Simvastatin inhibits cytokine production and nuclear factor-kB activation in interleukin 1β-stimulated synoviocytes from rheumatoid arthritis patients

P.E. Lazzerini[1], S. Lorenzini[2], E. Selvi[2], P.L. Capecchi[1], D. Chindamo[2], S. Bisogno[2], R. Ghittoni[1], M.R. Natale[1], F. Caporali[1], S. Giuntini[1], R. Marcolongo[2], M. Galeazzi[2], F. Laghi-Pasini[1]

[1]Department of Clinical Medicine and Immunological Sciences, Division of Clinical Immunology and [2]Division of Rheumatology, University of Siena, Italy.

Abstract

Objectives

Recent studies demonstrated in vivo *the effectiveness of statins in reducing the inflammatory response in rheumatic diseases, and still more recently, simvastatin has been reported to inhibit* in vitro *IL-6 and IL-8 production by unstimulated fibroblast-like-synoviocytes (FLS) from rheumatoid arthritis (RA) patients. However, no data are available on the effect of statins on the production of these cytokines induced by IL-1, which plays a crucial role in joint inflammation in the course of active RA* in vivo.

Methods

In 12 RA patients, synovial tissue specimens were taken to obtain cultures of FLS. Cultures were incubated with IL-1 ± simvastatin (5-50 μmol/l), and IL-6 and IL-8 production was evaluated (ELISA), also following the addition of mevalonate and its isoprenoid derivatives. Moreover, nuclear factor-kB (NF-kB) activation (immunocytochemistry and Western Blot analysis) were also evaluated.

Results

Culture incubation with IL-1 produced a dramatic increase (up to 40-fold) in cytokine production with respect to unstimulated cells. Simvastatin significantly inhibited (about 20%) IL-6 and IL-8 production from IL-1-stimulated FLS. This effect was completely reverted by the concomitant incubation with mevalonate or geranylgeraniol (but not farnesol or squalene). Moreover, simvastatin produced a clear-cut inhibition of IL-1-induced NF-kB activation.

Conclusion

Simvastatin significantly inhibits the production of IL-6 and IL-8 also in IL-1-stimulated FLS, even though to a lesser extent than in unstimulated cells, via a HMG-CoA-reductase block with an interference in prenylation process and NF-kB activation. Our results further support the rationale for the use of statins in the treatment of rheumatoid synovitis.

Key words

Simvastatin, rheumatoid arthritis, interleukin-6, interleukin-8, NF-kB, interleukin-1, inflammation, synoviocytes.

Pietro Enea Lazzerini, MD; Sauro Lorenzini, BiolD; Enrico Selvi, MD; Pier Leopoldo Capecchi, MD, PhD, Associated Professor; Daniela Chindamo, MD; Stefania Bisogno, MD; Raffaella Ghittoni, BiolD; Maria Rita Natale, BiolD; Francesca Caporali, Biol D; Serena Giuntini, BiolD; Roberto Marcolongo, MD, Professor; Mauro Galeazzi, MD, Professor; Franco Laghi-Pasini, MD, Professor.

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Please address correspondence to: Pietro Enea Lazzerini, MD, Department of Clinical Medicine and Immunological Sciences, Division of Clinical Immunology, University of Siena, Siena, Italy.
E-mail: pietroenea@yahoo.it

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Introduction

Synoviocytes play a relevant role in the development of cartilage damage in the course of chronic inflammatory joint diseases, particularly rheumatoid arthritis (RA), by producing many mediators including IL-6 and IL-8 (1), with relevant correlations between these cytokines and the clinical behaviour of the disease (2, 3).

Hydroxy-methylglutaryl-coenzymeA (HMGCoA)-reductase inhibitors (statins) reduce morbidity and mortality in cardiovascular diseases by means of hypolipidemic and non-hypolipidemic effects. Moreover, statins inhibit *in vitro* the production of IL-8 and IL-6 from macrophages, vascular smooth-muscle and endothelial cells (4-5). The inhibitory effect on cytokine production is reported to be related to the inactivation of nuclear factor-kB (NF-kB) (6-7), dependent on the inhibition of mevalonate synthesis and mevalonate isoprenoid derivatives, such as farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate. By modifying specific intracellular proteins by covalent attachment during the so-called prenylation process (8), these molecules play an essential role in the regulation of several cellular mechanisms, including transcription and synthesis of inflammatory cytokines.

Two recent studies demonstrated *in-vivo* the effectiveness of statins in reducing the inflammatory response in rheumatic diseases (9, 10), and still more recently, Yokota *et al.* (11) reported the *in vitro* inhibitory effect of simvastatin on the spontaneous production of IL-6 and IL-8 by unstimulated fibroblast-like synoviocytes (FLS). Indeed, in patients with active RA, an elevated degree of joint inflammation is present and the IL-6 and IL-8 production is highly enhanced at that site by several molecules, among which IL-1 plays a pivotal role.

On the basis of these considerations, and in order to better reproduce the inflammatory conditions really operating *in vivo* in RA patients, we tested whether the inhibitory effect of simvastatin on IL-6 and IL-8 production was present also in cultured human synoviocytes stimulated with IL-1. Putative intracellular mechanisms of action of the drug, such as the block of HMG-CoA-reductase, the interference in the prenylation process and the activation of NF-kB , were also investigated.

Materials and methods

Patients

Twelve patients with active RA were enrolled in our study (Table I). Synovial specimens for cell cultures were obtained in the course of diagnostic procedures by needle synovial biopsy of the knee joint.

Synoviocyte cultures

Synovial tissue was minced into 1 mm^3 pieces, washed in PBS and then digested for 24 hours with collagenase (1 mg/ml; Sigma-Aldrich Milan, Italy) in free serum Dulbecco's-Mod-Eagle-Medium (DMEM; Invitrogen, Milan, Italy). The obtained cells were washed with medium containing 10% foetal-calf-serum (FCS; Sigma-Aldrich Milan, Italy) and centrifuged (10 minutes at 700g). The resultant suspensions were plated out in DMEM supplemented with 30% of FCS. FLS were selected by adhesion to tissue culture plastics and were grown to confluence. Cells were used for experiments at the third passage.

Synoviocytes were plated out in 400 µl of complete medium in a 48-well tissue culture plate ($1x10^5$ cells/well) and allowed to attach for 24 hours.

The purity and phenotype of the isolated synovial fibroblasts were characterized by flow cytometry. Cultured cells were harvested with trypsin/EDTA and washed in PBS; 3 x 10^5 cells were stained with saturating amounts of monoclonal antibody CD90/Thy-1 (Calbiochem) and CD14 (BD) conjugated with fluorescein isothiocyanate fluorochrome (FITC) in buffer PBS, 1% FCS, 2mM EDTA for 30 minutes at 4°C. In the case antibodies were not conjugated with a fluorochrome, a secondary labelling for 30 minutes at 4°C with a FITC-labelled goat-anti mouse immunoglobulin (Calbiochem) was performed. The specificity of staining was confirmed using isotype-matched control mAbs at identical concentrations. Cells were analysed with the FACS Calibur flow cytometer (Becton

Table I. Demographic characteristics and ongoing therapy of RA patients.

Age (mean ± SD years)	53 ± 8
Sex (no. females /no. males)	8/4
Disease duration (mean ± SD months)	66 ± 39
Disease activity score (mean ± SD)	4.1 ± 0.5
Synovial fluid leukocyte count (mean ± SD cells/mm^3)	5,100 ± 2,200
ESR (mean ± SD mm/1st hour)	43 ± 14

All the patients were under steroid treatment (mean daily dose < 8 mg of prednison-equivalent) and/or NSAIDS. At the time of the study, the patients were neither under DMARDS, nor under TNF-blocking therapy, nor under statins.
Informed consent was obtained according to the Principles of the Declaration of Helsinki.

Dickinson). Forward and side scatter gates were set to include all viable cells. More than 90% of synoviocytes express CD90/Thy-1 antigen.

Study with simvastatin

Synoviocytes were incubated for 24 h with IL-1β (0.1 ng/ml) (Boehringer-Mannheim, Germany) ± simvastatin (5-10-50 μmol/l, Merck & Co., Inc., Rahway, NJ, USA) and. The concentration of IL-1 employed in the study was in the range of that commonly found in the synovial fluid of RA patients (12). The effect of the addition of mevalonate (100 μmol/l) and its isoprenoid derivatives, *i.e.*, farnesol (10 μmol/l), geranylgeraniol (10 μmol/l), or the cholesterol precursor squalene (10 μmol/l) (all Sigma) was also evaluated in 50 μmol/l simvastatin samples stimulated with IL-1β.

The cytokine concentration in culture supernatants was measured (ng/ml) by an ELISA-kit (Euroclone Lugano, Switzerland).

Evaluation of NF-kB activation

A. *Immunocitochemistry*

In order to qualitatively evaluate whether simvastatin could act via inhibition of NFkB activation, cultured FLS from three RA patients were immunostained for the NF-kB p65 subunit, *i.e.* the active form of the NF-kB transcription factor, in basal condition, after IL-1β incubation and after 50 μmol/l simvastatin+IL-1β co-incubation. Stimulated synoviocytes from RA patients were detached from culture plate by trypsine treatment, and then resuspended in PBS in order to obtain a final concentration of 300 cells/mm^3. 100 μl of the cell suspension were centrifuged onto poly-L-lisine coated slides, air-dried and 10 min fixed in pre-cooled acetone. Slides were incubated 1 hour at 4°C with the anti-human mouse monoclonal IgG NFkB p65 (Santa-Cruz-Biotechnology California, USA) diluted 1:400 in PBS. Immuno-reactions were then developed by using Vectastain ABC-Kit (Vector-Laboratories, Burlingame, CA, USA) according to the data sheet specifications and stained by diaminobenzidine solution (Sigma-Aldrich Milan, Italy).

B. *Immunoblot analysis*

NF-kB is normally present in the cytoplasm in an inactive form that is associated with an inhibitory protein named IkB (13). In the presence of various stimuli, IkB is rapidly phosphorylated (p-IkB), leading to ubiquitination and subsequent degradation of IkB as well translocation of NF-kB into the nucleus (activated NF-kB). On this basis, we studied p-IkB and IkB protein expression in the same patients and in the same conditions above depicted, to further evaluate, in an indirect manner, the effect of simvastatin on the activation state of NF-kB. In fact, the quantity of active NF-kB results proportional to the intracellular amount of p-IkB, directly, and, IkB, inversely.

FLS were washed with PBS and incubated with PBS + 0.04% EDTA for soft separation and centrifuged for 10 min at 700g. The cells than lysed in cold with lysis buffer, including protease inhibitor. The cells were centrifuged at 900g for 5 min at 4°C.

To assay p-IkB and IkB protein expression we performed a Western Blot analysis. Equal amount of protein from samples were separated by sodium dodecyl sulphate (SDS)-page on 12% bis/acrylammide gel and transferred the protein to nitrocellulose membrane (Bio-Rad Laboratories, Richmond VA). Equal lane loading was judged by non-permanent Ponceau-S staining of nitrocellulose membrane, after recording the images membrane was rinsed in buffer to remove the staining and processed for immunoblotting. The membrane was blocked with 5% non-fat milk and 1% bovine serum albumin for 1 h at room temperature and than incubated with polyclonal anti-p-IkB (1:100 in TBST milk 0.05%) and polyclonal anti-IkB (1:200 in TBST milk 0.05%) primary antibodies (Santa Crutz Biotechnology, USA) for 12 h at room temperature. After several washes in TBST, the membrane was incubated with goat anti-rabbit secondary antibody IgG conjugated to horsedish peroxidase (Jackson ImmunoResearch, USA) (1:10000) for 1 h (TBST milk 1%). The membranes were washed with TBST and developed with a Western blotting ECL kit (Amersham Bioscences, Buckinghamshire, UK).

Cell viability

Cell viability following treatment with simvastatin (50 μmol/l) was assessed by trypan-blue viable stain and methyl-thiazoletetrazolium (MTT)-assay.

Results

Cytokine production

Incubation of the cultures with IL1-β produced a dramatic increase in cytokine production, especially IL-6. The addition of 5-10-50 μmol/l simvastatin to IL-1-stimulated synoviocytes was associated with a significant and dose-dependent reduction in IL-6 production (Table II). The simvastatin-dependent inhibiting activity was completely reverted when mevalonate was added to the IL-1-stimulated cultures (Table II). Similar findings were observed for the effects of simvastatin and mevalonate on IL-8 production (Table II). The extent of the maximal inhibitory effect of simvastatin on IL-1-stimulated cultures was about 20% for both cytokines.

A similar effect was demonstrated for geranylgeraniol in IL-1-stimulated cells (Table III). On the contrary, we did not find any significant removal of simvas-

Table II. Effect of the 24 h incubation with 5, 10, and 50 μmol/l S, and 50 μmol/l S + 100 μmol/l mevalonate (M) on 0.1 ng/ml IL1β-induced IL-8 and IL-6 production by cultured synoviocytes from rheumatoid arthritis (RA, n = 12) patients. IL-8 and IL-6 values are expressed as ng/ml.

	Baseline	IL-1	IL1+S5	IL1+S10	IL1+S50	IL1+S50+M	RM ANOVA
IL-8	3.72 ± 0.89	34.02 ± 9.33	31.02 ± 9.28*	30.36 ± 9.29*	26.39 ± 9.36*	34.06 ± 8.70	§
IL-6	1.72 ± 0.50	74.51 ± 16.67	69.42 ± 15.34*	66.34 ± 15.28*	57.72 ± 13.67*	74.87 ± 17.32	§

Repeated measurements oneway analysis of variance on ranks (RM ANOVA on Ranks): §= $p < 0.001$. Student-Neuman-Keuls' test (IL-1-stimulated and S-treated *vs* IL-1-stimulated and S-untreated cells): * = $p < 0.05$. Data are expressed as mean ± SD.

Table III. Effect of the 24 h incubation with 50 μmol/l S, 50 μmol/l S + 100 μmol/l mevalonate (M), 50 μmol/l S + 10 μmol/l farnesol (F), 50 μmol/l S + 10 μmol/l geranylgeraniol (G), and 50 μmol/l S + 10 μmol/l squalene (Sq) on 0.1 ng/ml IL1β-induced IL-8 and IL-6 production by cultured synoviocytes from RA patients (n = 5). IL-8 and IL-6 values are expressed as ng/ml.

	IL-1	IL1+S50	IL1+S50+M	IL1+S50+G	IL1+S50+F	IL1+S50+Sq	RM ANOVA
IL-8	38.67 ± 9.39	29.41 ± 8.29*	37.53 ± 11.01	38.94 ± 9.58	30.61 ± 7.89*	29.94 ± 8.29*	†
IL-6	72.26 ± 17.25	57.76 ± 10.61*	72.42 ± 17.96	73.15 ± 18.31	58.18 ± 10.48*	57.46 ± 11.05*	†

Repeated measurements oneway analysis of variance (RM ANOVA): †= $p < 0.001$. Student-Neuman-Keuls' test (S-treated *vs* S-untreated cells): *= $p < 0.05$. Data are expressed as mean ± SD.

tatin effect when the cultures were supplemented with farnesol and squalene (Table III).

NF-kB activation

A. *Immunocytochemistry*

Positive reactions for the NF-kB p-65 subunit were observed in all the slides. Interestingly, a mild immunoreactivity was present also in cytospin preparations from unstimulated rheumatoid synoviocytes, as an expression of a constitutive activation of NF-kB (Fig. 1A). The cell immunoreactivity strongly increased, as expected, after IL-1β stimulation, since almost all the synoviocytes showed an evident immunostaining (Fig. 1 B). This positive reaction was markedly reduced after co-incubation of the cells with simvastatin 50 μmol/l (Fig. 1C).

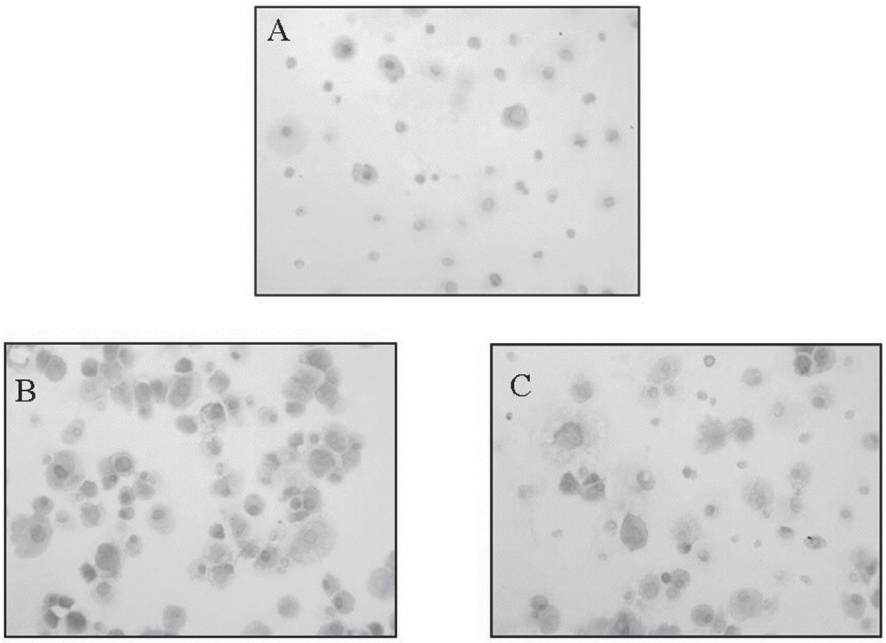

Fig. 1. Cytospin preparations of rheumatoid synovial cells immunostained by anti-human mouse monoclonal IgG NFkB p65. A) Basal condition; B) IL-1β stimulation; C) co-incubation with IL-1β + simvastatin 50 μmol/l.

B. *Immunoblot analysis*

In unstimulated FLS, only IkB protein was detectable, indirectly expressing a substantial inactivation of NF-kB (Fig. 2). Cell stimulation with IL-1β produced a clear-cut reduction in IkB expression, together with a concomitant increase in p-IkB (Fig. 2), as a reflection of a marked NF-kB activation. The addition of simvastatin 50 μmol/l to the cultures attenuated the effects of IL-1β leading to a partial restoration of the baseline IkB/p-IkB ratio. In fact, with respect to the incubation of the cells with IL-1 alone, co-incubation of FLS with simvastatin showed a reduced enhancement in p-IkB expression, concomitantly with a proportional less relevant decrease in IkB (Fig. 2).

Cell vitality

Ninety-five percent out of the simvastatin (50 μmol/l) incubated type-B fibroblast-like synoviocytes were vital by trypan-blue test and MTT-assay confirmed the good preservation of cell vitality.

Discussion

The study provides evidence of a dose-dependent inhibitory effect of simvastatin on IL-6 and IL-8 production in an *in vitro* model of cultured synoviocytes from RA patients stimulated with IL-1β. These data seem to confirm previous

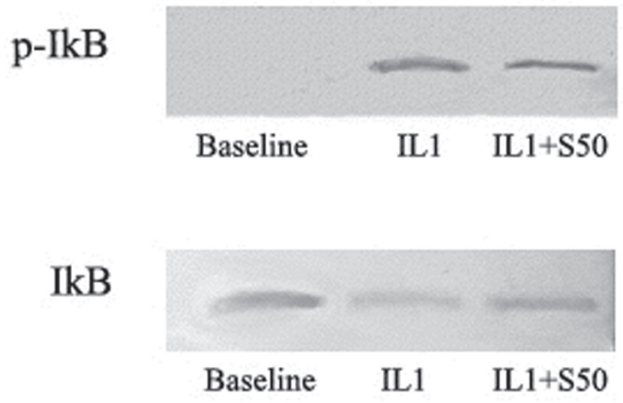

Fig. 2. Effect of simvastatin (S) 50 μmol/l on p-IkB and IkB expression (Western Blot analysis) in IL-1β-stimulated rheumatoid synoviocytes.

results obtained by Yokota *et al*. (11) in unstimulated RA synoviocytes, even if the extent of the maximal inhibitory effect found in our study is less relevant (20-25% *vs*. 50-60%) nothwistanding the employment of higher simvastatin concentrations (50 *vs*. 10 μmol/l). This finding seems to suggest that the degree of joint inflammation, reproduced in our *in vitro* study by the stimulation with IL-1 (increasing the cytokine production with respect to unstimulated cells up to 40-fold), is a crucial factor in the assessment of the effect of simvastatin on cytokine production in active RA patients.

The reversibility of the simvastatin effect on cytokine production observed with the addition of mevalonate and geranylgeraniol, similarly to that found by Yokota *et al*. (11), suggests the occurrence of a block of HMGCoA-reductase, with an interference in protein prenylation. More particularly, since the other isoprenoid derivative farnesol fails to revert the inhibiting activity of simvastatin on cytokine production, we can argue that the statin likely inhibits the function of intracellular proteins implicated in the regulation of the cytokine production, requiring the covalent attachment of a geranylgeranyl radical (8). Moreover, in our study, we also showed that the addition of squalene, a mevalonate derivative not involved in prenylation, does not restore cytokine production, thus ruling out the possibility that simvstatin interference on cholesterol synthesis may be also responsible for the inhibitory activity of the drug.

Recent data identified Rho as the main geranylgeranylated protein implicated in the activation of nuclear factor NF-kB (8, 14), which is the final stimulating effector of cytokine gene transcription (15). Moreover, it is well demonstrated that this pathway is crucial also in synovial cells (8). Thus, it seems conceivable that the observed inhibiting effect of simvastatin may be related to a specific interference of the drug on this metabolic cascade. The findings of this study seem to confirm this hypothesis. In fact, co-incubation of synoviocytes with IL-1 and simvastatin was associated with a net reduction in NF-kB activated subunit p65 together with an increase in the NF-kB inhibiting protein IkB, with respect to the incubation of the cells with IL-1 alone.

In conclusion, the study shows that simvastatin exerts an inhibitory effect also in IL-1-stimulated FLS from RA patients, even if to a lesser extent than that observed in unstimulated cells. The effect seems to be related to the HMG-CoA-reductase block with an interference in the prenylation process and NF-kB activation.

On the basis of our results, the rationale of a putative employment of simvastatin as an immuno-modulating drug in the treatment of RA appears strengthened by the evidence that the agent is also effective in an inflammatory context reproducing more exactly the *in vivo* conditions of the disease.

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