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Original Article

Simvastatin as a Modulator of Tissue Remodeling through Inhibition of Matrix Metalloproteinase (MMP) Release from Human Lung Fibroblasts

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Background: Statins can regulate the production of pro-inflammatory cytokines and inhibit MMP production or activation in a variety of types of cells. This study evaluated whether statins would inhibit MMP release from human lung fibroblasts, which play a major role in remodeling processes.

Methods: This study, using an in-vitro model (three-dimensional collagen gel contraction system), evaluated the effect of cytokines (tumor necrosis factor-α, TNF-a and interleukin-1β, IL-1b) on the MMP release and MMP activation from human lung fibroblasts. Collagen degradation induced by cytokines and neutrophil elastase (NE) was evaluated by quantifying hydroxyproline.

Results: In three-dimensional collagen gel cultures (3D cultures) where cytokines (TNF-a and IL-1b) can induce the production of MMPs by fibroblasts, it was found that simvastatin inhibited MMP release. In 3D cultures, cytokines together with NE induced collagen degradation and can lead to activation of the MMP, which was inhibited by simvastatin.

Conclusion: Simvastatin may play a role in regulating human lung fibroblast functions in repair and remodeling processes by inhibiting MMP release and the conversion from the latent to the active form of MMP.

Key Words: Simvastatin; Collagen; Matrix Metalloproteinases; Airway Remodeling

Introduction

Airway inflammation causes lung tissue damage or injury. Following lung injury, repair and remodeling processes are initiated. Insufficient repair may result in destructive lung diseases such as emphysema, while abnormal tissue remodeling may lead to the development of fibrotic lung disease. Understanding the mechanisms that mediate normal tissue repair and understanding the bases for altered tissue repair offer new opportunities designed to address the structural alterations that characterize chronic obstructive pulmonary disease.

Fibroblast-mediated collagen gel contraction and degradation is considered as an *in vitro* model of wound healing and tissue remodeling. Many factors can modulate the process of collagen gel contraction mediated by human lung fibroblasts.

The "protease-antiprotease" hypothesis for the development of emphysema suggested the concept that tissue destruction results when protease burden-for example, from neutrophil elastase-exceeds the protective screen provided by antiproteases, such as α_1-antitrypsin[1]. The concept has been expanded to include several classes of proteases and antiproteases as well as oxidants and antioxidants[2,3].

Statins, an inhibitor of 3-hydroxy-3-methylgluaryl coenzyme A (HMG-CoA) reducatse, is one of the powerful hypolipidemic drug currently used in the clinic. In addition to their effect on cholesterol levels, recent *in*

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vitro and *in vivo* studies demonstrated that statins inhibit production of inflammatory cytokines induce apoptosis in variety types of cells and inhibit Matrix Metalloproteinase (MMP)-1, -2, -3 and -9 production and activation[4]. Taken together, statins attenuate production of pro-inflammatory cytokines, inhibit MMP activation and induce apoptosis in variety types of cells[5].

We hypothesized that simvastatin can regulating human lung fibroblast function in wound repair and tissue remodeling following inflammatory lung injury through inhibiting MMP release from the fibroblast in collagen gel.

The aim of this study, using an *in vitro* model, was to evaluate the effect of simvastatin on MMP-2 and -9 production and activation from the human lung fibroblast in the presence or absence of cytokines together with NE.

Materials and Methods

1. Materials

Type I collagen was extracted from rat tail tendons by a previously published method[6,7]. Briefly, tendons were excised from rat tails, and the tendon sheath and other connective tissues were carefully removed. After repeated washes with Tris-buffered saline and 95% ethanol, type I collagen was extracted in 4 mM acetic acid at 4°C for 24 hours. Protein concentration was determined by weighing a lyophilized aliquot from each lot of collagen solution. SDS-PAGE routinely demonstrated no detectable proteins other than type I collagen.

Human neutrophil elastase was purchased from ECP (Owensville, MO, USA). Human recombinant TNF-a, human recombinant IL-1b purchased from R&D Systems (Minneapolis, MN, USA).

Tissue culture supplements and medium were purchased from GIBCO BRL (Life Technologies, Grand Island, NY, USA). Fetal calf serum (FCS) was purchased from BioFluids (Rockville, MD, USA).

2. Fibroblasts

Human fetal lung fibroblasts (HFL-1) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in 100-mm tissue culture dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA) with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 50 mg/mL of penicillin, 50 mg/mL of streptomycin, and 0.25 mg/mL of Fungizone.

The fibroblast were passaged every 3～5 days. Subconfluent fibroblasts were trypsinized (trypsin-EDTA; 0.05% trypsin, and 0.53 mM EDTA-4 Na) and used for collagen gel culture. Fibroblasts used in these experiments were between cell passages 12 and 18.

3. Preparation of collagen gels

Collagen gels were prepared by mixing the appropriate amounts of rat tail tendon collagen, distilled water, 4×concentrated DMEM, and cell suspension so that the final mixture resulted in 0.75 mg/mL of collagen, 4.5×10^5 cells/mL, and a physiological ionic strength[7]. Fibroblasts were always added last to minimize damage during the preparation of the collagen gels. The mixture (0.5-mL aliquots) was cast into each well of 24-well tissue culture plates (Falcon, Franklin Lakes, NJ). Gelation occurredin 20 minutes at room temperature, after which the gels were released and transferred to 60-mm tissue culture dishes containing 5 mL of serum-free DMEM and cultured at 37°C in 5% CO_2 for 4～5 days. To demonstrate the effects of cytokines and elastase on collagen gel contraction and collagen degradation, cytokines (5 ng/mL of TNF-a and 5 ng/mL of IL-1b)[8], 20 nM elastase, or a combination of both was added to the culture medium. Gel area was measured daily with an image analysis system (Optimax V, Burlington, MA, USA).

4. Hydroxyproline assay

Hydroxyproline, which is directly proportional to type I collagen content, was measured by spectrophotometric determination[9,10]. Briefly, the medium surrounding the gels was completely removed, and the gels were

transferred to a glass tube (KIMAX, Fisher Scientific, St. Louis, MO, USA) with 2 mL of 6 N HCl. O_2 was removed by ventilation with N2 for 30 seconds. The gels were hydrolyzed at 110°C for 12 hours. The samples were dried with a vacuum centrifuge and redissolved in distilled H_2O before measurement. Hydroxyproline in the samples was reacted with oxidant (1.4% chloramine T in acetate-citric acid buffer; Sigma, St. Louis, MO, USA) and Ehrlich's reagent (0.4% p-dimethylaminobenzaldehyde; Sigma) in 60% perchloric acid (Fisher Chemical, Fair Lawn, NJ, USA) at 65°C for 25 minutes, and hydroxyl -proline content was determined by spectrophotometer at 550 nm.

5. Gelatinase activity assay

To investigate the activity of gelatinase, gelatin zymography was performed. The supernatant-conditioned media were concentrated 10-fold by lyophilization and dissolved in distilled water.

Gelatin zymography was performed with a modification of a previously published procedure[11,12]. Samples were dissolved in 23 electrophoresis sample buffer (0.5 M Tris • HCl, pH 6.8, 10% SDS, 0.1% bromphenol blue, and 20% glycerol) and heated for 5 minutes at 95°C. Forty microliters of each sample were then loaded into each lane, and electrophoresis was performed at 45 mA/gel.

After electrophoresis, the gels were soaked with 2.5% (vol/vol) Triton X-100 and gently shaken at 20°C for 30 minutes. After this, the gels were incubated in the metalloproteinase buffer (0.06 M Tris • HCL, pH 7.5, containing 5 mM $CaCl_2$ and 1 mM $ZnCl_2$) for 18 hours at 37°C. The gels were then stained with 0.4% (wt/vol) Coomassie blue and rapidly destained with 30% (vol/vol) methanol, and 10% (vol/vol) acetic acid.

6. Statistical analysis

All data are expressed as mean±standard error of the mean (SEM). Statistical comparison of paired data was performed using Student's t-test, whereas multigroup data were analyzed by ANOVA followed by the Tukey's (one-way) or Bonferroni's (two-way) post-hoc analysis using PRISM4 software (GraphPad Prism, San Diego, CA, USA). $p<0.05$ was considered significant.

Results

1. Effect of simvastatin on collagen gel contraction in the Presence of cytokines and neutrophil Elastase

To investigate the effect of simvastatin on the collagen gel contraction mediated by human lung fibroblasts, HFL-1 cells were cast into collagen gels and floated in the serum free medium containing cytokines (IL-1b 5

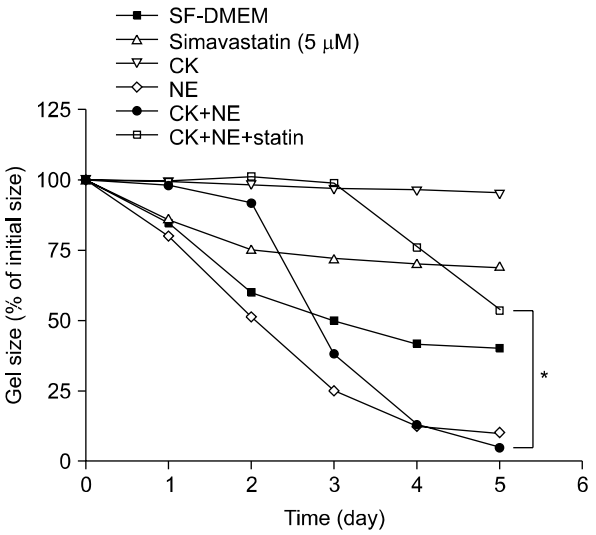

Figure 1. Effect of Simvastatin on Three-dimensional Collagen Gel (3D-gel) Contraction in the Presence or Absence of Cytokines and Neutrophil Elastase. Gels were released into tissue culture dishes containing 5 mL of serum-free DMEM with or without simvastatin (5 mM). After 1 hour incubation, cytokines (TNF-a 5 ng/mL, IL-1b 5 ng/mL) and NE (20 nM) were added in to the gel floating medium. Vertical axis presents gel size as % of initial area, and horizontal axis presents time after release (days). The data presented are mean±SEM from three separate experiments, each of which included triplicate gels for each condition. When simvastatin added together with the combination of the neutrophil elastase and cytokines, simvastatin (5 μM) significantly inhibit collagen gel contraction induced by the combination of the neutrophil elastase and cytokines. *$p<0.01$ compared with combination of neutrophil elastase and cytokines. CK: cytokines (TNF-α +IL-1b); NE: neutrophil elastase. Statin: simvastatin; SEM: standard error of the mean.

ng/mL and TNF-a 5 ng/mL), neutrophil elastase (NE, 20 nM), and simvastatin (5 μM) (Figure 1).

Under control culture conditions, fibroblasts contracted collagen gels. In five separate experiments, each performed in triplicate, gel size was reduced to 85.1±2.5% after 1 day of culture and 41.9±2.7% of the control level after 5 days in culture. Cytokines (TNF-a and IL-1b) consistently inhibited the contraction. NE consistently augmented fibroblast-mediated collagen gel contraction. Over the course of all five experiments in the presence of 20 nM NE, fibroblasts contracted to 78.3±4.5% of original size after 1 day and 11.7±1.7% of original size after 5 days ($p<0.01$ compared with control fibroblasts). When NE and cytokines were added concurrently, the degree of contraction after 1 day (95.2±2.6% of original size) was intermediate between the augmented contraction observed with NE alone and the inhibited contraction observed with cytokines alone. Beyond 1 day, however, the rate of contraction in cells incubated with cytokines and NE concurrently accelerated such that by day 5, the gels had contracted, on average for all experiments, to 3.5±0.5% of original size. This was significantly greater than the contraction seen with fibroblasts alone ($p<0.01$) and was also greater than that observed with fibroblasts incubated in the presence of NE alone ($p<0.01$). Simvastatin (5 μM) alone significantly inhibited collagen gel contraction mediated by HFL-1 cells. Simvastatin (5 mM) did not affect cell viability evaluated by MTT assay (data not shown).

When simvastatin added together with the combination of the NE and cytokines, simvastatin (5 μM) significantly inhibit collagen gel contraction induced by the condition of combination of the NE and cytokines, with 53.1±3.9% of original size after 5 days ($p<0.01$ compared with combination of NE and cytokines; Figure 1).

2. Effect of simvastatin on collagen gel degradation in the presence of cytokines and NE

To estimate whether the degradation of collagen gel was taking place during incubations with neutrophil elastase and/or cytokines, Hydroxyproline was measured both in the contracted gels and in the surrounding medium (Figure 2).

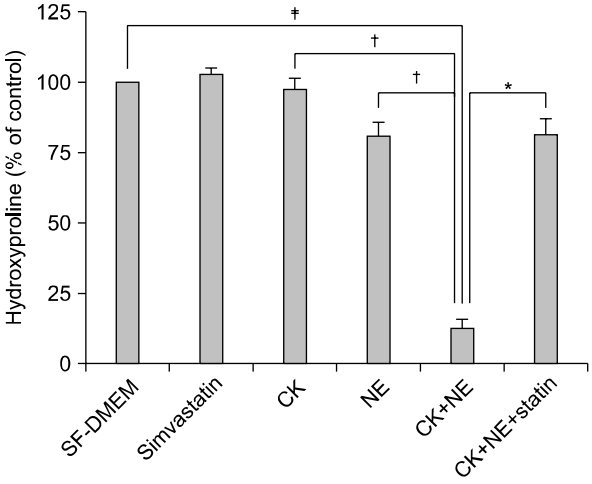

Figure 2. Effect of Simvastatin on Collagen Gel Degradation Induced by Cytokines and NE. Gels were prepared and cultured as shown above in figure 1. On day 5, gels were harvested and subjected to hydroxyproline assay. Vertical axis presents hydroxyproline (% of control), and horizontal axis presents culture conditions. The data presented are mean±SEM from three separate experiments, each per formed in duplicate. Cytokines (TNF-a 5 ng/mL, IL-1b 5 ng/mL) alone or NE (20 nM) alone slightly decreased the hydroxyproline content. The combination of cytokines and NE, however, resulted in a significant decrease of hydroxyproline content in collagen gels. Simvastatin (5 μM) significantly blocked collagen gel degradation induced by cytokines plus neutrophil elastase. *$p<0.01$ compared with combination of neutrophil elastase and cytokines, $^{\dagger}p<0.01$ compared with Cytokines alone or NE, $^{\ddagger}p<0.01$ compared with control cultures. CK: cytokines (TNF-a+IL-1b); NE: neutrophil elastase; SEM: standard error of the mean.

Control fibroblasts cultured over 5 days resulted in 93.0±1.4% of the hydroxyproline being recovered in the contracted gels. Over the 5 days of culture, neutrophil elastase resulted in an increase in solubilization of hydroxyproline, with 81.2±3.4% of the hydroxyproline recovered in the contracted gels ($p<0.05$ compared with control cultures). Cytokines alone to fibroblasts in three-dimensional collagen gels also increased collagen degradation slightly, with 96.9±1.8% of the hydroxyproline recovered in the contracted gels. In contrast with the modest degradation observed with either

neutrophil elastase or cytokines alone, the combination of the two resulted in 13.9±1.9% of the hydroxyproline being recovered in contracted gels ($p<0.01$ compared with both neutrophil elastase alone and cytokines alone). Over the 5 days of culture, simvastatin itself did not significantly affect the hydroxyproline content (Figure 2). When simvastatin added together with the combination of the neutrophil elastase and cytokines, simvastatin (5 μM) significantly blocked collagen gel degradation induced by the combination of the neutrophil elastase and cytokines, with 82.1±3.9% of the hydroxyproline being recovered in the contracted gels ($p<0.01$ compared with combination of neutrophil elastase and cytokines). The increase in hydroxyproline recovered in the surrounding medium was exactly matched by a decrease in the hydroxyproline recovered in the contracted gel assayed after 5 days.

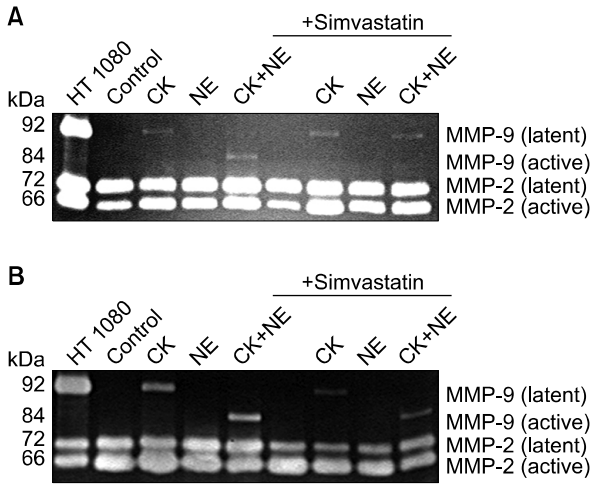

Figure 3. Effect of simvastatin on MMP-2 and -9 production and activation by the combination of cytokines and NE. Gels were prepared and cultured as shown above in figure 1. On day 2 (Figure 3A) and day 5 (Figure 3B), media (500 μL on day 2 and the rest on day 5) were harvested and subjected to gelatin zymography. Supernatant from HT1080 cell monolayer culture was used as a positive control. Under control conditions, HFL-1 fibroblasts cultured in three-dimensional collagen gels primarily released MMP-2 (gelatinase A) into surrounding media, as identified by its characteristic molecular weights of 72 kD (latent form) and 66 kD (active form) (Figure 3A and B). MMP-9 (gelatinase B) was not produced by HFL-1 cells under control conditions. In the presence of cytokines (TNF-a and IL-1b), however, latent form of MMP-9 (92 kD) was produced and this latent MMP-9 was converted to the active form (84 kD) by NE (10 nM, Figure 3). Simvastatin (5 μM) not only significantly blocked the MMP-9 conversion from latent to active form (Figure 3A), but also inhibited MMP-9 and MMP-2 production (Figure 3B). CK: cytokines (TNF-a+IL-1b); NE: neutrophil elastase.

3. Effect of simvastatin on MMP-2 and -9 production and activation by the combination of cytokines and NE

To determine if simvastatin could potentially modulate the production of gelatinases (MMP-2 and MMP-9) from the fibroblasts cultured in collagen gels, gelatin zymography was performed. Under control conditions, HFL-1 fibroblasts cultured in three-dimensional collagen gels primarily released MMP-2 (gelatinase A) into surrounding media, as identified by its characteristic molecular weights of 72 kD (latent form) and 66 kD (active form) (Figure 3). MMP-9 (gelatinase B) was not produced by HFL-1 cells under control conditions. In the presence of cytokines, however, latent form of MMP-9 (92 kD) was produced and this latent MMP-9 was converted to the active form (84 kD) by NE (20 nM; Figure 3). Simvastatin (5 μM) not only significantly blocked the MMP-9 conversion from latent to active form (Figure 3A), but also inhibited MMP-9 and MMP-2 production (Figure 3B).

Discussion

The present study demonstrates simvastatin may play a role in regulating human lung fibroblast functions in wound repair and tissue remodeling through inhibiting the MMP-9 conversion from latent to active form, but also simvastatin inhibited MMP-9 and MMP-2 production. Specifically, fibroblasts cultured in three-dimensional collagen gels are able to contract these gels, a system that has been used to model both normal wound healing and fibrosis[13,14]. Neutrophil elastase can augment this contractile process[15]. The cytokines (IL-1β and TNF-a) can inhibit this process[16]. However, when

neutrophil elastase added together with cytokines results in a marked contraction of collagen gels after an initial delay. This contraction is associated with a marked degradation of the collagen within the gel. This augmented degradation is due to induction of production of MMPs by the fibroblasts in culture as a result of their exposure to cytokines and by conversion of these MMPs by neutrophil elastase to lower molecular mass species that correspond to their active forms. The cytokines, IL-1β and TNF-α were effective at inducing MMP production and at augmenting collagen degradation in the presence of neutrophil elastase. When simvastatin added together with the combination of the cytokines and neutrophil elastase, simvastatin significantly blocked collagen gel degradation induced by cytokines plus neutrophil elastase. This effect of simvastatin on collagen gel degradation was caused by not only inhibition of MMP-9 and MMP-2 production, but also blocking the MMP-9 conversion from latent to active form.

The culture of fibroblasts in three-dimensional collagen gels has been utilized as an *in vitro* system to evaluate tissue repair and remodeling[13,14]. When cultured in three-dimensional gels composed of native type I collagen, fibroblasts orient themselves along the collagen fibers. Both fibroblast proliferation and protein production in three-dimensional collagen gel culture differ markedly from those in routine tissue culture conditions[13]. Through interactions that depend in part on $\alpha 2\beta 1$-integrins, fibroblasts can exert a tensile force on the collagen fibers. If the gels are unrestrained, for example in floating gel culture, the fibroblasts cause the gels to contract. This contraction can be modified by a variety of exogenous agents, which can either stimulate or inhibit collagen gel contraction[17,18].

Emphysema has been believed to develop when mediators of tissue injury exceed protective mechanisms within the lung. Evidence also supports the concept that tissue destruction represents a balance between tissue injury and tissue repair[19]. Repair processes are initiated as part of the inflammatory response in many tissues including the lung. If these repair responses can restore normal tissue architecture, function can be preserved. Efforts at repair, however, may result in disruption of normal tissue. In chronic obstructive pulmonary disease (COPD), both in the airways and in the alveolar structures, tissue dysfunction likely results from altered structure due to incompletely effective repair responses[20]. The net tissue destruction that characterizes emphysema represents an imbalance between tissue destruction and tissue repair processes, analogous to the protease-antiprotease balance.

The MMPs are a complex family of proteins[21,22]. More than 20 members have been identified. All share the structural similarity of a metal ion at the active site. They differ, however, in their mechanisms of activation and in their substrate specificity. Many MMPs are capable of degrading components of the extracellular matrix (ECM), hence the class name. Together they have been demonstrated to degrade all components of the extracellular milieu[21,22]. Other studies[23] have demonstrated that cytokines are capable of inducing MMPs by fibroblasts in routine tissue culture.

Several proteases can serve to activate the proteolytic cascade, which can lead to degradation of ECM. In this regard, several MMPs including, membrane-type MMPs, are constitutively active and are capable of activating MMP-2[24]. In addition, MMP-3, when activated, can activate MMP-2 and -9[25]. Finally, serine proteases are also capable of activating several of the MMPs. The MMPs produced in response to cytokines in the present study were observed in sizes that corresponded to their latent forms. Neutrophil elastase had no effect on MMP production. Elastase did, however, have a clear effect in converting the MMPs to lower molecular mass forms that corresponded to active MMPs. Thus the current study supports the concept that cytokines such as IL-1β and TNF-α can induce the production of MMPs but that maximal collagen degradation is achieved only in the presence of an activator such as neutrophil elastase[16].

There are several inflammatory processes in the lung that may be susceptible to the effects of statins. Statins could affect the chemokine and adhesion molecule directed migration of inflammatory cells from blood into

the airways[26]. The neutrophilia associated with a mouse model of acute lung injury is markedly reduced with lovastatin treatment[27] and this modulation of neutrophil apoptosis may prove beneficial in other inflammatory lung diseases, such as smokers with asthma or chronic obstructive pulmonary disease (COPD) where neutrophils are present and where corticosteroid treatment may be of limited benefit.

Statins also reduce the tissue damage and cellular changes associated with cigarette smoking. The mechanism of this appears to be related to the reduction by statins of the production of MMP-9 in smoking rats and in human macrophages and monocytes from smokers[28-31]. Other MMPs may also be reduced[32,33]. By targeting this key aspect of remodelling, this indicates a potential therapeutic role for statins in fibrotic lung diseases.

The current study supports the concept that simvastatin may play a role in regulating human lung fibroblast functions in wound repair and tissue remodeling through inhibiting the MMP-9 conversion from latent to active form, but also inhibited MMP-9 and MMP-2 production from the fibroblasts cultured with cytokines such as IL-1β and TNF-α and neutrophil elastase in three-dimensional collagen gels.

In conclusion, simvastatin could potentially work as a modulator of tissue remodeling process in emphysema through inhibition of matrix metalloproteinase release and activation from human lung fibroblasts cultured in three-dimensional collagen gels.

Acknowledgements

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