Statins inhibit MMP release from human lung fibroblasts

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Abstract

Rationale: Pleiotropic effects of statins have been reported to include inhibition of matrix metalloproteinase (MMP) release from macrophages and endothelial cells. We evaluated whether statins would inhibit MMP release from human lung fibroblasts, which play a major role in remodeling processes. ***Methods:*** Monolayer and 3D collagen gel cultures of fibroblasts were used. Cytokines (TNF-α and IL-1β) were used to induce MMP release and mRNA expression. Collagen degradation induced by cytokines and neutrophil elastase (NE) was evaluated by quantifying hydroxyproline. ***Results:*** Atorvastatin inhibited MMP-1 and -3 release and mRNA expression in both culture systems. Similar results were obtained with simvastatin and fluvastatin. In 3D cultures where cytokines also stimulated MMP-9 release, atorvastatin also inhibited MMP-9 release.   In 3D cultures, cytokines together with NE induced collagen degradation, which was also inhibited by atorvastatin. The effect of atorvastatin was reversed by mevalonate and geranylgeranyl-pyrophosphate (GGPP) but not by farnesyl-pyrophosphate (FPP).

Conclusion: The current data suggest that statins may modulate remodeling processes mediated by fibroblasts by inhibiting MMP release.

Introduction

Chronic obstructive pulmonary disease (COPD) is expected to become the third leading cause of death worldwide by the year 2020 [1]. The defining feature of COPD is loss of expiratory airflow that can result from at least two important structural alterations in the lungs, emphysema with its destruction of alveolar wall and loss of lung elastic recoil, and peribronchial fibrosis, which narrows airways. At present, therapy can partially alleviate symptoms, but has relatively little impact on the progressive structural alterations that compromise lung function [2]. Novel therapeutic strategies that have the potential to alter lung structure, therefore, are of interest as possible approaches to alter the long term natural history of COPD. One type of therapy that is attracting interest for its potential in COPD is the use of statins, which have been associated with reduced acute events and mortality [3-5] as well as with reduced decline in lung function in retrospective data base studies [6].

Statins are a class of cholesterol lowering drugs that inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA). Recently, pleiotropic effects of statins have been reported, including anti-inflammatory and anti-oxidant effects [7, 8], as well as the inhibition of MMP release from vascular cells and macrophages [9-11]. One potential mechanism for statin efficacy in COPD could be effects on inflammatory cells. Such effects could be a mechanism that could alter both acute events and long term progression. An alternate mechanism, which could be independent or could be interactive, is the effect of statins on MMPs. Through these effects, statins could

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directly modify lung extracellular matrix and, thereby, could modify the alterations in lung structure that compromise lung function.

The MMPs are a large family of proteolytic enzymes [12] that are produced by both inflammatory cells and structural cells in the lung including alveolar epithelial cells and fibroblasts [13]. Fibroblasts are believed to be major cells responsible for maintenance and repair of ECM [14]. This is accomplished by the production and assembly of ECM macromolecules, by the release of growth factors for other cells and by the release of proteolytic enzymes, particularly MMPs. This allows fibroblasts to modulate both ECM production and destruction as well as the response of other cells.

The current study was designed to determine if statins could modulate MMP release by human lung fibroblasts. To investigate this, we utilized two types of lung fibroblasts, the widely used fetal fibroblast strain HFL-1 [15], which allows our results to be compared with those of other investigations that have used identical or similar strains and, in addition, fibroblasts from normal appearing adult lung tissue that was removed during surgery. We have, moreover, used three statins, atorvastatin, fluvastatin and simvastatin to assure that the effects were not specific for a single agent. In addition, to determine if the effect of statins could modulate ECM degradation in addition to MMP release and to determine if the results obtained in the conventionally used monolayer culture would be observed in other culture conditions, the effect of atorvastatin was evaluated in 3D collagen gels in which collagen degradation was induced by cytokines and NE. Finally, the signaling pathway by which atorvastatin exerts its effect on MMP release was investigated in both culture systems.

Results

Effect of Statins on MMP Release in Monolayer Culture

      HFL-1 cells. To test if statins inhibit the release of MMPs in human fetal lung

fibroblasts (HFL-1), we first investigated three separate statins, not only to demonstrate

the reproducibility of our findings, but to be certain that any effects observed were not

specific for a single compound. To accomplish this, cells were cultured in 60-mm dishes

and treated with atorvastatin (2.5 μM), fluvastatin (1.0 μM) or simvastatin (0.5 μM) with

and without stimulation by cytokines (TNF-α 5 ng/ml and IL-1β 2 ng/ml) for 24 h. These

concentrations of statins were chosen as they are considered to be close to those detected

in the human body when administered therapeutically. Supernatants from each culture

condition were assessed for MMP-1 and MMP-3 release by Western blot analysis, and for

MMP-2 and MMP-9 by gelatin zymography. As expected, the cytokines stimulated

MMP-1 and MMP-3 release (Figures 1A-D). Bands corresponding to the latent forms

(55 kDa) were observed, but lower molecular weight bands corresponding to active

MMPs were not observed. All three statins inhibited both MMP-1 (Figures 1A and 1B)

and MMP-3 (Figures 1C and 1D) release stimulated by cytokines ($p<0.05$ compared to

the cytokine-treated controls). In monolayer culture, MMP-9 release was not observed

under any condition.   However, HFL-1 cells released a gelatinase corresponding to the

latent form of MMP-2 (Figure 1E and 1F).   The effect of atorvastatin on the release of

the latent form of MMP-2 stimulated by cytokines was modest (Figures 1E-and 1F).

None of the reagents affected cell viability evaluated by 3-(4,5-dimethylthiazolyl-2)-2,5-

diphenyltetrazoliumbromide (MTT) assay (data not shown).

To determine whether the inhibition of MMP-1 and MMP-3 expression by atorvastatin

was associated with changes in gene expression, quantitative real-time PCR was

performed after 6 hours of incubation with statins and cytokines (TNF-α 5 ng/ml and

IL-1β 2 ng/ml). Atorvastatin alone slightly stimulated both MMP-1 (Figure 2A) and

MMP-3 (Figure 2B) mRNA expression. However, this effect was modest compared to

that of the cytokines, which stimulated MMP-1 and MMP-3 mRNA 121.6 ± 3.8 and 46.9

± 1.0 fold of control, respectively. When cells were treated with atorvastatin before the

stimulation by cytokines, the effect of the cytokines on both MMP-1 and MMP-3 mRNA

was inhibited to 12.3 ± 1.4 and 7.1 ± 0.4 fold of control, respectively (p<0.01 compared

with the cytokine treated groups). Consistent with the results observed with gelatin

zymography, no MMP-9 mRNA was detected (data not shown).

Adult lung fibroblasts. Because fibroblasts may be functionally

heterogeneous, we also investigated the effect of atorvastatin on the inhibition of MMP

release from normal adult human lung fibroblasts (HLF) after 24 hours of culture. In

contrast to HFL-1 cells, which did not release any detectable MMP-1 or MMP-3 in

control conditions, HLF cells released a small amount of MMP-1 and MMP-3

corresponding in molecular size to the latent form of 55 kDa (Supplemental Figure 1).).

As with HFL-1 cells, the cytokines also stimulated both MMP-1 and MMP-3 release from

HLF cells (Supplemental Figure 1 A-D). Also, as with HFL-1 cells, atorvastatin

inhibited HLF release of both MMP-1 (Supplemental Figures 1A and 1B) and MMP-3

(Supplemental Figures 1C and 1D) release stimulated by cytokines (p<0.05 compared to

the cytokine-treated controls). The release of MMP-2 was similar to that of HFL-1 cells

and, as with HFL-1 cells, MMP-9 was not detected in the supernatant of HLF cells in

monolayer cultures (Supplemental Figures 1E and 1F).

Role of the Mevalonate Pathway

Inhibition of MMP-1 and MMP-3 Protein Release. Incubation of cells

with HMG-CoA reductase inhibitors such as the statins blocks production of mevalonate

[16, 17]. Mevalonate metabolism yields a series of isoprenoids, including FPP and GGPP. To

confirm that this is the pathway by which atorvastatin inhibits MMP-1 and MMP-3

release, we tested whether mevalonate, FPP or GGPP could reverse atorvastatin

inhibition of cytokine-stimulated HFL-1 MMP-1 and MMP-3 release. HFL-1 cells were

cultured in monolayer and treated with atorvastatin (2.5 μM) with or without mevalonate

(100 μM), FPP (5 μM) or GGPP (5 μM) followed by stimulation with cytokines (TNF-α

5 ng/ml and IL-1β 2 ng/ml) for 24 hours. In the presence of mevalonate, GGPP or FPP

alone, there was no detectable level of MMP-1 release (Figure 3A) and this corresponded

to the results under control conditions (data not shown). The addition of mevalonate and

GGPP almost completely blocked the inhibitory effect of atorvastatin on the release of

MMP-1 (Figures 3A and 3B; $p < 0.05$ compared to cytokine-treated groups after

incubation with atorvastatin). In contrast, FPP had no effect.

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No MMP-3 was detected when cells were incubated under control conditions

(data not shown) or with mevalonate, GGPP or FPP added alone (Figure 3C).

Mevalonate and GGPP blocked the inhibitory effect of atorvastatin on MMP-3 release

that was stimulated by cytokines (Figures 3C and 3D; $p < 0.05$ compared to

cytokine-treated groups after incubation with atorvastatin). FPP was without effect.

Inhibition of MMP-1 and MMP-3 mRNA Expression. To determine

whether the effects of mevalonate and GGPP were associated with changes in gene

expression, quantitative real-time PCR assay was performed. HFL-1 cells were treated

with atorvastatin (2.5 µM) with or without mevalonate (100 µM) or GGPP (5 µM), and

then stimulated with cytokines (TNF-α 5 ng/ml and IL-1β 2 ng/ml) for 6 hours. Neither

mevalonate nor GGPP alone altered MMP-1 or MMP-3 mRNA expression levels

(Figures 4A and 4B, respectively; $p < 0.01$ compared with cytokines plus atorvastatin

treated groups). However, the addition of mevalonate or GGPP blocked the inhibitory

effect of atorvastatin on MMP-1 and MMP-3 mRNA expression significantly (Figures

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4A and 4B, respectively).   FPP did not have any effect on MMP-1 or MMP-3 mRNA

expression (data not shown).

Culture in 3D Collagen Gels

   Effect of Atorvastatin on Collagen Degradation.   The response of

fibroblasts to exogenously added mediators can vary depending on the culture condition.

In three-dimensional (3D) cultures in floating collagen gels, which are thought to more

closely mimic *in vivo* conditions, we have previously shown that stimulation of

fibroblasts with cytokines in the presence of neutrophil elastase (NE) can result in

degradation of the collagen gel matrix [18]. To determine if atorvastatin inhibition of

fibroblast MMP release could lead to inhibition of matrix degradation, we prepared 3D

collagen gels populated with HFL-1 fibroblasts.   When stimulated by cytokines (TNF-α

5 ng/ml and IL-1β 2 ng/ml) to induce MMP release in the presence of NE (10 nM), which

leads to activation of MMPs, the collagen gels were degraded as we have reported

previously [18].   The hydroxyproline content of the gels was significantly reduced after 3

days (hydroxyproline content $38 \pm 4\%$ of control, Figure 5; $p < 0.05$ compared with

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control).   This decrease was almost completely inhibited by atorvastatin

(hydroxyproline content $83 \pm 8\%$ of control, Figure 5; $p < 0.05$ compared with CK + NE).

Hydroxyproline content of gels treated with atorvastatin + cytokines (without NE) or

atorvastatin + NE (without cytokines) was the same as that of control (data not shown).

Effect of Atorvastatin on MMP Release.   To confirm the results obtained in

monolayer culture on plastic, we also assessed the effect of atorvastatin on MMP release

in 3D culture in collagen gels.   There were modest differences in MMP release in control

conditions.   For example, detectable MMP-1 was released in latent form under control

conditions in 3D culture, which contrasts with the undetectable levels in monolayer

culture.   Importantly, as with monolayer culture, there was marked increase in release of

MMP-1 following cytokine stimulation (Supplemental Figure 2).   As in monolayer

culture, MMP-3 was undetectable under control conditions in 3D culture (Supplemental

Figure 3).   MMP-2, which was released in the latent form in monolayer culture, was

released in both latent and active forms under control conditions in 3D culture

(Supplemental Figure 4).   MMP-9, which was not released under any condition tested in

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monolayer culture, was not released under control conditions in 3D culture, but its release

was stimulated by cytokines (TNF-α 5 ng/ml and IL-1β 2 ng/ml)   (Supplemental Figure

4).   Importantly, the cytokines stimulated the release of all the MMPs assessed, MMP-1,

3 and 9 and this release was inhibited ($p < 0.05$ compared to CK) in all cases by

atorvastatin (Supplemental Figures 2, 3 and 4). There was, moreover, a reduction in the

conversion of latent MMPs to the active forms in the presence of neutrophil elastase for

MMP-1 ($p < 0.05$), MMP-3 ($p < 0.01$) and MMP-9 ($p < 0.001$).   A modest effect, which was

not quantified was also observed in inhibiting MMP-2 release (Supplemental Figure 4A).

Role of the Mevalonate pathway in inhibition of Collagen Gel

Degradation.   To confirm that atorvastatin inhibited collagen gel degradation by the

same pathway by which it inhibited MMP release in monolayer culture, we assessed the

ability of mevalonate (100 μM), FPP (5 μM) and GGPP (5 μM) to block atorvastatin

inhibition of collagen gel degradation induced by the combination of cytokines and NE.

After 3 days of culture in the presence of cytokines and NE, collagen gel hydroxyproline

content was reduced by more than half (Supplemental Figure 5; $p < 0.05$ compared with

control), indicating degradation of the collagen gels. Atorvastatin almost completely

inhibited this degradation ($p < 0.05$ compared with CK + NE). Mevalonate and GGPP

had no effect when added alone, but almost completely reversed atorvastatin inhibition of

collagen degradation caused by cytokines and NE (Figure 3; $p < 0.05$ compared with

control). In contrast, FPP had no effect when added alone and did not reverse the

inhibitory effect of atorvastatin on collagen gel degradation.

Role of the Mevalonate Pathway in 3D culture. Because fibroblasts in 3D

culture release MMP somewhat differently than in monolayer culture, we also

investigated whether mevalonate or isoprenoids blocked the effect of atorvastatin on

MMP release in 3D gel culture (Supplemental Figure 6). Although statistical significance

was not achieved, there was a trend for both mevalonate and GGPP to block the

inhibitory effect of atorvastatin on the amount of detectable active MMP-1 released in the

presence of cytokines and NE (Supplemental Figures 6A and 6B). Both mevalonate and

GGPP significantly blocked the inhibitory effect of atorvastatin on MMP-3 release

(Supplemental Figures 6C and 6D; $p < 0.01$) and on the release of MMP-9 and its

conversion to an 84 kDa size that corresponds to the active form MMP-9 (Supplemental

Figures 6E and 6F; $p < 0.01$). FPP was consistently without effect.

Adult Human Lung Fibroblasts (HLF) in 3D culture

Collagen degradation. To confirm that collagen gel degradation occurs in

the gels populated with adult human lung fibroblasts (HLF) and that MMP release by

these cells is also blocked by atorvastatin, cultured HLF cells were cast into 3D cultures

and treated with cytokines and NE to induce degradation. As observed with HFL-1 cells,

in the presence of cytokines and NE, hydroxyproline content of the gels was significantly

reduced after 3 days of culture (Supplemental Figure 7; $p < 0.05$ compared with control).

This decrease was inhibited by atorvastatin ($p < 0.05$ compared with CK + NE). The role

of the mevalonate pathway also appeared to be the same in HLF cells as mevalonate and

GGPP had no effect when added alone, but reversed the effect of atorvastatin on the

inhibition of collagen degradation caused by cytokines and NE (Supplemental Figure 7; p

< 0.05 compared with control).

Discussion

The current study demonstrates that HMG-CoA reductase inhibitors can inhibit

cytokine-induced release of MMPs from human lung fibroblasts cultured both in

monolayer and in 3D collagen gels. Release of both MMP-1 and MMP-3 from fibroblasts

in monolayer culture was inhibited by atorvastatin, simvastatin and fluvastatin.

Atorvastatin also inhibited the cytokine-induced expression of MMP-1 and MMP-3

mRNA expression. In 3D gel culture, atorvastatin inhibited the degradation of collagen

gels that was induced by cytokines in the presence of NE. In contrast to monolayer

culture, in 3D gel culture fibroblasts were induced by cytokines to release MMP-9, and

these were also inhibited by atorvastatin. All of these changes were reversed by the

addition of mevalonate and GGPP, which are intermediates in the HMG-CoA reductase

pathway that bypass the step inhibited by statins. Interestingly, the intermediate FPP did

not reverse these effects of atorvastatin. Taken together, the current data suggest that

statins may modulate remodeling processes mediated by lung fibroblasts by inhibiting the

release of MMPs.

Since the classic studies of Laurell and Eriksson [19], proteases have been believed to play a major role in the pathogenesis of COPD. In particular, the matrix metalloproteinases (MMPs) have been suggested to play a prominent role. In support of this concept, mice deficient in the macrophage elastase MMP-12 have been reported to be resistant to the development of cigarette smoke-induced emphysema [20], although MMP-12 is not prominent in human emphysema [21]. Increased MMP-9 expression in the lungs has been reported to be associated with emphysema, both in experimental [22-26] and clinical [27] studies. Moreover, immunohistochemical analysis of COPD lungs showed an increased expression of collagenase 1 (MMP-1) and collagenase 2 (MMP-8) [28], and alveolar epithelial cells from patients with emphysema express MMP-1 mRNA and protein, and exhibit collagenase activity [29].

MMPs are expressed in low to undetectable levels in normal lung, but are readily detected in many lung diseases in addition to emphysema. In this context, the expression of MMPs is not restricted to inflammatory cells; virtually all cells including alveolar epithelial cells and fibroblasts can make MMPs [29, 30]. Alveolar fibroblasts, which comprise approximately 40% of alveolar cells [31], are believed to be the major cells

responsible for the production and remodeling of ECM. They are one of the major

sources of MMPs, which are secreted following lung injury. Therefore, inhibition of

MMP release from fibroblasts could confer a new therapeutic approach to prevent the

lung destruction observed in emphysema. Whether inhibition by statins could lead to

toxicities, e.g. by inhibiting lung tissue turnover in fibrotic areas, of course, is a potential

toxicity that would need to be considered.

Statins were originally developed for their cholesterol-lowering properties and

efficacy in cardiovascular disease. However, evidence of pleiotropic effects of statins has

been accumulating and suggests immunomodulatory, antioxidant, antithrombogenic and

vascular actions [7, 17]. Several statins have been reported to inhibit the expression of

MMPs in vascular cells and macrophages *in vitro* [9-11]. Consistent with this effect on

MMPs, statins have been reported to inhibit the development of emphysema in model

systems. In a rat model of cigarette smoking-induced emphysema, Lee and coworkers

found that simvastatin inhibited lung parenchymal destruction and MMP-9 expression [32].

In a mouse model of emphysema induced by elastase, simvastatin reduced mRNA

expression for IFN-γ, TNF-α and MMP-12 in the whole lung [33].

The current study extends these observations by providing a basis for statins to alter lung structural remodeling by a direct effect on fibroblast MMP release.   Further, this study demonstrates that the effect of the statins is mediated through the mevalonate pathway, likely involving GGPP but not FPP.   This observation is similar to other studies that also observed that the addition of FPP did not reverse the effect of statins [34, 35]. The mechanism for the failure of FPP to reverse these statin effects remains unexplained.

As with the normal lung, fibroblasts in culture release very low amounts of MMP-1, -2, -3 and -9.   The release of these MMPs, however, can be readily induced by the inflammatory cytokines TNF-α + IL-1β, and it is the induced MMP release that statins were demonstrated to inhibit in the current study.   This inhibition occurred both in the more conventionally used monolayer culture system (on plastic dishes) and in culture in 3D collagen gels, a system that is believed to more closely resemble tissue conditions [14, 36, 37].   In this system, degradation of the extracellular matrix can be quantified, and statins also inhibited this process.   Some modest differences were observed between cells grown in monolayer and 3D culture.   Part of these differences

could be related to MMP retention in the collagen gel in the 3D system. This is unlikely, however, to account for the production of MMP-9 in 3D culture, which was not observed in monolayer culture. While there were some differences between the culture systems, the consistency of the inhibitory effect of statins, which were also observed in both the fetal strain of fibroblasts HFL-1 that has been widely used to explore lung fibroblast biology and in a strain of normal adult fibroblasts suggests that the inhibitory effects of statins are robust.

In the current study, statins slightly increased the release of MMP-1 when added alone, and this was confirmed at both the mRNA and protein levels. Atorvastatin also slightly increased MMP-3 mRNA when added alone; however, MMP-3 was not detected at the protein level. The mechanisms that lead to mild stimulation when added alone and marked inhibition when added together with cytokines are unclear, but similar effects have been observed in other regulatory networks when agents can have multiple effects. These observations, therefore, suggest that statins might be modulating MMP release at several levels.

In summary, the current study demonstrates that statins can inhibit MMP release from human lung fibroblasts both in monolayer and 3D collagen gel cultures. This effect is mediated through the inhibition of the HMG-CoA reductase pathway. Through actions on fibroblast MMP release, statins may modulate remodeling processes mediated by fibroblasts. The use of statins, therefore, may have therapeutic potential in diseases characterized by alterations in tissue architecture particularly diseases characterized by tissue destruction such as emphysema.

Materials and Methods

Materials

Native type I collagen (rat tail tendon collagen [RTTC]) was extracted from rat-tail

tendons by a previously published method [36, 38]. Commercially available reagents were

obtained as follows: Dulbecco's Modified Eagle's Medium (DMEM) and fetal calf serum

(FCS) were from Invitrogen Life Technologies (Grand Island, NY); human neutrophil

elastase (NE) was from Elastin Products Company, Inc. (Owensville, MO, USA);

recombinant human MMP-1 and MMP-3 Western blotting standards, recombinant

human TNF-α and IL-1β, and monoclonal anti-human pro/active MMP-1 (Clone 36665)

and MMP-3 (Clone 10D6) antibodies were from R&D Systems, Inc. (Minneapolis, MN);

HRP-conjugated goat anti-mouse IgG was from Rockland (Gilbertsville, PA); fluvastatin

and simvastatin were from Calbiochem (San Diego, CA); mevalonate, FPP, GGPP,

Chloramine-T, p-dimethylaminobenzaldehyde and 3-(4,5-dimethylthiazolyl-2)-2,5-

diphenyltetrazoliumbromide (MTT) were from Sigma (St. Louis, MO).

Atorvastatin was a kind gift from Pfizer Inc. (New York, NY) and was dissolved in

methanol to a stock solution of 2.5 mM.

Cell Culture

HFL-1 (lung, diploid, human) was obtained from the American Type Culture

Collection (ATCC) (#CCL-153; Rockville, MD). HT1080 cells (connective tissue

fibrosarcoma, diploid, human) were also purchased from ATCC (#CCL-121). HLF were

isolated from alveolar lung tissue removed at surgery for suspected malignancy that

appeared to be free of cancer under a protocol approved by the Human Studies

Committee of the University of Michigan. The cells were cultured on 100-mm tissue

culture dishes (Falcon; Becton-Dickinson Labware, Lincoln Park, NJ) with DMEM

supplemented with 10% FCS, 100 μg/ml penicillin, 250 μg/ml streptomycin sulfate

(penicillin-streptomycin; Invitrogen), and 2.5 μg/ml amphotericin B (Geneva

Pharmaceuticals, Inc., Dayton, NJ). Cells were cultured at 37°C in a humidified

atmosphere of 5% CO_2 and passaged every 3 to 5 days at a 1:4 ratio. In all experiments,

HFL-1 cells between the 14th and 18th passage were used. For HLF, passages between the

7th and 11th were used. HT1080 cell culture media were used as a positive control for

gelatin zymography.

To evaluate the release of MMP in monolayer culture, fibroblasts were cultured in

DMEM with 10% FCS in 60-mm dishes (Falcon) at 0.5×10^6 cells for 24 h. The medium

was then changed to serum free (SF)-DMEM. After 24 h, cells were washed with PBS

once and treated with statins for 1 h before stimulation with cytokines   (TNF-α 5 ng/ml

and IL-1β 2 ng/ml) in SF-DMEM. After 24 h incubation, the media were harvested for

gelatin zymography and Western blot analysis.

Collagen Gel Culture

Collagen gel cultures were conducted according to the previously published methods

[38] with a cell density of 3×10^5 fibroblasts/ml gel. The gels were incubated in SF-DMEM

with or without designated reagents at 37°C, 5% CO_2 for 3 days.

Hydroxyproline Assay

Hydroxyproline, which is directly proportional to type I collagen content, was

measured by spectrophotometry according to the previously published methods [39].

Briefly, the collagen gels were solubilized by heating in 0.1 N HCl (50 μl/3 gels).

Samples (20 μl) were mixed with 30 μl 3.3N NaOH and autoclaved at 120°C for 20 min.

After oxidization with 0.056 M Chloramine-T for 20 min, samples were reacted with

Ehrlich's reagent at 65°C for 20 min. The absorbance was measured at 540 nm with

BenchMark Microplate Reader and Microplate Manager III software (Bio-Rad, Hercules,

CA).

Western Blot Analysis of Metalloproteinase

To assess the release of MMP-1 and MMP-3, Western blot analysis was performed

according to the previously published method [18, 40] . Briefly, the supernatants (4 ml per

condition) from monolayer and 3D collagen gel cultures were precipitated with 50%

(vol/vol) cold ethanol and re-suspended in 50 μl ddH$_2$O. Because secreted MMPs were

assessed, no internal standard to allow for variable dilution was used.   Rather, a constant

volume, thirty-five μl of each sample was subjected to 10 % SDS-PAGE under

nonreducing conditions and then proteins were transferred to polyvinyl difluoride

(PVDF) membranes (Bio-Rad). This then allowed comparison of the intensity of the final

bands in a manner similar to immunoassay [41, 42].   After blocking with 5% nonfat milk,

blots were incubated overnight at 4°C with primary antibodies (1 μg/ml mouse

anti-human MMP-1 or MMP-3 mAb). Target proteins were detected using

HRP-conjugated goat anti-mouse IgG in conjunction with an enhanced

chemiluminescence detection system (ECL; Amersham Biosciences UK Limited, Little

Chalfont, Buckinghamshire, UK) using a Typhoon Scanner (Amersham Biosciences).

Band intensity was quantified using ImageJ software version 1.34s (National Institute of

Health: NIH, USA).

Gelatin Zymography

Supernatant media from monolayer and 3D collagen gel cultures (500 μl per culture

condition) were concentrated 10-fold by precipitation with cold ethanol and re-suspended

in 50 μl ddH$_2$O. Gelatin zymography was performed by a modification of previously

published procedures [40, 43, 44]. Briefly, 25 μl of each sample were separated in 10%

SDS-PAGE containing 0.1% gelatin (Invitrogen, Carlsbad, CA, USA) under

nonreducing conditions. After electrophoresis, the gels were soaked in 2.5% Triton-X

100 and incubated in metalloproteinase activation buffer containing CaCl$_2$ and ZnCl$_2$ for

16 h at 37°C. The gels were stained with 0.4% Coomassie blue (Bio-Rad) and rapidly

destained with destaining buffer (30% methanol, 10% acetic acid). The gels were dried

between cellophane sheets (Amersham, Piscataway, NJ). Zones of proteolysis appeared

as clear bands against a blue background were scanned by Gel Doc 2000 (Bio-Rad). Band

intensity was quantified using ImageJ software.

RNA Preparation and Complementary DNA Synthesis

To determine whether changes in MMP mRNA levels were present, quantitative

real-time PCR was performed. HFL-1 fibroblasts were cultured in 60-mm dishes at 1.5 ×

10^6 cells for 24 h. Cells were then washed by PBS twice and treated with statins for 1 h

before the stimulation by cytokines (TNF-α 5 ng/ml and IL-1β 2 ng/ml) in SF-DMEM

for 6h. Total RNA was isolated by a single-step

guanidinium-thiocyanate-phenol-chloroform extraction procedure designed by

Chomcynski [45] and the total amount of RNA was quantified spectrophotometrically

(Shimadzu Scientific Instruments, Inc., Columbia, MD). One μg of total RNA was

transcribed according to the previously published methods [40, 44].

Quantitative Real-Time PCR

Gene expression was measured with the use of an ABI Prism 7500 Sequence

Detection System (Applied Biosystems) by a slight modification of previously published

methods [40, 46]. Same primers and probes for MMP-1, -3 and -9 were used as described by

Fang [40]. rRNA was simultaneously tested using TaqMan Ribosomal RNA Control

Reagents (Applied Biosystems).

Cell Viability Assay

Cell viability was evaluated by MTT assay using previously described methods [47]. Cells were treated with various concentrations of reagents used in this study with MTT solution at a final concentration of 1 mg/ml for 6 h at 37°C. After incubation, DMSO was added into each well. The absorbance of each sample at 540 nm was determined by BenchMark Microplate Reader (Bio-Rad).

Statistical Analysis

Data are expressed as mean ± SEM. Each experiment was repeated at least three times.   The number of replicates is specified in each figure.   Experiments with multiple comparisons were evaluated by one-way ANOVA followed by post hoc analysis by pair-wise comparisons of values with Tukey's test to adjust for multiple comparisons. Probability values < 0.05 were considered significant.

Acknowledgement

Abbreviations

COPD; chronic obstructive pulmonary disease

FPP; farnesyl-pyrophosphate

GGPP; geranylgeranyl-pyrophosphate

HFL-1; Human fetal lung fibroblast

HLF; Human lung fibroblast

HMG-CoA; 3-hydroxy-3-methylglutaryl coenzyme A

Isoprenoids; FPP and GGPP

MMP; matrix metalloproteinase

MTT; 3-(4,5-dimethylthiazolyl-2)-2,5- diphenyltetrazoliumbromide

NE; neutrophil elastase

SF-DMEM; serum free-DMEM

Figure legends

Figure 1

Effect of statins on MMP release by fibroblasts in monolayer culture. HFL-1 cells

cultured in monolayer were treated with atorvastatin (2.5 μM), fluvastatin (1.0 μM) and

simvastatin (0.5 μM). One hour later, cytokines (TNF-α 5 ng/ml and IL-1β 2 ng/ml)

were added. Supernatant media harvested after 24 hours were subjected to Western blot

analysis for MMP-1 and MMP-3, and gelatin zymography for MMP-2 and MMP-9.

Panel A: MMP-1 western blot. The band corresponding to the latent form is indicated by

the *arrow*. Panel B: Quantification of MMP-1 staining. Panel C: MMP-3 western blot.

The band corresponding to the latent form of MMP-3 is indicated by the *arrow*. Panel

D: Quantification of MMP-3 staining. Panel E: Gelatin zymography for MMP-2 and

MMP-9. Panel F: Quantification of MMP-2 staining. Densitometry data are shown as

mean ± SEM from three separate experiments performed on different occasions. * $p <$

0.05compared to the cytokine-treated controls. STD: MMP-1 and MMP-3 standards,

CTR: control, CK: cytokines (TNF-α + IL-1β), Atr: atorvastatin, Flu: fluvastatin, Sim:

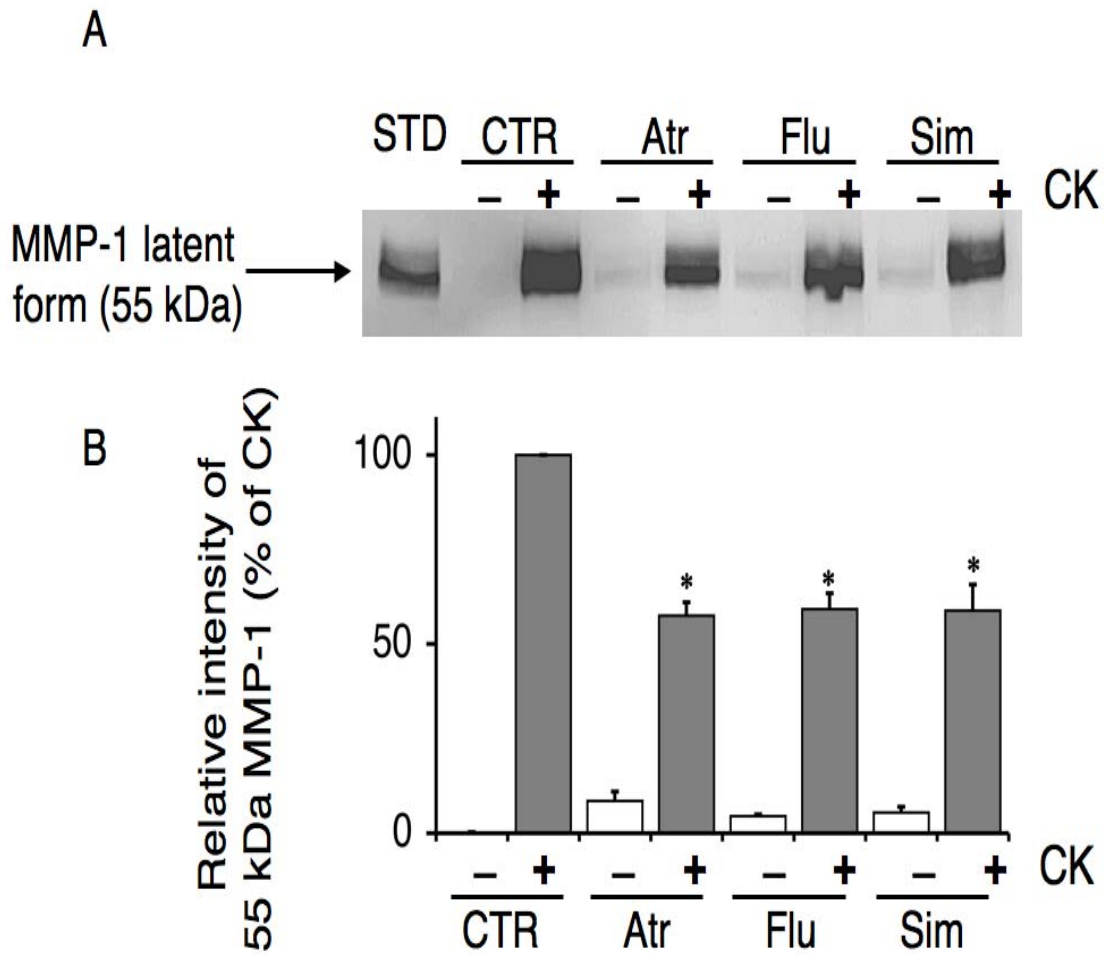simvastatin, HT1080: supernatant from HT1080 cell monolayer culture as a positive
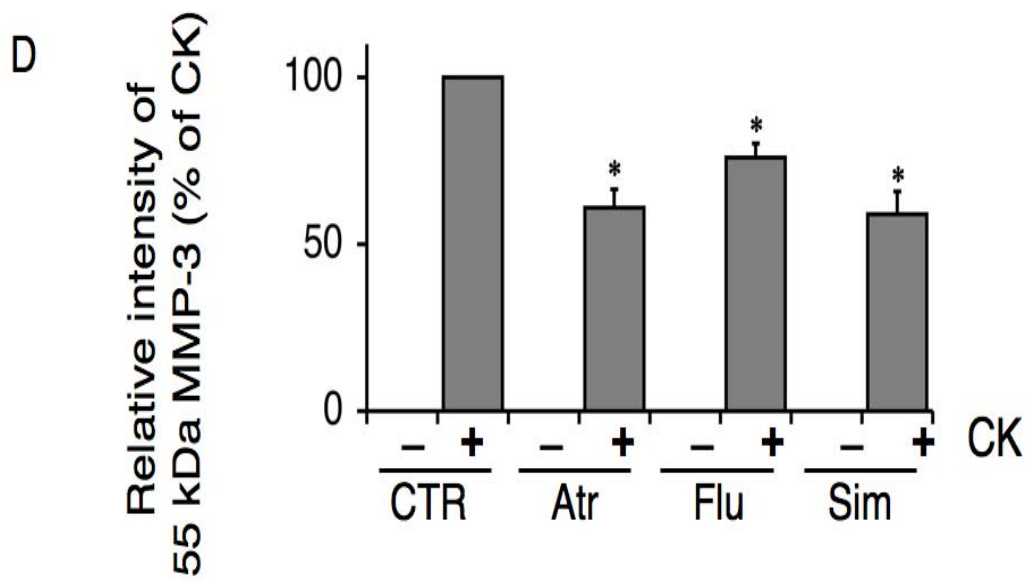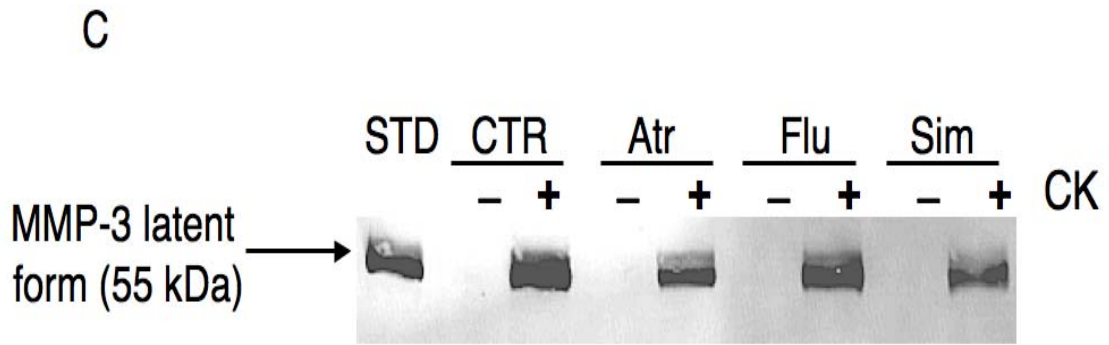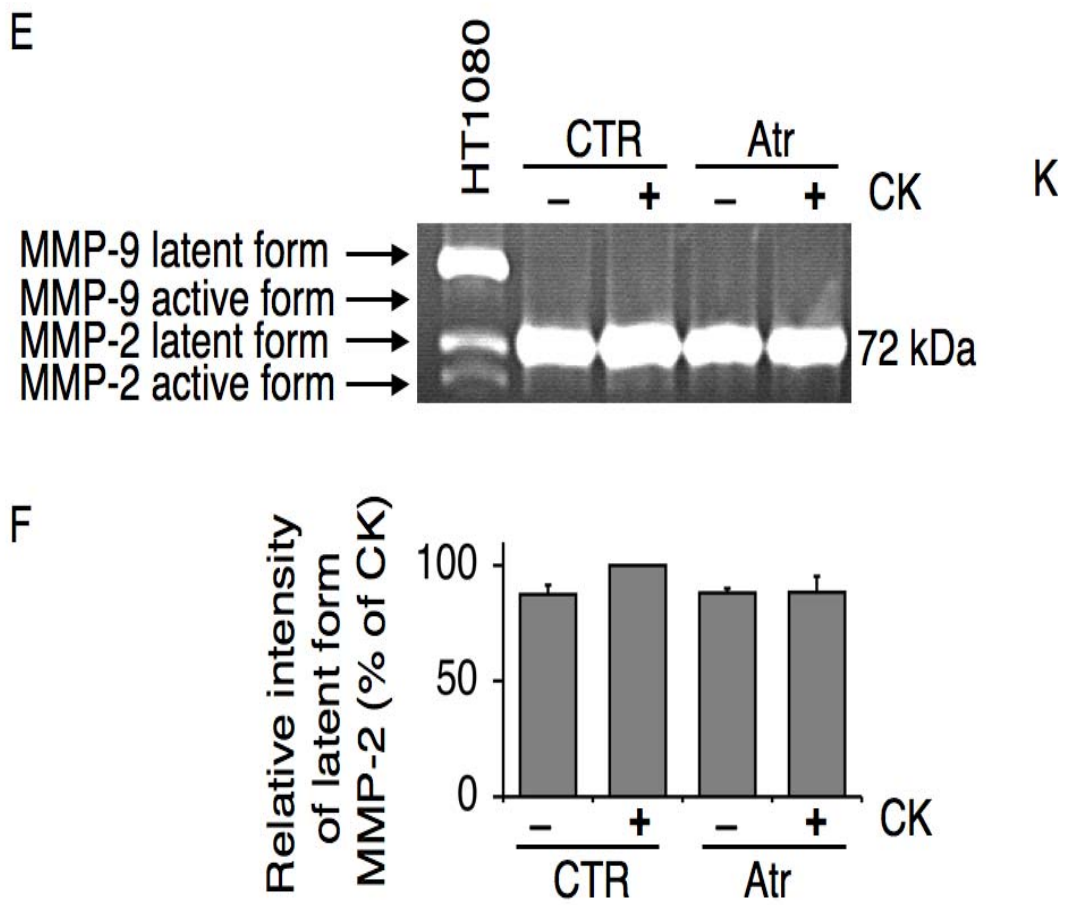
control.

Figure 1

C

STD CTR Atr Flu Sim
     − + − + − + − + CK

MMP-3 latent →
form (55 kDa)

D

Relative intensity of
55 kDa MMP-3 (% of CK)

100

50

0

− +   − +   − +   − + CK
CTR   Atr   Flu   Sim

Figure 1

E

MMP-9 latent form →
MMP-9 active form →
MMP-2 latent form →
MMP-2 active form →

K

72 kDa

F

Figure 1

Figure 2

Effect of atorvastatin on MMP-1 and MMP-3 mRNA expression. HFL-1 fibroblasts

cultured in monolayer were treated with and without atorvastatin (2.5 µM). One hour

later, cytokines (TNF-α 5 ng/ml and IL-1β 2 ng/ml) were added and then cultured for 6

hours.   mRNA levels were quantified by Taqman RT-PCR.   Panel A: MMP-1; Panel B:

MMP-3.   *Vertical axes*: mRNA expression level normalized to the amount of rRNA and

expressed as fold of control; *horizontal axes*: culture conditions. The data presented are

mean ± SEM from three separate experiments, each performed in duplicate. * $p < 0.01$

compared with cytokine treated groups. CTR: control, CK: cytokines (TNF-α + IL-1β),
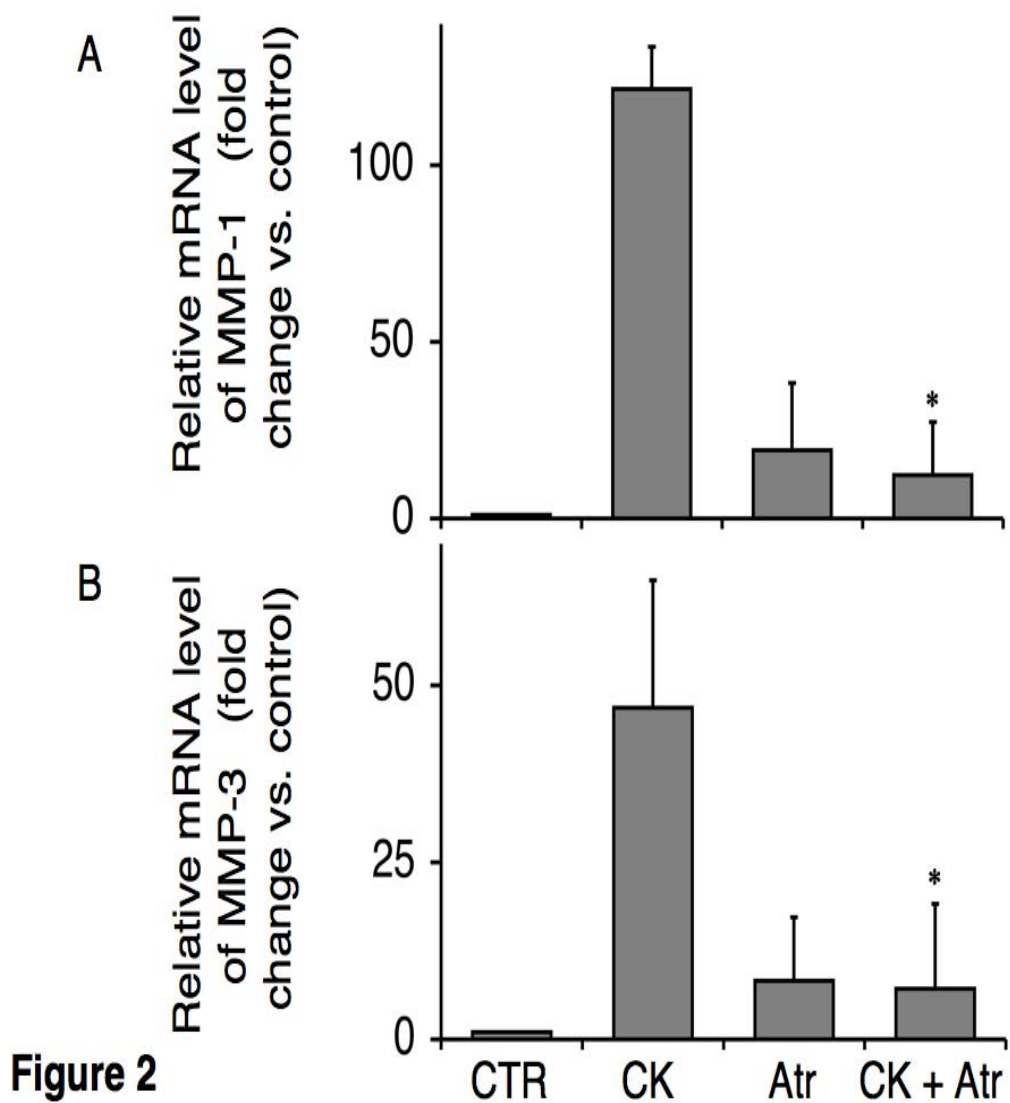
Atr: atorvastatin.

Figure 2

Figure 3

Effect of mevalonate and isoprenoids on atorvastatin inhibition of MMP-1 and MMP-3

release. HFL-1 cells were cultured in monolayer and treated with atorvastatin (2.5 μM)

with or without mevalonate, GGPP or FPP. One hour later, cytokines (TNF-α 5 ng/ml

and IL-1β 2 ng/ml) were added. Supernatant media harvested after 24 hours were

subjected to western blot analysis for MMP-1 and MMP-3. Panel A: MMP-1 western

blot. The band corresponding to the latent form is indicated by the *arrow*. Panel B:

Quantification of MMP-1 staining. Panel C: MMP-3 Western blot. The band

corresponding to the latent form of MMP-3 is indicated by the *arrow*. Panel D:

Quantification of MMP-3 staining. Densitometry data are shown as mean ± SEM for

three separate experiments performed on separate occasions. * $p < 0.05$. CK:

cytokines (TNF-α + IL-1β), Mev: mevalonate.

A

Atorvastatin

CK

Standard Mev GGPP FPP Mev GGPP FPP

MMP-1 latent form (55 kDa) →

B

Relative intensity of latent form MMP-1 (% of CK)

Mev GGPP FPP Mev GGPP FPP

CK

Atorvastatin

Figure 3

C

MMP-3 latent form (55 kDa) →

D
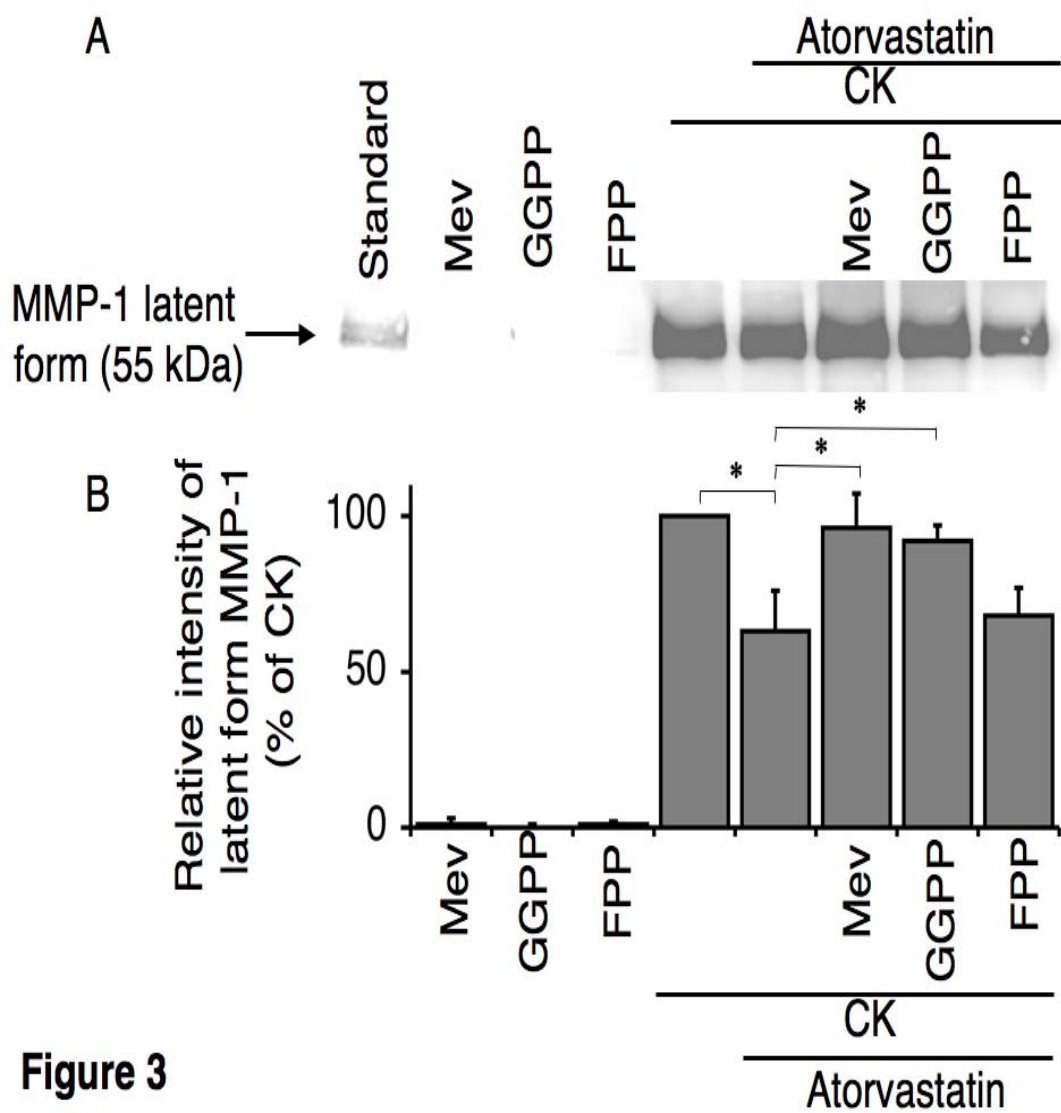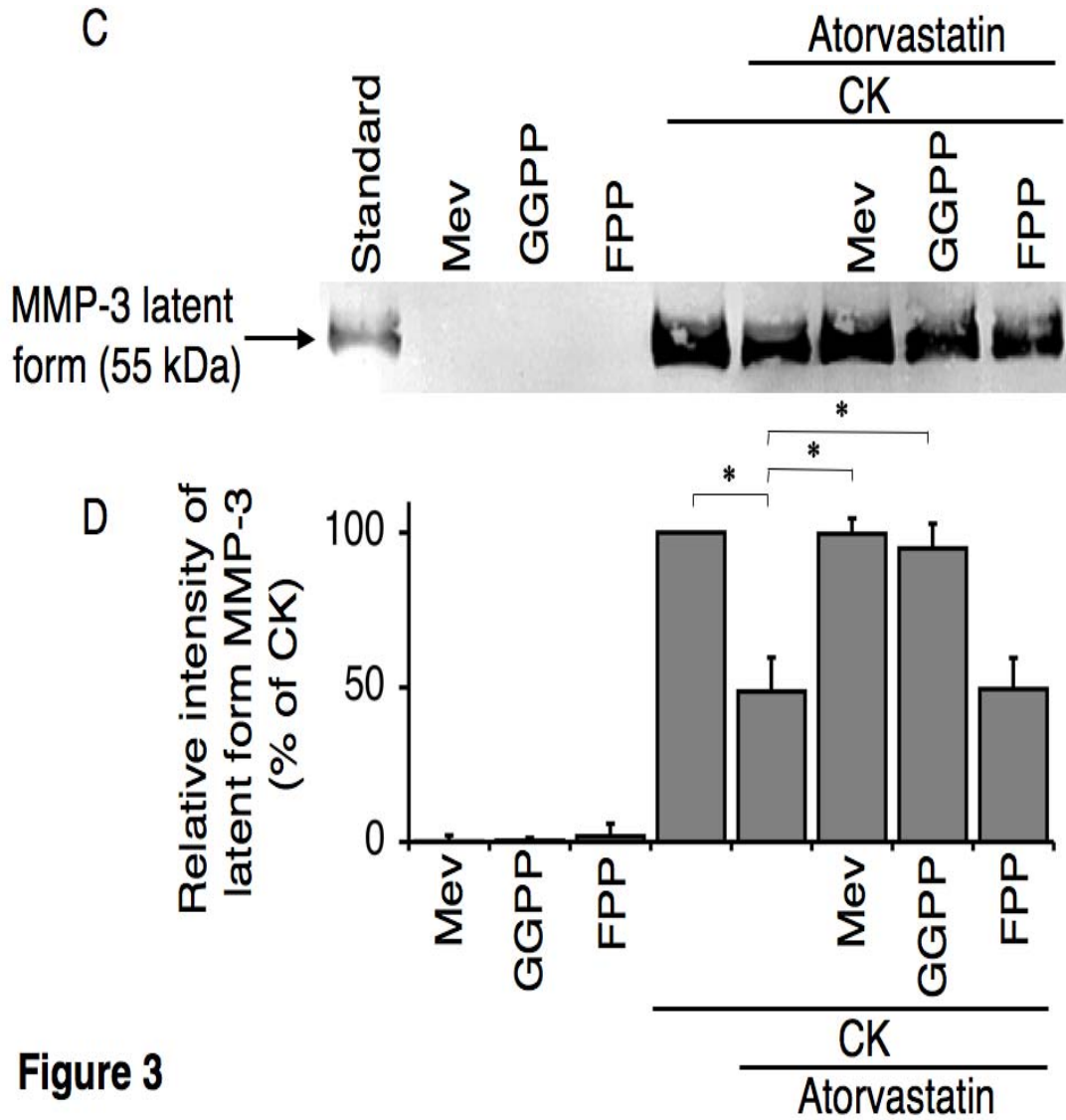
Relative intensity of latent form MMP-3 (% of CK)

Figure 3

Figure 4

Effect of mevalonate and isoprenoids on atorvastatin inhibition of MMP-1 and MMP-3

mRNA expression.   HFL-1 cells were cultured in monolayer and treated with

atorvastatin (2.5 μM) with or without mevalonate, GGPP or FPP.   One hour later,

cytokines (TNF-α 5 ng/ml and IL-1β 2 ng/ml) were added and cultured for 6 hours.

mRNA levels were quantified by Taqman RT-PCR.   Panel A: MMP-1; Panel B:

MMP-3.   *Vertical axes*:   mRNA expression level normalized to the amount of rRNA

and expressed as fold of control; *horizontal axes*:   culture conditions.   The data

presented are mean ± SEM from three separate experiments, each performed in duplicate.

* $p < 0.01$.   Mev: mevalonate, CK: cytokines (TNF-α + IL-1β) added.
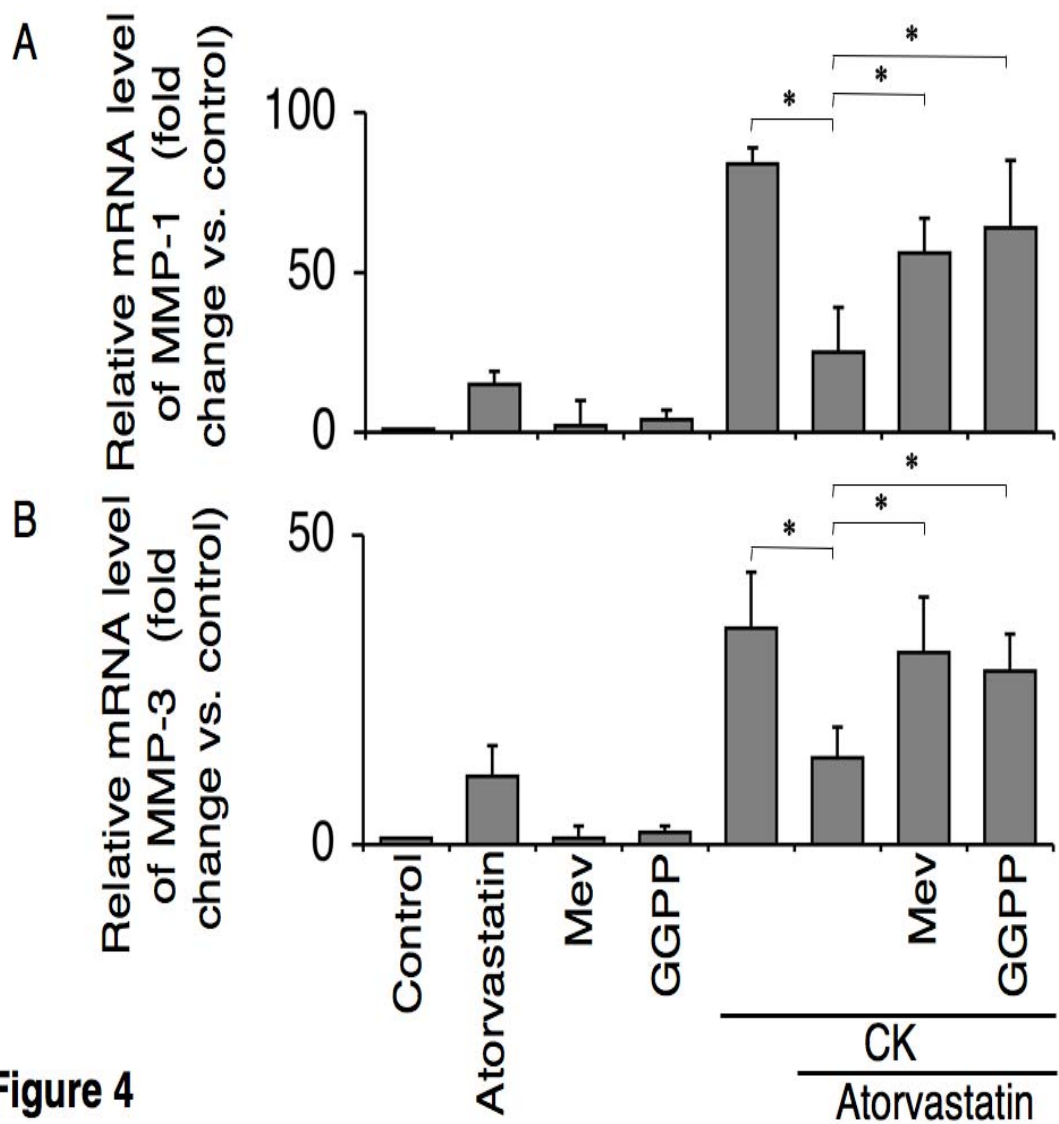
Figure 4

Figure 5

Effect of atorvastatin on collagen gel degradation induced by cytokines together with NE.

HFL-1 fibroblasts (3×10^5 cells/ml) were cast into 3 dimensional collagen gels and

released into floating media (SF-DMEM) containing atorvastatin (2.5 μM). One hour

later cytokines (TNF-α 5 ng/ml and IL-1β 2 ng/ml) were added, NE was added the next

day. The gels were cultured for 3 more days and hydroxyproline content in the gels was

determined. *Vertical axis*: hydroxyproline (% of control); *horizontal axis*: culture

conditions. The data presented are mean ± SEM from three separate experiments, each of

which included triplicate gels for each condition. * p <. CK: cytokines (TNF-α +
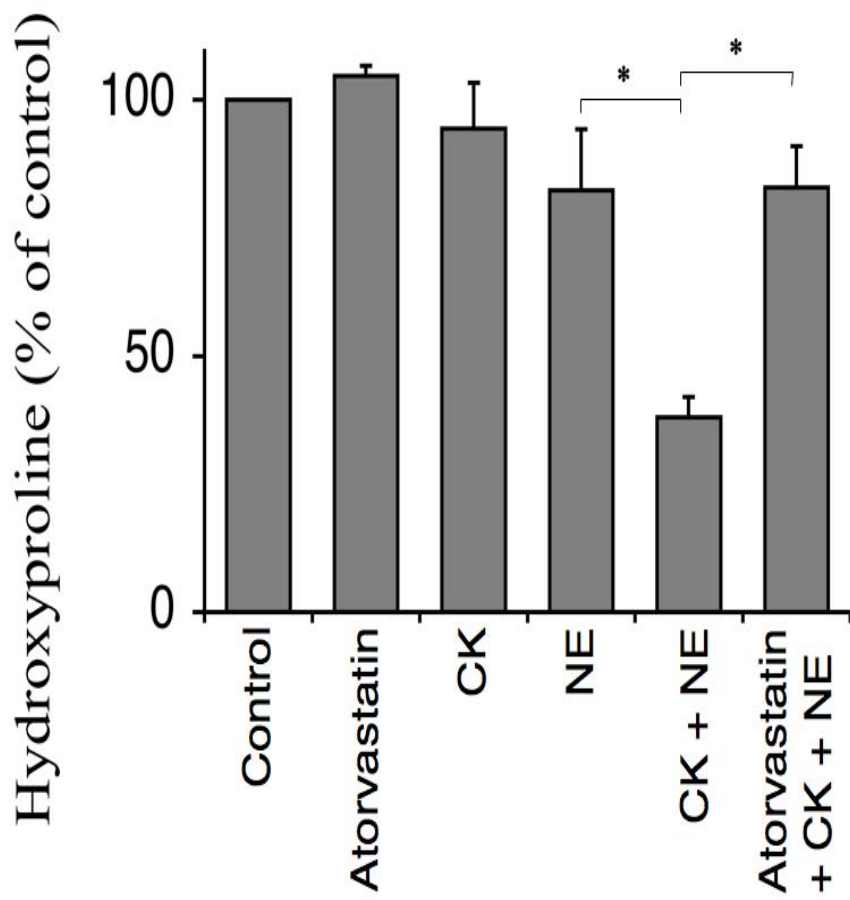
IL-1β), NE: neutrophil elastase.

Figure 5

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