

## EXTENDED REPORT

Simvastatin reduces MMP-3 level in interleukin 1 $\beta$  stimulated human chondrocyte culture

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**Objectives:** Matrix metalloproteinases (MMPs) produced by chondrocytes play a role in the development of cartilage degradation in joint diseases. Moreover, inhibition of MMP secretion by macrophages accumulating in arteriosclerotic plaques would account for the plaque stabilising activity of statins in cardiovascular patients. Recently, simvastatin has been shown to inhibit both developing and established collagen induced arthritis in a murine model. We thus decided to investigate the effect of simvastatin on the production of MMP-3 from cultured interleukin (IL)1 stimulated human chondrocytes.

**Methods:** Cells from human cartilage, obtained from eight subjects with osteoarthritis undergoing surgery for total hip prostheses, were cultured in the presence of different concentrations of simvastatin (5, 10, and 50  $\mu\text{mol/l}$ ) with and without IL1 $\beta$  (5 ng/ml). MMP-3 level was measured in the culture medium after 48 h of incubation.

**Results:** IL1 $\beta$  stimulation of chondrocytes increased MMP-3 concentration in the cultures (from 0.69 (0.09) to 1.94 (0.12) ng/ $\mu\text{g}$  protein). Incubation with simvastatin was associated with a dose dependent reduction in MMP-3 increase, both in the presence (–15%, –17%, and –26% with 5, 10, and 50  $\mu\text{mol/l}$ , respectively) and in the absence (–32% with 50  $\mu\text{mol/l}$ ) of IL1 $\beta$ . The inhibiting effect of simvastatin was completely reversed by the addition of mevalonate (100  $\mu\text{mol/l}$ ) or farnesol (10  $\mu\text{mol/l}$ ).

**Conclusions:** Our data show that simvastatin, by blocking HMGCoA-reductase and interfering in the prenylation processes, is able to inhibit MMP-3 production from cultured human chondrocytes that have been either unstimulated or stimulated with IL1 $\beta$ , thus suggesting a possible additional mechanism for statins in counteracting chronic joint disease related cartilage damage.

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Several studies have provided evidence for a significant role of matrix metalloproteinases (MMPs), particularly MMP-3 or stromelysin-1, produced by chondrocytes, in the development of cartilage degradation in joint diseases.<sup>1,2</sup> MMP release by chondrocytes may be enhanced in conditions of physical and chemical stress. Among the mediators of tissue injury in the course of osteoarthritis and inflammatory joint diseases, one cytokine, interleukin (IL)1, is actively involved in the development and progression of cartilage damage by different mechanisms, including enhancement of MMP production.<sup>3,4</sup>

HMG-CoA reductase inhibitors (statins) are able to reduce mortality in cardiovascular diseases by means of hypolipidaemic and non-hypolipidaemic pharmacological effects. The latter include a stabilising activity on the arteriosclerotic plaque, possibly by increasing collagen content in the fibrous cap. This effect is referred to as the statin dependent inhibition of MMP secretion by macrophages accumulating in the plaque.<sup>5</sup> Macrophages are capable of degrading extracellular matrix by phagocytosis or by secretion of proteolytic enzymes that may weaken the fibrous cap, predisposing to its rupture<sup>6</sup> and to secondary thrombosis and embolisation. Furthermore, recent data suggest that the effect of statins on MMPs is dependent on the inhibition of mevalonate synthesis and, as a consequence, the production of mevalonate isoprenoid derivatives, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate. These molecules, by modifying specific intracellular proteins by covalent attachment during the so-called prenylation process, play an essential role in the regulation of several cellular mechanisms, including cytoskeletal dynamics and endocytotic/exocytotic transport, which are involved in MMP

secretion.<sup>7</sup> Moreover, a recent paper showed that simvastatin markedly inhibited not only developing but also established collagen induced arthritis in a murine model.<sup>8</sup>

Based on the above observations, we tested the possibility that the statin dependent inhibition of MMP production could also be reproduced in a different cell model such as human chondrocytes. Thus, the effect of increasing concentrations of simvastatin on the production of MMP-3 from cultured human chondrocytes stimulated with IL1 $\beta$  was studied. In addition, proteoglycan (PG) production was also measured in order to rule out a possible unfavourable inhibitory effect of simvastatin on the biosynthetic activity of chondrocytes.

## MATERIALS AND METHODS

## Chondrocyte culture

Human cartilage was obtained from the femoral heads of eight subjects with osteoarthritis undergoing surgery for total hip prostheses (5M, 3F; mean (SD) age 63.7 (10.1) years). Cartilage was cut aseptically and minced into 2 mm<sup>2</sup> pieces. The cartilage fragments were then digested by clostridial collagenase (Sigma, St Louis, MO, USA) 1 mg/ml in PBS. Collagenase digestion was carried out at 37°C for 14–18 h with moderate stirring.

Cells obtained after digestion were cultured in 24 well microplates at a density of  $3 \times 10^5$  cells/well and overlaid with 1 ml of medium containing 10% fetal calf serum, 200 U/ml penicillin, 200  $\mu\text{g/ml}$  streptomycin, 2 mmol/l glutamine, and 50  $\mu\text{g/ml}$  ascorbate in Dulbecco's minimum essential

**Abbreviations:** MMP, matrix metalloproteinase; PG, proteoglycan

medium. Cells were maintained in an atmosphere of 5% CO<sub>2</sub> in air at 37°C for 48 hours.

### Study with simvastatin

The effect of simvastatin +/- IL1 $\beta$  was analysed on chondrocytes cultured as described above.

Cells were grown in culture medium with 5 ng/ml human recombinant IL1 $\beta$  (Boehringer Mannheim, Germany) and simvastatin (5, 10, and 50  $\mu$ mol/l; Merck & Co Inc, Rahway, NJ, USA). Cells were also cultured in the presence of 50  $\mu$ mol/l simvastatin alone. A control sample of chondrocytes cultured in the absence of simvastatin and IL1 $\beta$  was also evaluated in all experiments. Finally, the effect of the addition of exogenous mevalonate (100  $\mu$ mol/l), farnesol (10 mol/l), and geranylgeraniol (10 mmol/l) (all Sigma) was evaluated in the 50  $\mu$ mol/l simvastatin samples, with or without IL1 $\beta$ .

After 48 h the medium was removed, and stored at -70°C pending determination of its MMP-3 content. Cell viability was evaluated by Trypan blue and methylthiazolotetrazolium (MTT) (both Sigma).

### MMP-3 assays

The amount of total MMP-3 in the culture medium was measured by an immunoenzymatic method on microplates (Biotrak Matrix MMP-3 ELISA System, Amersham, Buckinghamshire, UK), and expressed as ng/ $\mu$ g protein.

### Proteoglycan assays

The amount of PG in the culture medium was evaluated by a solid phase enzyme amplified sensitivity immunoassay (Biosource PG EASIA kit, BioSource Europe SA, Belgium) performed in a microtitre plate, and expressed as  $\mu$ g/ml. Cells from three of the eight patients with osteoarthritis were studied in duplicate. In this part of the study, the concentration of simvastatin employed was 50  $\mu$ mol/l.

### Statistical analysis

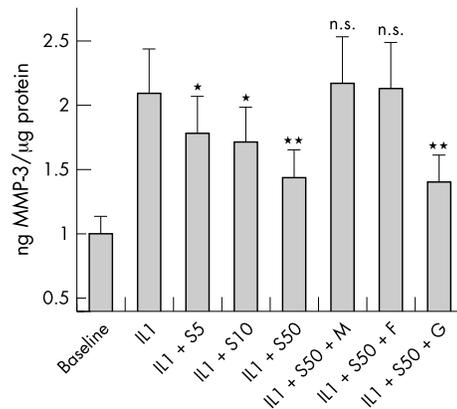
Results were statistically evaluated by Student's *t* test for paired data. Values of  $p < 0.05$  were considered significant.

## RESULTS

IL1 $\beta$  stimulation of chondrocytes was able to increase MMP-3 production (from 0.69 (0.09) to 1.94 (0.12) ng/ $\mu$ g protein). Incubation with simvastatin was associated with a dose dependent significant reduction of MMP-3 production, both in the presence (-15%, -17%, and -26% with 5, 10, and 50  $\mu$ mol/l, respectively) and absence (-32% with 50  $\mu$ mol/l) of IL1 $\beta$  (fig 1-2). In the 50  $\mu$ mol/l samples, with or without IL1 $\beta$ , the inhibiting effect of simvastatin was completely reversed by the addition of mevalonate (100  $\mu$ mol/l) and farnesol (10  $\mu$ mol/l) (fig 1-2). In contrast, no reversal effect was observed when the culture was co-incubated with geranylgeraniol (10  $\mu$ mol/l) (fig. 1-2).

The addition of simvastatin 50  $\mu$ mol/l did not significantly affect proteoglycan synthesis from IL1 $\beta$  treated and untreated cell cultures. Levels of proteoglycans were 2.38 (0.86)  $\nu$  3.10 (1.04)  $\mu$ g/ml, and 1.31 (0.48)  $\nu$  1.69 (0.77)  $\mu$ g/ml for control sample  $\nu$  control + 50  $\mu$ mol/l simvastatin, and control + IL1 $\beta$   $\nu$  control + IL1 $\beta$  + 50  $\mu$ mol/l simvastatin, respectively.

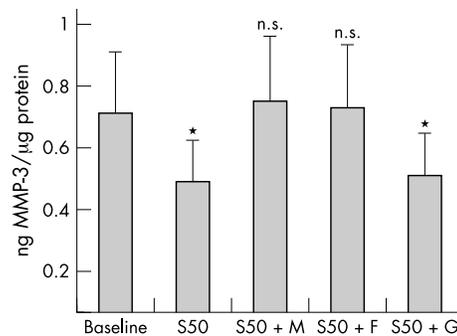
Viability of the cells was maintained, as demonstrated by the evaluation with Trypan blue, which indicated 90-95% viability with simvastatin 50  $\mu$ mol/l. Longer term preservation of cell viability was confirmed with MTT (88-92%) in the same conditions.



**Figure 1** Effect of the 48 h incubation with 5, 10, and 50  $\mu$ mol/l simvastatin (S) and 50  $\mu$ mol/l S+100  $\mu$ mol/l mevalonate (M), 50  $\mu$ mol/l simvastatin + 10  $\mu$ mol/l farnesol (F), and 50  $\mu$ mol/l simvastatin + 10  $\mu$ mol/l geranylgeraniol (G) on 5 ng/ml IL1 $\beta$  dependent MMP-3 production (expressed as ng/ $\mu$ g protein) from cultured chondrocytes from osteoarthritis patients. All experiments were performed in duplicate. Data are expressed as mean (SD). Baseline sample represents the blank control. Student's *t* test for paired data (simvastatin treated samples + IL1 $\beta$   $\nu$  untreated sample + IL1 $\beta$ ). \* $p < 0.05$ ; \*\* $p < 0.01$ ; n=8.

## DISCUSSION

Recent evidence has shown that statins possess a variety of biological properties, other than lipid lowering abilities, including immunomodulating and anti-inflammatory effects. In particular, these agents have been shown to inhibit the production of cytokines such as MCP-1,<sup>9</sup> IL8,<sup>10</sup> and IL6;<sup>11</sup> to block LFA-1 mediated adhesion and co-stimulation of lymphocytes;<sup>12</sup> and to reduce the expression of MHC-II molecules, thus interfering with T cell activities.<sup>13</sup> Interestingly, a very recent work showed that simvastatin markedly inhibited not only developing but also established collagen induced arthritis in a murine model. Ex vivo analysis demonstrated a significant suppression of collagen specific Th1 humoral and cellular immune responses and a reduction of anti-CD3/anti-CD28 proliferation and IFN- $\gamma$  release from mononuclear cells derived from peripheral blood and synovial fluid.<sup>8</sup> Thus, statins may reasonably play a role in chronic inflammatory conditions, such as rheumatic diseases.<sup>14</sup> Moreover, recent studies have demonstrated that



**Figure 2** Effect of the 48 h incubation with 50  $\mu$ mol/l simvastatin (S), 50  $\mu$ mol/l simvastatin + 100  $\mu$ mol/l mevalonate (M), 50  $\mu$ mol/l simvastatin + 10  $\mu$ mol/l farnesol (F), and 50  $\mu$ mol/l simvastatin + 10  $\mu$ mol/l geranylgeraniol (G) on spontaneous MMP-3 production (expressed as ng/ $\mu$ g protein) from cultured chondrocytes from osteoarthritis patients. All experiments were performed in duplicate. Data are expressed as mean (SD). Student's *t* test for paired data (simvastatin treated samples  $\nu$  untreated sample). \* $p < 0.05$ ; n=8.

some statins inhibit MMP production both in vitro<sup>5, 15</sup> and in vivo.<sup>16, 17</sup> This effect of statins provides further evidence for a possible additive cartilage protective mechanism of action of these agents in joint diseases.

Our data show that simvastatin is able to inhibit MMP-3 production from cultured human chondrocytes stimulated with IL1 $\beta$ , and from unstimulated cells. The complete prevention of the inhibitory effect of the drug seen after co-incubation with mevalonate suggests that simvastatin influences chondrocyte MMP-3 production through its blocking activity on HMGCoA reductase, a key enzyme in the cholesterol biosynthetic pathway, which synthesises the isoprenoid precursor mevalonate. In the same way, the pivotal role of prenylated, and more particularly farnesylated proteins, in the process leading to MMP-3 production is further confirmed by the peculiar ability of farnesol (but not geranylgeraniol) to completely revert the effect of simvastatin. Bellosta *et al*<sup>5</sup> demonstrated that statins reduce MMP-9 production by cultured macrophages without any interference in MMP-9 gene expression or in the activation process after the secretion of the protease. Moreover, a recent study reported that statins are able to inhibit the production of other MMPs, including MMP-3, in different cell lines without producing any change in MMP mRNA levels.<sup>15</sup> Based on these data, and because the covalent addition of isoprenoids to proteins (the prenylation process) plays an important role in regulating membrane traffic, it seems conceivable that statin interference in isoprenoid synthesis may lead to a reduced MMP-3 secretion by chondrocytes.

It seems remarkable that this potential anti-resorptive effect of simvastatin is not associated with a concomitant inhibition of chondrocyte biosynthetic activity; as a result, the overall activity of simvastatin on cartilage metabolism should produce favourable effects. In fact, proteoglycan synthesis was not significantly modified by simvastatin in our study. Indeed, simvastatin seems to produce a slight increase in proteoglycan concentration in the medium from chondrocyte cultures, also partially reversing the well known IL1 induced proteoglycan downregulation.<sup>18</sup> This effect was not statistically significant, which may be due to the fact that the sample size was small. A study of the effects of simvastatin on proteoglycan metabolism was beyond the scope of the present paper; nevertheless, the possibility that simvastatin may increase proteoglycans in resting and stimulated chondrocytes deserves further studies, which are now in progress.

These novel findings would suggest that statins may play a protective role in rheumatic diseases by acting on either the basic immunological mediators, or the final effectors of tissue damage. Our results, although very preliminary, may help further our understanding of the possible therapeutic role of statins in the prevention of irreversible cartilage damage associated with osteoarthritis and inflammatory joint diseases. More studies are now in progress in order to give further insight into the mechanism of action of these drugs.

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