Anti-inflammatory Effect of Pitavastatin on NF-κB Activated by TNF-α in Hepatocellular Carcinoma Cells

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As nuclear factor-kappa B (NF-κB) is essential for promoting inflammation-associated cancer, it is a potential target for cancer prevention in chronic inflammatory diseases. Here we examined the anti-inflammatory effect of pitavastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, on NF-κB activated by TNF-α in hepatocellular carcinoma (HCC) cells. Western blot revealed that the treatment of Huh 7 cells with pitavastatin at 0.1 μM inhibited the nuclear expression of NF-κB p65 induced by TNF-α. Furthermore, electrophoretic mobility shift assay showed that after the cells were incubated with pitavastatin alone or with pitavastatin and TNF-α for 24 h, pitavastatin significantly decreased the DNA binding activity of NF-κB induced by TNF-α. Subsequently, luciferase assay revealed that pitavastatin suppressed the transcriptionsal activity of the NF-κB promoter, which was clearly related to the HMG-CoA reductase activity because the addition of mevalonic acid (MEV) elevated the TNF-α activity. Moreover, the Rho kinase inhibitor Y27632 had no major effect on the NF-κB inhibitory activity of pitavastatin. The inhibitory effect of pitavastatin is possibly independent of the Rho kinase pathway in inflammation-associated HCC cells. Finally, the addition of TNF-α significantly increased IL-6 protein production, which was suppressed by the addition of pitavastatin. These results suggest that pitavastatin at a low dose (0.1 μM) inhibits NF-κB activation and decreases IL-6 production induced by TNF-α, and is therefore expected to be a new strategy for treating HCC.

Key words nuclear factor (NF)-κB activation; inflammation; hepatocellular carcinoma cell; pitavastatin; IL-6

A causal relationship between inflammation and cancer has long been suspected. Rudolf Virchow demonstrated the presence of leukocytes in malignant tumors and claimed that tumors arise from regions of chronic inflammation. The microenvironment in and around tumors contains cells of the innate immune system that secrete pro-inflammatory cytokines and chemokines, such as TNF-α, IL-1, IL-6 and IL-8. This environment enhances cell proliferation, survival and migration, as well as angiogenesis, thereby promoting tumor development. When the inflammation is more severe and of longer duration, hepatocellular carcinoma (HCC) develops more frequently. Therefore, chronic inflammation may have a causative role in hepatocarcinogenesis.

The molecular and cellular mechanisms linking chronic inflammation to tumorigenesis remain largely unresolved. Nuclear factor-kappa B (NF-κB) is a ubiquitous transcription factor that is activated by a variety of cytokines and mitogens, and is a key regulator in the inflammatory response to infection. The activation of NF-κB is thought to be part of a stress response as it is activated by a variety of stimuli that include growth factors, cytokines, lymphokines, UV, pharmacological agents, and other stresses. Moreover, the activation of NF-κB has been shown to play an important role in enhancing the expression of several inflammatory cytokine genes, including TNF-α, IL-6 and IL-8. That phenomenon is observed in various cell types upon stimulation with such agonists as IL-1 and TNF-α.

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly referred to as statins, are potent inhibitors of cholesterol biosynthesis and widely prescribed for the treatment of hypercholesterolemia. Several clinical trials have demonstrated that statins are effective for both primary and secondary prevention of coronary artery diseases, acting by markedly decreasing serum cholesterol levels and reducing the incidence of myocardial infarction and stroke. Interestingly, recent studies have emphasized that some non-lipid-related effects of statins present potential benefits in certain diseases. Pitavastatin is a novel, highly potent inhibitor of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. Previously, we reported that pitavastatin shows protective action on vascular endothelial cells (ECs) by inducing the activation of eNOS, thereby protecting the vascular ECs from injury due to the inflammatory reaction induced by TNF-α, and increasing NO production, which is dependent on post-transcriptional regulation, and involves phosphoinositide 3-kinase and the Akt pathway.

In addition, the anti-inflammatory action, as well as the reduction of pro-inflammatory signaling by statins, such as cytokine and oxygen radical formation, has been reported. Other groups have suggested that statins may also play a beneficial role in cancer therapy. Pravastatin prolonged the survival of patients with advanced HCC in a randomized clinical trial. However, the mechanisms of the anti-cancer effect of statins are little known. Therefore, the purpose of the present study was to examine the effect of pitavastatin treatment on inflammation-associated hepatocarcinoma cells. Here we show that pitavastatin inhibits NF-κB activation and IL-6 protein production induced by TNF-α in hepatocarcinoma cells. Its inhibitory effect is possibly independent of the Rho kinase pathway, indicating its potential use in cancer prevention and therapy.

MATERIALS AND METHODS

Reagents Pitavastatin (trade name: LIVALO®, code name: NK-104) was kindly provided by Kowa Co., Ltd. (Nagoya, Japan) and Nissan Chemical Industries, Ltd. (Tokyo, Japan). Recombinant TNF-α and the Rho kinase inhibitor Y27632 were purchased from CALBIOCHEM.

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Cell Culture  The human HCC cell line Huh 7 was originally obtained from the American Type Culture Collection (ATCC) and maintained in DMEM medium (Sigma) containing 10% FBS. The cells were maintained at 37 °C and 5% CO2. For experiments, the cells were seeded onto six-well culture plates and grown in complete medium to 90% confluence. Then, the cells were washed with phosphate-buffered saline (PBS) and incubated for the indicated times at 37 °C in 2 ml of medium containing pitavastatin, TNF-α or vehicle, in the presence or absence of MEV or Y27632.

**WST-8 Assay** Cytotoxicity was evaluated with the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] colorimetric assay. Huh 7 cells (1×10⁴ cells/well) were seeded onto 96-well plates and counted for 30 min and then centrifuged at 15000 rpm for 5 min at 4 °C. Next, 10 μl of WST-8 reagent (Cell Counting Kit, Dojindo Laboratories, Japan) was added and incubation was conducted for 2 h. Cell viability was determined according to the manufacturer’s instructions.

**Extraction of Nuclear Protein** After appropriate treatment of the cells with pitavastatin in the absence or presence of TNF-α as described above, nuclear protein was extracted. Briefly, the cells were harvested with 1 ml of ice-cold PBS and centrifuged for 1 min at 5000 rpm at 4 °C. The cell pellet was lysed with 0.4 ml of buffer A containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM diethiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF), for 15 min on ice. Then, 25 μl of 10% Nonidet P-40 solution was added and the samples were vortexed for 15 s before centrifuging at 15000 rpm for 5 min at 4 °C. The pellet was washed once with 0.5 ml of buffer A and resuspended in 50 μl of buffer B, which was composed of 20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF. The lysed nuclei were left on ice for 30 min and then centrifuged at 15000 rpm for 5 min at 4 °C. The nuclear protein concentration was determined by the DC Protein Assay (Bio-Rad, Hercules, CA, U.S.A.).

**Western Blotting** Aliquots of the nuclear extracts (20 μg of protein each) were separated by 8.5% SDS-PAGE, electrotransferred to a polyvinyldene difluoride (PVDF) membrane (Bio-Rad), blocked with 5% nonfat milk in TBS-Tween buffer (20 mM Tris–HCl, pH 7.4, 135 mM NaCl, 0.1% Tween) for 1.5 h at room temperature, and incubated with the appropriate antibody overnight at 4 °C, and then with horseradish peroxidase conjugated secondary antibody for 30 min at room temperature. After extensive washing, immunoreactive proteins were detected with an Enhanced Chemiluminescence Detection System (ECL; Amersham Biosciences Corp., Piscataway, NJ, U.S.A.). The following primary antibodies were used: polyclonal antibody for NF-κB p65 from Santa Cruz; and monoclonal antibody for TFIIB from BD Transduction Laboratories.

**Electrophoretic Mobility Shift Assay (EMSA)** The following reagents were used for the phosphorylation reaction: 3 pmol of consensus oligonucleotide 5'-AGTTGAGGG-GACCTTCCAGGC-3’ NF-κB DNA binding sequence (Promega, Madison, WI, U.S.A.), 5× T4 polynucleotide kinase buffer, T4 polynucleotide kinase, nuclelease-free water and 10 μCi of [γ-32P]-ATP. The reaction mixture was incubated for 10 min at 37 °C. Eight micrograms of nuclear extract was incubated in binding buffer (20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl, pH 7.5) and 1.4 mg/ml poly (dl)pyloric (dC) containing 32P-labeled NF-κB oligonucleotide with or without the unlabelled oligonucleotide probe at room temperature for 20 min. The sample was loaded on a pre-run 5% polyacrylamide gel. The gel was dried, exposed overnight and analyzed by autoradiography scanning densitometry.

**NF-κB Luciferase Reporter Assay** The human 3×κB promoter constructs: NF-κB-dependent luciferase reporter (3×κB-luc) was kindly provided by Dr. Shigeki Miyamoto (University of Wisconsin, WI, U.S.A.). Huh 7 cells were seeded onto six-well plates and cotransfected with 1.0 μg of firefly-luciferase reporter plasmid and 0.1 μg of renilla-luciferase transfection control (phR-CMV; Promega, Southampton, U.K.) using Lipofectamine™ with Plus Reagents (Invitrogen). After transfection for 24 h, the cells were treated with the reagents for the indicated times, and then harvested for the luciferase assay. Luciferase activity was measured with the Dual-Luciferase Assay System according to the manufacturer’s instructions (Promega) and a luminometer (Gene-Light 55, Microtech Nichion, Chiba, Japan). The firefly-luciferase activity of NF-κB was normalized to the renilla-luciferase activity and expressed as fold induction compared with the control value.

**Enzyme Linked Immunosorbent Assay (ELISA)** Cytokine concentrations in the supernatants of the control and IL-6 treated with reagents were measured using commercial enzyme linked immunosorbent assay (ELISA) kits for IL-6 (Pierce Chemical, Rockford, IL, U.S.A.), according to the manufacturer's instructions. Values were expressed as pg per milligram protein (pg/mg prot).

**Statistical Analysis** Data are presented as means±S.D. Statistical analysis was performed with the Student’s t test or ANOVA, followed by Tukey’s test.

**RESULTS**

**Inhibition of Huh 7 Cell Growth by Pitavastatin** To determine the optimal concentration of pitavastatin for inhibiting Huh 7 cell growth, the effect of pitavastatin at concentrations ranging from 0.01 to 10 μM on Huh 7 cell growth was examined with the WST-8 assay. Growth inhibitory effects were observed with pitavastatin at 1 μM and 10 μM, and 60% of the cells were lysed by pitavastatin at 10 μM compared with the control (Fig. 1). In addition, the clinically used concentrations of pitavastatin in plasma range from 0.1 to 1 μM. Therefore, the anti-inflammatory effect in Huh 7 cells was detected at low concentrations of pitavastatin.

**Pitavastatin Suppresses NF-κB Expression by TNF-α** A key player in inflammatory processes is NF-κB. There is evidence that the survival of hepatocytes and their progression to malignancy are regulated by NF-κB. We examined whether pitavastatin inhibits NF-κB by immunoblotting with an antibody specific for NF-κB p65. TNF-α strongly induced the expression of NF-κB by approximately 1.8-fold.
further investigate the effect of pitavastatin on NF-κB.

The nuclear protein was extracted and NF-κB expression was examined by immunoblotting with an antibody specific for NF-κB or TFIIB. The two panels on top show the protein expression levels of NF-κB and TFIIB (A); bar graphs at the bottom show the expression levels of NF-κB relative to those of TFIIB (B). Data are means±S.D. (n=3), **p<0.01 vs. control.

compared with the control in the nuclei of Huh 7 cells, as shown by western blotting conducted three times. However, the induced NF-κB expression was significantly suppressed by the addition of pitavastatin at 0.1 μM (p<0.01, Fig. 2).

**Pitavastatin Decreases DNA Binding Activity of NF-κB Induced by TNF-α** Because the transcription factor NF-κB is crucial for pro-inflammatory cytokine regulation, to further investigate the effect of pitavastatin on NF-κB activation in Huh 7 cells, the cells were stimulated with pitavastatin alone or with pitavastatin and TNF-α for 24 h. Nuclear extracts were prepared and analyzed by EMSA. As shown in the EMSA fluorograms (Fig. 3A) and the densitometric patterns (Fig. 3B), NF-κB activation in the Huh 7 cells was increased by approximately 4-fold after TNF-α stimulation compared with the control and the addition of pitavastatin significantly inhibited NF-κB binding to DNA induced by TNF-α (p<0.01).

**Pitavastatin Reduces NF-κB Promoter Transcriptional Activity Induced by TNF-α** NF-κB is an important transcription factor that controls cell survival by regulating programmed cell death, proliferation and growth arrest. We performed the dual-luciferase assay with a reporter vector containing the NF-κB promoter in Huh 7 cells. As shown in Fig. 4A, upon stimulation with TNF-α, the luciferase reporter activity was increased by approximately 2.6-fold compared with the control. Pitavastatin at 0.1 and 1 μM significantly inhibited the NF-κB promoter transcriptional activity induced by TNF-α.

**Inhibitory Effect of Pitavastatin on NF-κB Induced by TNF-α Possibly Independent of Rho Kinase Pathway** We further delineated the potential mechanism underlying the inhibitory effect of pitavastatin on NF-κB activity by luciferase assay. Generated by HMG-CoA reductase, 1,mevalonate serves as a key intermediate of cholesterol synthesis from acetyl-CoA. To determine whether the pitavastatin inhibition of NF-κB activity occurred through blockage of the HMG-CoA reductase activity or suppression of 1,mevalonate synthesis, we added exogenous MEV (1 mM) to the cultures of Huh 7 cells with TNF-α in the presence or absence of pitavastatin (0.1 μM). The pitavastatin-suppressed NF-κB transcriptional activity was clearly related to the HMG-CoA reductase activity because the addition of MEV elevated NF-κB activity (Fig. 4B).

In addition, the Rho family of small GTP-binding proteins consists of three subfamilies, Rho, Rac and Cdc42, which play important roles in signal transduction and inflammation. Treatment with statin reduces Rho GTPase activation, thereby diminishing monocytes stable in vascular endothelium.25 The Rho kinase inhibitor Y27632 specifically inactivates the p160 Rho-associated protein kinase p160ROCK, which is known to regulate NF-κB activity.25
We tested whether Y27632 could mimic the inhibitory effect of pitavastatin on NF-κB activity induced by TNF-α in Huh 7 cells. Y27632 showed no major effect similar to that of pitavastatin on NF-κB activity (Fig. 4B). In addition, Y27632 did not influence NF-κB activity because there was no difference between the cells treated with Y27632 and the untreated cells in the presence of TNF-α (Fig. 4B). These results suggest that the Rho signaling pathway may not participate in pitavastatin inhibition of NF-κB activity.

**Pitavastatin Decreases IL-6 Protein Production**

The activation of NF-κB has been shown to play an important role in enhancing the expression of several cytokine genes, including IL-6 and IL-8. IL-6, which has diverse biological effects on immune and inflammatory responses, is produced in response to infection or injury in a variety of cells. To evaluate the effect of pitavastatin on the production of the inflammatory marker, we measured IL-6 protein production in the supernatant of Huh 7 cells by ELISA. The addition of TNF-α significantly increased IL-6 protein production in the Huh 7 cells by approximately 3.5-fold compared with the control (Fig. 5A); however, the increase was suppressed by 50% upon the addition of pitavastatin. As expected, MEV also elevated low-dose pitavastatin-reduced production of IL-6 (Fig. 5B). By contrast, Y27632 did not influence the effect of pitavastatin on IL-6 production (Fig. 5B).

**DISCUSSION**

That there is a connection between inflammation and cancer has been suspected for a long time. Inflammatory cells have been largely ignored as a promoter of tumor development. However, epithelial cell turnover is regulated by inflammation. More importantly, proliferation in the setting of chronic inflammation predisposes humans to carcinoma of the large bowel, liver, breast, urinary bladder, gastric mucosa, prostate, ovary, and skin. As infections by Helicobacter pylori in HCC have a very high cancer-associated risk of 75%, chronic inflammation may have a causative role in hepatocarcinogenesis.

There is growing evidence that statins exert anti-inflammatory and antioxidative vascular actions that are independent of their serum lipid lowering effects. The anti-inflammatory action, as well as the reduction of pro-inflammatory signaling, such as cytokines and oxygen radical formation, by statin treatment has been reported.

Others have suggested that statins may also potentially play a beneficial role in cancer therapy. Simvastatin (1—30 μM) was demonstrated to have cancer preventing ability. Fluvastatin inhibited the proliferation of Huh 7 cells by inducing apoptosis and G1/S cell cycle arrest. A maximal decrease in cell number of 90% was observed at 50 μM and the IC_{50} of fluvastatin was 10±3 μM in Huh 7 cells. However, the systemic bioavailability of a single oral dose of 20 mg of fluvastatin was found to be in the range of 0.1—1 μM. In the present study, pitavastatin at a low dose (0.1 μM) induced an anti-inflammatory effect through the inactivation of NF-κB in Huh 7 cells without producing any anti-proliferative effects.
A key player in inflammatory processes is the transcription factor NF-κB. Activated NF-κB has been detected in different tumor cell lines and primary tumor samples. NF-κB activation is often observed in human HCC, particularly following hepatitis. Moreover, the activation of NF-κB has been shown to play an important role in enhancing the expression of several cytokine genes, including IL-6 and IL-8. That phenomenon has been observed in various cell types upon stimulation with such agonists as IL-1 and TNF-α. It has been reported that statin treatment inhibits NF-κB activation in differential cell lines. For example, lovastatin (10 μM) inhibited NF-κB activation and the expression of four pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, and MCP-1) induced by lipopolysaccharide (LPS) in human mesangial cells (HMCs). Gel shift assay revealed that cerivastatin (25 ng/ml) displayed a potent anti-inflammatory effect on human breast cancer cells (MDA-MB-231 cells), which could be related to NF-κB inhibition. The anti-inflammatory action of simvastatin (3 μM) on human C-reactive protein (huCRP) expression in hepatocytes is based on the up-regulation of the cytosolic inhibitor of NF-κB, IκBα, resulting in reduced NF-κB activity. Pretreatment with lovastatin (20 μM) decreased the TNF-α-induced activation of the DNA binding activity of NF-κB in Hela cells. Pitavastatin at 5—15 μM decreased NF-κB p65 expression as shown by western blotting and inhibited NF-κB activity as shown by EMSA, in macrophages. Our data showed that pitavastatin at 0.1 μM markedly inhibited the TNF-α-induced NF-κB activation in Huh 7 cells. Furthermore, pitavastatin significantly decreased the DNA binding activity of NF-κB. Subsequently, we analyzed the effect of pitavastatin on NF-κB-dependent gene expression by reporter gene analysis using an NF-κB-specific minimal promoter construct, and found that the increase in NF-κB-regulated gene expression was largely blocked upon treatment of the cells with pitavastatin, closely approximating the results of western blot.

The Rho families of GTPases are involved in the regulation of multiple cellular functions, including cell migration, proliferation and inflammation. It has been reported that treatment with statin reduces Rho GTPase activation, thereby diminishing monocyes stable in vascular endothelium. Pitavastatin (10 μM) was suggested to play an anti-inflammatory role through the RhoA-dependent pathway in monocytic THP-1 cells. Other studies have demonstrated that statins may regulate cytokine-induced IL-6 level through different signal pathways that involve both Y27632-sensitive and -insensitive molecular signaling, in particular the NF-κB pathway. Our results demonstrate that the effect of pitavastatin at a low dose appears to be specific to the inhibition of HMG-CoA reductase, as the addition of MEV abolishes the inhibitory effect of pitavastatin. Furthermore, the pitavastatin-reduced NF-κB activity is possibly independent of the Rho kinase pathway. To establish the exact role of the small GTP-binding proteins in the regulation of NF-κB activity by pitavastatin in Huh 7 cells, it is essential to examine the effect of dominant-negative mutants of the small GTP-binding proteins.

IL-6, which has diverse biological effects on immune and inflammatory responses, is produced in response to an infection or an injury in a variety of cells, including monocytes, lymphocytes, fibroblasts, ECs, and keratinocytes. The activation of NF-κB has been shown to play an important role in enhancing IL-6 expression. To evaluate the effect of pitavastatin on the production of the inflammatory marker, we measured the amount of IL-6 protein produced in the supernatant of Huh 7 cells. The TNF-α-induced IL-6 production was suppressed by the addition of pitavastatin, which was abolished by MEV and was not mediated by the Rho kinase pathway. Our findings demonstrate that pitavastatin not only inhibits the activation of NF-κB, but also decreases the expression level of the pro-inflammatory cytokine IL-6, which is possibly independent of the Rho kinase pathway and may influence the evolution of the HCC inflammatory process.

Previously, we found that NF-κB expression is necessary for the maintenance of the malignant phenotype and serves as a target for cancer treatment. It has been reported that pitavastatin is a powerful statin and has favorable pharmacokinetic properties. Our study provides important in vitro evidence that pitavastatin at a low dose (0.1 μM) exerts an anti-inflammatory effect by inhibiting NF-κB activation independent of cholesterol reduction. This dose is very similar to the expected plasma levels of it in patients, and may also potentially play a beneficial role in cancer therapy.

In summary, as NF-κB is essential for promoting inflammation-associated cancer, it is a potential target for cancer prevention in chronic inflammatory diseases. Pitavastatin at 0.1 μM decreased the production of the pro-inflammatory cytokine, IL-6, through the inhibition of NF-κB activation in Huh 7 cells. Our results suggest that pitavastatin may be a potent agent for the prevention of HCC progression, aside from its serum lipid lowering effect.

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