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Simvastatin prevents the induction of interleukin-6 gene expression by titanium particles in human osteoblastic cells

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ABSTRACT

One of the most important complications of total joint arthroplasty is failure associated with periprosthetic osteolysis, a process mainly initiated by the biological response to wear-derived products from the biomaterials in service. The inflammatory mediator interleukin-6 (IL-6) plays a key role in the establishment and progression of aseptic loosening. Metal particles specifically up-regulate IL-6 production in bone-forming cells and implant–bone interfacial tissues. The use of statins has been recently associated with a significantly reduced risk of revision in patients that undergo total hip arthroplasty. We hypothesized that simvastatin (Simv) could modulate the osteoblastic response to titanium particles (Ti) by attenuating the production of IL-6. Pre-treatment of human osteoblastic cells with Simv down-regulated Ti particle-induced *IL-6* gene expression at mRNA and protein levels. The effect of Simv on Ti-induced IL-6 production in osteoblastic cells could not be explained by inhibition of the internalization of metal particles. The mechanism involved in this down-regulation is based in the inhibition of the HMG-CoA/GGPP/RhoA/ROCK pathway, independently of Simv effects in the cholesterol synthesis. The cytokine-lowering property of Simv has been observed in Saos-2 cells and human primary osteoblasts (hOBs) exposed to Ti particles, and was further enhanced when hOBs were co-cultured with macrophages.

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1. Introduction

Joint prosthetic failure is demanding a growing rate of revision surgeries, which are usually much more challenging, expensive and with worse outcomes than the primary procedure. Although failures associated with infections or defective surgical procedures have been greatly reduced, aseptic loosening associated with periprosthetic osteolysis remains one of the most important complications influencing the long-term survival of total joint arthroplasty. This process is mainly initiated by the biological response to wear-derived products from the prosthetic biomaterial in service [1,2]. Wear particles induce an inflammatory response that leads to the formation of a periprosthetic granulomatous tissue and bone erosion around the implant. This facilitates the access of fluid-containing particles and soluble factors to the effective joint space, stimulating further bone resorption and propagating this devastating effect. Among other cellular targets, particles and molecules may affect the functions of bone-forming cells located at the tissue–implant interface which are primarily responsible for the maintenance of the osseointegration of the implant [1–3]. Although the mechanisms involved in aseptic loosening are not completely understood, the key role played by several inflammatory mediators such as interleukin-6 (IL-6) has been reported [4–6]. IL-6 is a multifunctional cytokine which has important local and systemic physiological effects. Locally, increased expression levels of IL-6 have been detected in retrieved periprosthetic membranes and associated with areas exhibiting more aggressive osteolysis [6,7]. Moreover, raised IL-6 levels have been found in joint fluid from patients with failed total hip arthroplasty (THA), supporting the proposal of this cytokine as a useful marker to predict prosthesis failure [4,8]. In the bone environment, IL-6 is mainly produced by stromal and osteoblastic cells, amplifying the effects of several pro-resorptive agents through paracrine and autocrine mechanisms and collaborating in the production of pro-resorptive factors [2,9]. Experimental approaches employing animal models of wear-particle osteolysis have confirmed that metal particles specifically up-regulate IL-6 production in bone and interfacial tissues [10,11]. Furthermore, in vitro experiments have reported that

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treatment with titanium particles (Ti) increases osteoblastic IL-6 secretion [3,12,13]. Interestingly, incubation of osteoblasts with conditioned media obtained from periprosthetic tissues stimulated the production of IL-6 [5]. Thus, IL-6 is produced not only in response to wear particles, but also to local soluble factors, being eventually responsible for signaling processes leading to osteolysis.

Recently, it has been reported that the use of statins is associated with a significant reduced risk of revision in patients with primary THA [14]. The beneficial effects of statins on bone formation have been reported in vitro and in vivo, including experimental models of particle-induced osteolysis [15–17]. Statins are inhibitors of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of the cholesterol synthesis pathway [15,18]. Statins exhibit pleiotropic or cholesterol-independent effects as a result of the depletion of mevalonate-derived intermediates of the sterol pathway. The blockade in the biosynthesis of these isoprenoid intermediates is largely associated with the clinical benefits of statins [19,20]. These pleiotropic effects include the modulation of proliferation, adhesion, differentiation and apoptosis of different cell types, including osteoblasts [15,16,19,20]. Interestingly, one of the most used statins, simvastatin (Simv), has shown anti-inflammatory effects mediated by decreasing IL-6 production on different cell types [21–23]. To our knowledge, there is no available information on the ability of statins to modulate IL-6 secretion upon stimulation of osteoblasts with wear particles. In this work, we hypothesized that simvastatin could modulate the osteoblastic response to micrometric Ti particles by attenuating the production of IL-6.

2. Materials and methods

2.1. Reagents

Simv, mevalonate (Mev), geranylgeranylpyrophosphate (GGPP), farnesylpyrophosphate (FPP) and squalene (Sq) were obtained from Sigma (Madrid, Spain). Geranylgeranyl transferase I (GGTaseI) inhibitor GGTI-286, farnesyl transferase (FTase) inhibitor FTI-277 and Rho kinase inhibitors, hydroxyfasudil (HF) and Y-27632, were from Calbiochem (Darmstadt, Germany). Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO) (Simv, 10 mM; GGPP, 2.5 mM; GGTI-286 10 mM; FTI-277 10 mM) or phosphate-buffered saline (PBS) (Mev, 100 mM; Y-27632 5 mM) and stored at −20 °C. Immediately before use, the compounds were diluted in incubation medium to the final test concentration.

2.2. Particles

Commercially pure Ti particles (Johnson Matthey Chemicals, Ward Hill, MA, USA) were characterized in the course of our previous works [12,24]. Seventy-five per cent of the Ti particles were <4 μm, which is the size range considered clinically relevant [25,26]. Ti particles were sterilized by incubation in isopropanol at room temperature and dried under UV light in a laminar flow hood, as previously described [24]. Immediately prior to the addition to the cells, particles were resuspended at 20 mg ml^{-1} in the appropriate culture medium, sonicated at maximum power for 10 min in a bath sonicator (Bransonic 12, Branson Ultrasonidos SAE, Barcelona, Spain) and thoroughly vortexed to obtain a homogeneous particle suspension.

2.3. Cell culture and treatments

All components for cell culture were obtained from Cambrex Bio Science (Verviers, Belgium). Cells were maintained at 37 °C under 5% CO_2 and 95% air in a humidified incubator.

Human osteosarcoma Saos-2 cells (ECACC; Salisbury, UK) were grown in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 500 IU ml^{-1} of penicillin and 0.1 mg ml^{-1} of streptomycin. Culture media were changed every 3 days until confluence was reached. For treatments, 5×10^4 cells were seeded into 24-well plates and cultured for 24 h. Then, cells were washed with PBS and supplemented with 0.5 ml of fresh medium containing or not (controls) the corresponding drug at the desired final concentration (1 μM Simv, 1 mM Mev, 20 μM Sq, 10 μM FPP, 10 μM GGPP, 10 μM FTI-277, 10 μM GGTI-286, 10 μM HF, 10 μM Y-27632). After 24 h, appropriate volumes of particle suspensions prepared in fresh media, supplemented with the corresponding tested drugs, were added to cells to achieve a dose of 50 ng cell^{-1} in a final volume of 0.5 ml. We selected this dose on the basis of our previous work studying the effects of Ti particles on human osteoblastic cells [12,27]. Cells were incubated in the presence of particles for 3 h for IL-6 mRNA measurements or for 24 h for quantification of IL-6 secretion and analysis of cell morphology. Parallel sets of control Saos-2 cells were incubated with the corresponding final concentrations of DMSO, 0.01–0.4%, in the absence of drugs and/or Ti particles. Preliminary comparison experiments with untreated and DMSO-treated cells showed that the solvent did not exert any effect on the cellular parameters evaluated (cell viability and internalization or IL-6 production).

Primary human osteoblasts (hOBs) were obtained from bone specimens aseptically collected during orthopedic surgery of the knee and cultured as previously described [24]. Each bone sample was processed in a separated primary culture and experiments were performed using cultures obtained from six independent patients (mean age 67.8 ± 2.6 years). Patients enrolled in this research gave informed consent and all procedures using human tissue designated "surgical waste" were approved by the Human Research Committee of Hospital La Paz (date of approval: 14 January 2010). hOBs were cultured in DMEM containing 15% (v/v) FBS, 500 IU ml^{-1} of penicillin and 0.1 mg ml^{-1} of streptomycin. Culture media were changed every 3 days until confluence was reached. For treatments, hOBs were seeded into 24-well plates at a density of 5×10^4 cells well^{-1} and cultured for 24 h. Then, cells were washed with PBS and supplemented with 0.5 ml of fresh medium containing or not 1 μM Simv. After 24 h, appropriate volumes of particle suspensions prepared in fresh media, supplemented or not with 1 μM Simv, were added to cells to achieve a dose of 50 ng cell^{-1} in a final volume of 0.5 ml. Cells were incubated in the presence of particles for 24 h. Parallel sets of control cells were incubated with 0.01% DMSO, in the absence of Simv and/or Ti particles. hOBs were co-cultured with TPA-differentiated THP-1 cells using a transwell insert system (Corning, Life Sciences, MA, USA) with a 0.4 μm porous membrane, thus preventing direct cell–cell contact. THP-1 cells (ECACC) were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 500 IU ml^{-1} of penicillin and 0.1 mg ml^{-1} of streptomycin. THP-1 cells were seeded into 6-well plates at a density of 3×10^5 cells well^{-1}, treated with 10 ng ml^{-1} 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) (Sigma) for 12 h, thoroughly washed with PBS and recovered in fresh medium for a further 24 h. THP-1 cells treated with TPA according this procedure express high levels of the surface antigens CD11b and CD14, characteristic of the monocyte/macrophage lineage. In addition, these cells are responsive to treatment with Ti particles, which significantly increased tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 secretion in a similar trend to that observed in human primary macrophages treated under the same experimental conditions [24]. After treatment with TPA for 12 h, THP-1 cells adhered to the bottom of the wells were washed with PBS and supplemented with 2.5 ml of a mixture of 50% RPMI and 50% DMEM, containing 12.5% (v/v) heat-inactivated FBS, 500 IU ml^{-1} of penicillin and 0.1 mg ml^{-1} of streptomycin. Parallel

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