Simvastatin, an HMG-CoA Reductase Inhibitor, Reduced the Expression of Matrix Metalloproteinase-9 (Gelatinase B) in Osteoblastic Cells and HT1080 Fibrosarcoma Cells

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Received November 4, 2003; Accepted February 7, 2004

Abstract. MMP-9 or Gelatinase B, a member of the matrix metalloproteinase family (MMPs), plays important roles in physiological events such as tissue remodeling and in pathological processes that lead to destructive bone diseases, including osteoarthritis and periodontitis. In addition to its effect on the increase of total bone mass, statin (an HMG-CoA reductase inhibitor) suppresses the expression of MMPs. In this study, we proposed that simvastatin reduces MMP-9 expression in osteoblasts and HT1080 fibrosarcoma cell line. Gelatin zymography, Western blot analysis and reverse transcriptase-PCR were used to investigate the effects of simvastatin on MMP-9 in primary calvaria cells, U2-OS osteosarcoma cells, and HT1080 fibrosarcoma cells. The results from gelatin zymography and Western blot analysis revealed that simvastatin suppressed MMP-9 activity in these cells in concentration- and time-dependent manners. The effective concentrations of simvastatin were 100 – 500 nM, 5 – 15 μM, and 2.5 – 10 μM in primary calvaria, U2-OS, and HT1080 cells, respectively. Collectively, these results suggest that simvastatin is a potent drug for inhibition of MMP-9 expression in osteoblastic cells and HT1080 fibrosarcoma cells.

Keywords: HT1080, matrix metalloproteinase-9, osteoblast, simvastatin, tumor necrosis factor-α

Introduction

Osteoporosis, osteoarthritis, and periodontitis are bone diseases that impact human health and economics. Although the etiology of these diseases are unknown, increases in enzyme matrix metalloproteinase expression has been reported in all of these pathologies (1 – 6). Matrix metalloproteinases (MMPs) are a family of enzymes that play important roles in both physiological and pathological processes of the bone (6, 7). MMPs can be classified into 4 subgroups: collagenase, stromelysin, gelatinase, and membrane-type MMP (6, 7). The 92-kDa gelatinase B (MMP-9) appears to significantly contribute to the pathological bone destruction in osteo- and rheumatoid arthritis, osteosarcoma, and squamous cell carcinoma (1, 2, 8 – 10). Substrates for MMP-9 include collagens type I, IV, and V; laminin; and fibronectin (6, 7).

Statin, an HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitor, has been used to control cholesterol levels in serum by inhibiting HMG-CoA reductase (11). This reductase is the rate-limiting enzyme of endogenous cholesterol synthesis. There have been reports of the effectiveness of statin in increasing the total bone mass both in human and animal models (12 – 15). A recent study suggests that statin inhibits bone resorption and stimulates bone formation (15). Several members of the statin family have been reported to suppress the expression of MMPs in macrophage and endothelial cells (16, 17). In this study, we investigated the effect of simvastatin, a member of the statin family, on MMP-9 expression in osteoblastic cells, including calvarial-derived primary osteoblasts, U2OS osteosarcoma cells, and HT1080 fibrosarcoma cells. We demonstrated that simvastatin inhibited MMP-9 expression by downregulating the steady level of mRNA and protein secretion in time- and concentration-
Materials and Methods

Materials

Dulbecco’s Modified Eagle’s Medium (DMEM), collagenase type II, L-glutamine, Trypsin-EDTA; penicillin/streptomycin/amphotericin (10,000 U/ml, 10,000 µg/ml, and 2,500 µg/ml, respectively); fetal bovine serum (FBS); oligonucleotides; and TRIzol reagent were obtained from Gibco-Life Technologies, Ltd. (Paisley, UK). Simvastatin was purchased from Apins Chemical Limited (Abingdon, UK). Tumor necrosis factor (TNF)-α was obtained from R&D Systems (Minneapolis, MN, USA). Immuno-Blot PVDF membrane and Bio-Rad Protein assay kit were obtained from Bio-Rad Laboratories (Hercules, CA, USA). The chemiluminescence kit was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). RT-PCR kits were purchased from Promega (Madison, WI, USA). The rabbit monoclonal antibody anti-human MMP-9 (M5302) and the anti-rabbit secondary antibody conjugated to horseradish peroxidase (A6154) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell cultures

Primary calvaria cells were isolated from the parietal bones of one-day-old newborn rats by using the sequential digestion technique as previously described (18, 19). Briefly, periosteum and sutural fibrous tissue were removed from the parietal bones. The bones were then cut into small pieces (2 mm) and sequentially digested with 0.5% collagenase type II at 37°C for 30 Min. The calvaria cells from fraction numbers 3 – 5 were pooled together and plated on 35-mm plates in DMEM supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml amphotericin, 2 mM L-glutamine, and 10% FBS. The medium was changed after 3 days and then every 48 h. When cells reached confluence, they were subcultured using 0.05% trypsin with 0.01% EDTA in a 60-mm plate, and these cells were considered the first-passage cells. The second- and third-passage cells were used for the experiments.

The U2-OS human osteosarcoma cell line (ATCC number: HTB-96) was a generous gift from Dr. Masa-aki Ikeda (Tokyo Medical Dental University, Tokyo). The HT1080 fibrosarcoma cell line (ATCC number: CCL-121) was obtained from the American Type Culture Collection (Rockville, MD, USA). The cell lines were maintained in DMEM supplemented as described above. All cells were maintained in humidified 95% air 5% CO₂ atmosphere at 37°C.

To obtain the conditioned media, nearly confluent cells were incubated for 24 and 48 h at 37°C with serum-free DMEM supplemented with 0.2% BSA and increasing concentrations of simvastatin. Simvastatin was dissolved by dimethyl sulfoxide (DMSO). The final concentration of DMSO in the culture medium did not exceed 0.2% (v/v). At the end of the incubation period, the conditioned media were collected, and the activity of secreted MMP-9 was analyzed using gelatin zymography. Total secreted cellular protein was measured using the Bio-Rad Protein assay kit and compared to the standard curve of the BSA.

Zymographic assay for gelatinase

Gelatinase activity in the conditioned media was determined by SDS-PAGE zymography as previously described (20). The conditioned media were concentrated by centricon, and a 5-µg sample of total proteins was electrophoresed in a 10% SDS-PAGE containing 0.1% (w/v) gelatin under non-reducing conditions and without boiling. Gels were washed twice with 2.5% (v/v) Triton X-100 for 20 min at room temperature, and subsequently incubated overnight at 37°C in a developing buffer containing 10 mM CaCl₂, 150 mM NaCl, and 50 mM Tris-HCl, pH 8.0. The gels were then stained with 0.5% Coomassie brilliant blue R-250 in 10% acetic acid (v/v) and 50% methanol. Clear zones against the blue background indicate the presence of gelatinolytic activity. It is important to note that in this SDS-containing gel, both the latent form and the activated form of MMP-9 develop gelatinolytic activity. Therefore, “activity” indicates the total gelatinolytic capacity measured in the conditioned media.

Western blot analysis

The conditioned media (50 µg of total protein) were analyzed on 10% SDS-PAGE under the reducing condition. The proteins were blotted on an Immuno-Blot PVDF membrane and incubated in 5% non-fat skim milk in PBS containing 0.1% Tween 20 (PBS-T) for at least 1 h. Subsequently, the rabbit monoclonal antibody anti-human MMP-9 was added to the PBS-T (1:1000 dilution) and incubated at 4°C overnight. The protein binding of MMP-9 was detected using the anti-rabbit secondary antibody conjugated to horseradish peroxidase and enhanced using chemiluminescence according to the manufacturer’s instructions.

RNA isolation and RT-PCR analysis

Total cellular RNA was extracted from treated cells using TRIzol reagent. Changes in the steady-state concentration of mRNA for MMP-9 were assessed by RT-PCR. Briefly, total RNA (2 µg) was converted to single stranded cDNA using a Reverse Transcription
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System (Promega). The target cDNA was amplified using the following sense primer and antisense primers: for MMP-9, sense -CGC AGA CAT CGT CAT CCA GT and antisense -GGA TTG GCC TTG GAA GAT GA; for GAPDH, sense -GCC ACC CAG AAG ACT GTG GAT and antisense -TGG TCC AGG GTT TCT TAC TCC. The amplification cycles were 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. After 30 cycles, the PCR products were separated by electrophoresis on 1.5% agarose (400 bp for MMP-9 and 500 bp for GAPDH).

Statistical analyses

For quantification of zymogram and Western blotting, densitometric scanning was performed using the Molecular Analyst Software version 1.5, Bio Rad. Zymogram gels were photographed and the negatives were scanned using the GS-700 Imaging Densitometer, Bio Rad. Data are presented as the mean ± S.D. Group comparison was performed using ANOVA followed by post-hoc analysis with Duncan’s multiple range test. Significance was assumed at a P level of 0.05.

Results

Simvastatin suppressed the release of MMP-9 in U2-OS and HT1080

To investigate the effect of simvastatin on enzyme MMP-9 activity and protein levels, gelatin zymography and Western blotting were performed. Gelatin zymography is a highly sensitive method for detecting gelatinolytic enzyme activity. Upon renaturation of the enzyme, gelatinase digests the gelatin in the gel and gives clear bands in an intensely stained background. Gelatin zymography revealed that simvastatin significantly reduced MMP-9 activity in U2-OS in a concentration-dependent manner at 48 h (Fig. 1A), but simvastatin did not change the activity of MMP-9 at 24 h (data not shown). The active bands of MMP-9 gradually decreased when U2-OS cells were treated with higher concentrations of simvastatin (from 5 to 15 μM, Fig. 1A).

To exclude the possible direct effect of simvastatin on the degradation of secreted MMP-9 in U2-OS, this drug was incubated in serum-free conditioned media collected from U2-OS cells at 24 and 48 h. Gelatin zymography revealed that MMP-9 activity was not altered under these experimental conditions (data not shown).

To confirm that the clear bands were truly MMP-9, Western blotting was used. Anti-MMP-9 antibody detects a pro-MMP-9 (92 kDa) and an active MMP-9 (84 kDa). The results from Western blotting were consistent with the results found in gelatin zymography as previously described. The levels of MMP-9 were significantly suppressed by simvastatin at 5, 10, and 15 μM (Fig. 1: B and C). Simvastatin also inhibited MMP-9 expression and activity in HT1080 fibrosarcoma cells at 24 h (Fig. 2) and UMR 106-01 rat osteosarcoma cells at 72 h (data not shown). Gelatin zymography and Western blotting revealed that the expression of MMP-9 in HT1080 decreased with increasing concentrations of simvastatin at 24 h. Conversely, the activity of a 72-kDa gelatinase, whose size is identical to MMP-2, was slightly changed by simvastatin in all cell types.

Fig. 1. Simvastatin decreased the gelatinolytic activity and level of enzyme MMP-9 in U2-OS. The cells were treated with increasing concentrations of simvastatin for 48 h. A: Gelatinolytic activity of conditioned media from U2-OS tested with increasing concentrations of simvastatin. The enzyme activity was measured by gelatin zymography. B: Western blot analysis of MMP-9 expression. Conditioned media from simvastatin-treated cells were separated by SDS-PAGE, transferred to PVDF membrane, incubated with monoclonal MMP-9 antibody, and then analyzed by ECL Western blotting detection reagent kit as described in Materials and Methods. C: Graph presents the relationship between percentage of MMP-9 protein and the concentration of simvastatin. Data are depicted as the mean ± S.D. The data are representative of at least two separate experiments. *Significantly different from the control group at P<0.05.
Expression of MMP-9 mRNA in U2OS was downregulated by simvastatin

The concentration- and time-dependent effects of simvastatin on MMP-9 mRNA levels in U2-OS were investigated by using RT-PCR analysis. Treatment with 5, 7.5, 10, and 15 μM of simvastatin significantly decreased the levels of MMP-9 mRNA nearly 40 – 50% (Fig. 3A). When exposed to 10 μM of simvastatin, the levels of MMP-9 mRNA were significantly decreased at 16 and 24 h (Fig. 3B). There was no significant difference in MMP-9 mRNA levels between the control group measured at 0 and 24 h.

Simvastatin also suppressed the release of MMP-9 in primary calvaria

To confirm the effect of simvastatin on MMP-9 expression in osteoblasts, primary calvaria cells were tested. Cells were incubated for 24 h with increasing concentrations of simvastatin. Similar to a previous report (21), primary calvaria released both MMP-9 and MMP-2 when cultured in a serum-free medium. Although the effective concentrations of simvastatin in primary calvaria were somewhat different from those used in U2-OS, the results of gelatin zymography and Western blotting were consistent with those of U2-OS (Fig. 4: A and B, respectively). Data analysis revealed that the increasing concentrations of simvastatin (100 to 500 nM) resulted in the reduction of MMP-9 activity (30% to 48%) and secretion (30% to 70%) in a concentration-dependent manner.

Simvastatin reduced TNF-α enhanced MMP-9 expression in HT1080 and primary calvaria

The expression of MMP-9 is upregulated by TNF-α, an inflammatory cytokine involved in many destructive bone diseases. To investigate the effect of simvastatin on TNF-α-enhanced MMP-9 expression, HT1080 and
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primary calvaria cells were treated for 24 h with TNF-α (with and without simvastatin). TNF-α dramatically increased MMP-9 expression in both cells, similar to several previous reports (21, 22). Co-incubation of TNF-α with the increasing concentrations of simvastatin resulted in a gradual decrease of MMP-9 activity and secretion in the HT1080 and primary calvaria cells (Fig. 5).

**Discussion**

In this study, we have demonstrated that simvastatin, a HMG-CoA reductase inhibitor, reduced the expression of MMP-9 in primary calvaria, U2-OS human osteosarcoma cells, and HT1080 fibrosarcoma cells. Simvastatin suppressed both basal and TNF-α-induced expression of MMP-9 in primary calvaria and HT1080 cells. Gelatin zymography, Western blot analysis, and RT-PCR clearly revealed that this drug downregulated MMP-9 mRNA levels, the amount of proteinase released into the incubation media, and the gelatinolytic activity.

The effective concentrations of simvastatin that reduced MMP-9 expression in primary calvarial cells and U2-OS cells were strikingly different from each other. Primary calvaria cells are normal osteoblasts, whereas U2-OS cells were originally isolated from osteosarcoma (23, 24). To rule out the hypothesis of simvastatin’s toxicity, we performed a toxicity test of simvastatin on primary calvaria and U2-OS using MTT analysis. The toxicity concentrations were 2 and 20 μM, respectively (data not show). The results from the test confirmed that simvastatin had a direct effect on MMP-9 expression. Interestingly, in primary calvaria, the effective concentrations of simvastatin on MMP-9 expression (100 – 500 nM) were in the range found in patients’ serum that received simvastatin for therapy (25). Therefore, our data suggest that primary calvaria may be an appropriate model for studying the effect of simvastatin on MMP-9 expression in bone forming cells.

Previous studies have been reported the non-cholesterol effect of simvastatin on the synthesis of important isoprenoid intermediates (farnesyl pyrophosphate and geranylgeranyl pyrophosphate) (26, 27). Isoprenoid proteins serve as lipid attachment for a variety of intracellular signaling molecules such as the small GTP-binding proteins Ras and Ras-like proteins (Rho, Rap, and Rab) (28). These proteins were found to mediate their effects on MMP-9 expression by activation of the linear cascade of the protein kinase pathway (29 – 32).
Therefore, it may possible that simvastatin suppresses Ras and Ras-like proteins activities via isoprenoid intermediates and subsequently inhibits MMP-9 expression. However, future studies are necessary to verify and elucidate the details of this hypothesis.

MMP-9 plays important roles in bone development. MMP-9 knockout mice were found to have a delay in bone marrow formation and an impaired development of endochondral ossification (33, 34). Osteoblasts exposed to bone-resorbing hormones or cytokines such as PTH and TNF-α show an increased expression of MMPs including MMP-1, MMP-13, and MMP-9 (21, 22, 35). Although the precise roles of MMP-9 secreted from osteoblasts are not clear, studies suggest that MMP-9 is involved in bone surface preparation for the recruitment of osteoclasts at the beginning of bone resorption (35–38).

Recent studies show that simvastatin stimulates bone mineral deposition and alkaline phosphatase expression in MC3T3-E1 (39). Treatment of fractured femur male mice with simvastatin improves healing (40). Furthermore, simvastatin and compactin, another member of the statin family, promoted the expression of BMP-2 in more, simvastatin and compactin, another member of the statin family, promoted the expression of BMP-2 in human osteoblast cell lines (15, 41). BMP-2 stimulates mineral deposition and alkaline phosphatase expression from osteoblasts are not clear, studies suggest that MMP-9 is involved in bone surface preparation for the recruitment of osteoclasts at the beginning of bone resorption (35–38).

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