer effect of statins, hoping to apply them in the prevention and treatment of colon cancer.

Corresponding author: Zhigang Gao, MD. Department of General Surgery; Beijing Chaoyang Hospital, Capital Medical University, 5 Jingyuan Rd, Shijingshan District, Beijing, China. Tel: +8601051718352; Email: ebi9779482@163.com

Received: 15/05/2021; Accepted: 03/06/2021



Colon cancer is associated with many signaling pathways, such as cyclooxygenase-1 (COX-1) and COX-2, two subtypes of COX [10]. COX-1 is responsible for keeping the normal functions, which are constitutively expressed in most tissues. However, COX-2 is a major inflammatory factor inducing the development of cancer, which is overexpressed in all metastatic cancer cells and involved in the initiation of angiogenesis and epithelial-mesenchymal transition (EMT) [11-13]. It is reported that the COX-2-induced production of prostaglandin E2 (PGE2) contributes to the migration and EMT of human breast cancer cells [14]. In addition, in vivo studies have confirmed that the specific inhibition or deletion of COX-2 reduces tumor metastasis in the mouse model [15]. Therefore, it can be seen that COX-2/PGE2 not only regulates EMT, but also plays an inestimable role in tumor proliferation and invasion. As an important component of intercellular adhesion, β -catenin forms a dynamic link between E-cadherin and cytoskeleton, which is also a key component of Wnt signals [16]. β-catenin regulates the expressions of multiple target genes mediating cellular processes, including cell proliferation and migration. It is reported in the literature that colon cancer and skin cancer are closely related to COX-2 and β -catenin [17-20]. Therefore, this paper aims to explore the effects of atorvastatin (ATST) on colon cancer cell proliferation and apoptosis through regulating the COX-2/PGE2/β-catenin signaling pathway.

Methods

Materials

ATST was from Pfizer (New York, NY, USA), Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) from Gibco (Rockville, MD, USA), penicillin-streptomycin solution from Ambionic, Western blotting (WB) goat anti-rabbit primary antibody and horse radish peroxidase (HRP)-labeled rabbit anti-mouse IgG secondary antibody from Abcam (Cambridge, MA, USA), propidium iodide (PI) dye and Annexin from ABM (Richmond, Canada), enzyme-linked immunosorbent assay (ELISA) kit from Abcam (Cambridge, MA, USA), and HCT116 cell lines and colon cancer tissues from Beijing Chaoyang Hospital, Capital Medical University.

HCT116 cell culture, transfection, ATST treatment and grouping

HCT116 cells were cultured with DMEM containing 11.0% FBS and 1.0% penicillin-streptomycin in an incubator with 5% CO_2 at 37°C, followed by passage upon reaching 70-80% confluence. According to the instructions of the TurboFect transfection reagent, HCT116 cells were transfected with COX-2-knockdown plasmids to lower the protein expression of COX-2. HCT116 cells were treated with ATST at different concentrations for different times, and were divided into blank control group (Con group), ATST group (ATST added), and ATST + anti-COX-2 group (COX-2 knockdown and ATST added).

ELISA

The culture supernatant of HCT116 cells was collected and incubated in serum-free RPMI 1640 medium for 24 h. After the medium was concentrated to remove cell debris, the supernatant was frozen at -80°C for later detection of PGE2 level via ELISA. In addition, the serum of 20 patients undergoing resection of colon cancer was separated via centrifugation at 3,000 rpm for 15 min, and stored at -70°C for later detection. The concentration of PGE2 was determined using the commercially-available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Detection of protein expressions of COX-2 and β -catenin via WB

After activation for a prescribed time, the cells were washed with 1 mL of ice-cold phosphate buffered saline (PBS), centrifuged at 3,000 g for 5 min, and resuspended with 100 μ L of ice-cold hypotonic buffer (10 mmol/L HEPES/KOH, 2 mmol/L MgCl₂, 0.1 mmol/L EDTA, 10 mmol/L KCl, and 1× protease inhibitor, pH 7.9). Then the cells were placed on ice for 10 min, centrifuged at 15,000 g for 30 s, washed with 1 mL of PBS for 3 times, and resuspended again with 50 μL of ice-cold saline buffer (50 mmol/L HEPES/KOH, 50 mmol/L KCl, 300 mmol/L NaCl, 0.1 mmol/L EDTA, 10% glycerol, and 1× protease inhibitor, pH 7.9). After that, the cells were placed on ice for 2 h, ultrasonically treated for 30 s and centrifuged at 15,000 g and 4°C for 5 min. The supernatant containing nucleoprotein was frozen in liquid nitrogen and stored at -70°C. Finally, the protein expressions of COX-2 and β -catenin were detected via WB.

Detection of HCT116 cell proliferation using cell counting kit-8 (CCK-8) assay

The HCT116 cell proliferation was detected using CCK-8 assay, as follows. First, the cells were diluted into cell suspension $(1 \times 10^6 \text{ cells/mL})$, and incubated with 10% CCK-8 solution in an incubator with 5% CO₂ at 37°C for 4 h. Then the optical density (OD) of the cell suspension was measured at 490 nm. Finally, the number of proliferating HCT116 cells was calculated, and the cell growth curves were plotted.

Statistics

All experiments were performed in triplicate, and data were analyzed using SPSS 18.0 software package (SPSS Inc., Chicago, IL, USA). All data in quantitative analysis were expressed as mean \pm standard deviation. Comparison between multiple groups was done using One-way ANOVA test followed by *post-hoc* test (least significant difference). The survival rate was calculated using the Kaplan-Meier method, and compared using log-rank test. * suggested the statistical significance (*p<0.05, **p<0.01).

Results

Effect of ATST on survival rate of HCT116 cells with treatment time

As shown in Figure 1, the survival rate of HCT116 cells had a significant difference at 24 h compared with that at 0 h (p<0.01), but it had no difference at 72 h compared with that at 48 h (p>0.05). It can be seen that ATST can inhibit the proliferation of HCT116 cells. Hence, the cells were treated with ATST for 24 h in the subsequent experiments.

HCT116 cells

As shown in Figure 2, there was a significant difference in the survival rate between HCT116 cells treated with 30 µM ATST and those treated with 0 μ M ATST (p<0.01), and the same was true between HCT116 cells treated with 30 μ M ATST and those treated with 10 µM ATST (p<0.01). There-



Figure 1. Effect of ATST on survival rate of HCT116 cells with treatment time. The survival rate of HCT116 cells had a significant difference at 24 h compared with that at 0 h (**p<0.01), but it had no difference at 72 h compared with that at 48 h (p>0.05).

fore, the optimal concentration of ATST was 30 μ M in the treatment of HCT116 cells.

Results of Kaplan-Meier survival analysis

The survival time was obviously longer in patients with low expressions of COX-2 and PGE2 than that in patients with high expressions of COX-2 and PGE2 (p<0.01) (Figure 3). It can be seen that low expressions of COX-2 and PGE2 in colon cancer tissues indicate a longer survival time, and there may be a positive correlation between COX-2 and PGE2.

Effect of ATST concentration on survival rate of A positive correlation between COX-2 and PGE2 expressions in HCT116 cells

A positive correlation was found between HCT116 cell density and COX-2 level (linear regression, r=0.9545, p<0.001), HCT116 cell density and PGE2 level (linear regression, r=0.9734, p<0.001), and COX-2 and PGE2 levels (linear regression, r=0.9690, p<0.001) (Figure 4). The re-



Figure 2. Effects of different ATST concentrations on survival rate of HCT116 cells. There was a significant difference in the survival rate between HCT116 cells treated with 30 μM ATST and those treated with 0 μM ATST (**p<0.01), and the same was true between HCT116 cells treated with $30 \,\mu\text{M}$ ATST and those treated with $10 \,\mu\text{M}$ ATST (**p<0.01).



Figure 3. Kaplan-Meier survival analysis. The overall survival time was obviously longer in patients with low expressions of COX-2 and PGE2 than that in patients with high expressions of COX-2 and PGE2 (p<0.01).

have a positive correlation.

Protein expressions in cells detected using WB

It was found that the protein expressions of COX-2 and β-catenin were obviously different between ATST + anti-COX-2 group and Con group (p<0.001), while they notably declined in ATST + anti-COX-2 group compared with those in ATST group (p<0.05) (Figure 5). The above findings suggest that ATST can down-regulate the protein expressions of COX-2 and β -catenin, and knocking down COX-2 can lower the expression of β -catenin, further confirming the positive correlation between expressions of COX-2 and β-catenin.

Effects of ATST and anti-COX-2 treatment on apoptosis-related molecules detected by WB

As shown in Figure 6, the activity of cleaved caspase-9, caspase-3 and poly-ADP-ribose-polymerase (PARP) was remarkably enhanced in HCT116 cells after treatment with ATST and

sults demonstrate that COX-2 and PGE2 levels ATST + anti-COX-2 (p<0.01), with β -actin as an internal reference protein. The activity of cleaved caspase-9, caspase-3 and PARP had no remarkable difference between ATST group and ATST + anti-COX-2 group. The results demonstrate that ATST and anti-COX-2 treatment can induce apoptosis of HCT116 cells.

ATST and anti-COX-2 treatment could markedly in*hibit cell proliferation*

COX-2 was knocked down in cells via lentiviral transfection, and HCT116 cells were treated with ATST. The proliferation of HCT116 cells was detected using MTT assay. The results manifested that the proliferation of HCT116 cells was evidently weakened in the ATST group and the ATST + anti-COX-2 group compared with that in Con group (p<0.01), while it had no significant changes between ATST group and ATST + anti-COX-2 group (p>0.05) (Figure 7). It can be seen that ATST and ATST + anti-COX-2 treatment can not only inhibit the proliferation, but also promote the apoptosis of HCT116 cells.



Figure 4. Correlations of HCT116 cells with COX-2 and PGE2. A positive correlation was found between HCT116 cell density and COX-2 level (linear regression, r=0.9545, p<0.001), HCT116 cell density and PGE2 level (linear regression, r=0.9734, p<0.001), and COX-2 and PGE2 levels (linear regression, r=0.9690, p<0.001).



Figure 5. Protein expressions in cells detected using WB. A: Protein bands. B: Protein expressions. The protein expressions of COX-2 and β -catenin were obviously different between ATST + anti-COX-2 group and Con group (***p<0.001), while they notably declined in ATST + anti-COX-2 group compared with those in ATST group (*p<0.05).



Figure 6. Effects of ATST and anti-COX-2 treatment on apoptosis-related molecules detected by WB. **A:** Protein expressions of apoptosis-related molecules detected by WB. **B:** Protein expressions of related molecules. The activity of cleaved caspase-9, caspase-3 and PARP was remarkably enhanced after treatment with ATST and ATST + anti-COX-2 (**p<0.01).



Figure 7. ATST and anti-COX-2 treatment could distinctly inhibit cell proliferation. The proliferation of HCT116 cells was remarkably weakened in ATST group and ATST + anti-COX-2 group compared with that in Con group (**p<0.01), while it had no significant changes between ATST group and ATST + anti-COX-2 group (p>0.05).

Discussion

Colon cancer, the third most common malignancy worldwide, is characterized by a high mortality rate [21]. As a malignant tumor caused by gastrointestinal lesions, colon cancer mainly has 3 types: adenocarcinoma, undifferentiated carcinoma, and mucinous adenocarcinoma, with the ulcerative and polypous manifestations [22]. In recent years, colorectal cancer features are gradually increasing morbidity and mortality rates mainly in developing countries [23]. Colon cancer progresses through the following 5 steps: 1) malignancies first develop in the colonic mucosa or the inner wall of the colon, 2) cancer cells then invade the colonic submucosa, 3) colon cancer invades the colonic wall, 4) the cancer cells further spread to lymph nodes, and 5) finally, tumor nodules are generated in the tissues around the colon, and the cancer cells invade the liver, lungs and other tissues [24]. Since early-stage colon

cancer has many symptoms that are inconspicuous, the overwhelming majority of patients have not been definitely diagnosed with colon cancer until the middle-advanced stage. Due to blood circulation and lymphatic infiltration around the intestinal wall, colon cancer metastasizes to the peri-intestinal lymph nodes and liver tissues, so it is very difficult to treat this disease [25]. Only 5-10% of cases of colorectal cancer are confirmed at the early stage in China. The colorectal cancer patients at the early stage can be cured mainly through surgically removing cancer tissues, while chemotherapy and immunotherapy are performed for the patients at the advanced stage to control the spread of tumor cells, thereby extending their life [26]. At present, it has been held that the morbidity rate of colon cancer can be raised by hyperlipidemia that is caused by increases in cholesterol and lipoprotein content. Clinical studies have demonstrated that statins can reduce the content of cholesterol and lipoprotein in the blood. Therefore, delving into the molecular mechanisms of colon cancer progression, proliferation and apoptosis and searching for novel efficacious treatment schemes are of important clinical significance, with the development of molecular pathology and the research and development as well as the application of targeted drugs.

In the present study, the influence of ATST treatment time on HCT116 cells was first studied. According to the findings, ATST had an inhibitory effect on the proliferation of HCT116 cells, and the inhibitory effect was the most potent at the treatment time of 24 h and the concentration of 30 μ M. In tissues, COX-1 can regulate normal physiological functions, while COX-2 is able to modulate the release of major inflammatory factors related to cancer progression, and it is over-expressed during the spread of cancer cells to activate angiogenesis and epithelial-mesenchymal

transition. Some studies have found that the colon cancer patients with lowly expressed COX-2 and PGE2 survive longer, and that COX-2 may be positively correlated with PGE2 therein. The results of this study further showed that the density of HCT116 cells was positively correlated with the protein expressions of both COX-2 and PGE2, and that there was a positive correlation between the levels of COX-2 and PGE2 as well. Therefore, the level of COX-2 is positively correlated with that of PGE2. Additionally, it was found through the WB that ATST down-regulated the expressions of the $COX-2/\beta$ -catenin signaling pathway-associated proteins and that the knockdown of COX-2 decreased the expression of β -catenin. It can be inferred that the expression of COX-2 is positively associated with that of β -catenin. Multiple studies have suggested a positive correlation between the protein expressions of COX-2 and β -catenin in colon cancer and skin cancer [19]. In the present study, no obvious differences in the activation of cleaved Caspase-9, cleaved Caspase-3 and cleaved PARP were observed after separate treatment with ATST and ATST + anti-COX-2, suggesting that both ATST and

anti-COX-2 can promote the apoptosis of HCT116 cells. Besides, the anti-COX-2 HCT116 cells were constructed via lentivirus infection, and treated with ATST. Based on these results, ATST and ATST + anti-COX-2 repressed the proliferation of colon cancer cells and facilitated their apoptosis. Hence, it is believed that ATST down-regulates the COX-2/ PGE2/ β -catenin signaling pathway to restrain the proliferation and promote the apoptosis of colon cancer HCT116 cells.

Conclusions

In conclusion, the results of the present study corroborate that ATST inhibits the proliferation of colon cancer cells and induces their apoptosis through the down-regulation of the COX-2/PGE2/ β -catenin signaling pathway, thereby providing a medical theoretical basis for the prevention and treatment of colon cancer.

Conflict of interests

The authors declare no conflict of interests.

References

- 1. Baena R, Salinas P. Diet and colorectal cancer. Maturitas 2015;80:258-64.
- 2. Lippi G, Mattiuzzi C, Cervellin G. Meat consumption and cancer risk: a critical review of published metaanalyses. Crit Rev Oncol Hematol 2016;97:1-14.
- Keshk WA, Zineldeen DH, Wasfy RE, El-Khadrawy OH. Fatty acid synthase/oxidized low-density lipoprotein as metabolic oncogenes linking obesity to colon cancer via NF-kappa B in Egyptians. Med Oncol 2014;31:192.
- Zhou X, Jin J, Wang et al. Efficacy and adverse reactions of combination therapy of bevacizumab and 5-fluorouracil in patients with metastatic colon cancer. JBUON 2019;24:494-500.
- 5. Reverter M, Rentero C, Garcia-Melero A et al. Cholesterol regulates Syntaxin 6 trafficking at trans-Golgi network endosomal boundaries. Cell Rep 2014;7:883-97.
- Sheng R, Kim H, Lee H et al. Cholesterol selectively activates canonical Wnt signalling over non-canonical Wnt signalling. Nat Commun 2014;5:4393.
- Nielsen SF, Nordestgaard BG, Bojesen SE. Statin use and reduced cancer-related mortality. N Engl J Med 2012;367:1792-802.
- Poynter JN, Gruber SB, Higgins PD et al. Statins and the risk of colorectal cancer. N Engl J Med 2005;352:2184-92.

- 9. Lytras T, Nikolopoulos G, Bonovas S. Statins and the risk of colorectal cancer: an updated systematic review and meta-analysis of 40 studies. World J Gastroenterol 2014;20:1858-70.
- 10. Mao Y, Poschke I, Wennerberg E et al. Melanoma-educated CD14+ cells acquire a myeloid-derived suppressor cell phenotype through COX-2-dependent mechanisms. Cancer Res 2013;73:3877-87.
- 11. Gomes M, Teixeira AL, Coelho A, Araujo A, Medeiros R. The role of inflammation in lung cancer. Adv Exp Med Biol 2014;816:1-23.
- 12. Mattsson JS, Bergman B, Grinberg M et al. Prognostic impact of COX-2 in non-small cell lung cancer: a comprehensive compartment-specific evaluation of tumor and stromal cell expression. Cancer Lett 2015;356:837-45.
- 13. Sicking I, Rommens K, Battista MJ et al. Prognostic influence of cyclooxygenase-2 protein and mRNA expression in node-negative breast cancer patients. BMC Cancer 2014;14:952.
- 14. Bocca C, Ievolella M, Autelli R et al. Expression of Cox-2 in human breast cancer cells as a critical determinant of epithelial-to-mesenchymal transition and invasiveness. Expert Opin Ther Targets 2014;18:121-35.
- 15. Chulada PC, Thompson MB, Mahler JF et al. Genetic disruption of Ptgs-1, as well as Ptgs-2, reduces intestinal tumorigenesis in Min mice. Cancer Res 2000; 60:4705-8.

- 16. Drees F, Pokutta S, Yamada S, Nelson WJ, Weis WI. Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. Cell 2005;123:903-15.
- 17. Castellone MD, Teramoto H, Williams BO, Druey KM, Gutkind JS. Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis. Science 2005;310:1504-10.
- Smith KA, Tong X, Abu-Yousif AO et al. UVB radiationinduced beta-catenin signaling is enhanced by COX-2 expression in keratinocytes. Mol Carcinog 2012;51:734-45.
- 19. Xiao H, Zhang Q, Lin Y, Reddy BS, Yang CS. Combination of atorvastatin and celecoxib synergistically induces cell cycle arrest and apoptosis in colon cancer cells. Int J Cancer 2008;122:2115-24.
- 20. Yang Z, Xiao H, Jin H, Koo PT, Tsang DJ, Yang CS. Synergistic actions of atorvastatin with gamma-tocotrienol and celecoxib against human colon cancer HT29 and HCT116 cells. Int J Cancer 2010;126:852-63.
- 21. Byrne M, Saif MW. Selecting treatment options in

refractory metastatic colorectal cancer. Onco Targets Ther 2019;12:2271-8.

- 22. Zenger S, Gurbuz B, Can U, Balik E, Bugra D. Clinicopathological features and prognosis of histologic subtypes in the right-sided colon cancer. JBUON 2020;25:2154-9.
- 23. Tang X, Qiao X, Chen C, Liu Y, Zhu J, Liu J. Regulation Mechanism of Long Noncoding RNAs in Colon Cancer Development and Progression. Yonsei Med J 2019;60:319-25.
- 24. Pandurangan AK, Divya T, Kumar K, Dineshbabu V, Velavan B, Sudhandiran G. Colorectal carcinogenesis: Insights into the cell death and signal transduction pathways: A review. World J Gastrointest Oncol 2018;10:244-59.
- 25. Weinberg BA, Marshall JL. Colon Cancer in Young Adults: Trends and Their Implications. Curr Oncol Rep 2019;21:3.
- Mody K, Bekaii-Saab T. Clinical Trials and Progress in Metastatic Colon Cancer. Surg Oncol Clin N Am 2018;27:349-65.