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Simvastatin exerts antiproliferative and differentiating effects on MG63 osteoblast-like cells: Morphological and immunocytochemical study

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Background and Objective: Current evidence suggests that statins exert an anabolic effect on bone and may therefore impact on osteogenic differentiation and proliferation. These effects can be useful for their use in guided bone regeneration. The objective of this study was to determine the in vitro effects of simvastatin on the differentiation and proliferation of MG63 human osteoblast tumor cells.

Material and Methods: MG63 human osteosarcoma cells were cultured in the presence of simvastatin or solvent alone for 72 hours, and their proliferation was assessed by MTT assay. Cells from the culture were prepared for light, transmission and scanning electron microscopy studies. immunocytochemical was used to analyze the differentiation and proliferation markers Musashi-1, Ki-67, CD56 and CD44.

Results: Cultured MG63 control cells showed spheroid morphology with numerous secretion vesicles accumulated on the surface, observing no cytoplasmic projections with intercellular connections. However, cells cultured with simvastatin had a polygonal and spindle-shaped morphology, with cytoplasmic projections that interconnected cells. There were numerous microvilli-like filamentous projections on the surface with no defined pattern. At 72 hours of culture, CD56, Ki-67 and Musashi-1 expression was significantly reduced ($P < .001$) in simvastatin-treated cells. CD44 expression was intense in both groups and was not affected by simvastatin treatment.

Conclusion: MG63 cells cultured with simvastatin for 72 hours undergo morphological and surface changes. Simvastatin treatment exerts antiproliferative and differentiating effects on these cells as well as promoting recovery of cellular homeostasis.

KEYWORDS
cell differentiation, cell proliferation, electron microscopy, immunohistochemistry, osteoblasts, Simvastatin

1 | INTRODUCTION

Statins are hypolipidemic drugs that inhibit hydroxy-methyl-glutaryl co-enzyme A reductase of the mevalonate pathway, which is involved in hepatic cholesterol synthesis, and they are responsible for the production of steroid and non-steroid isoprenoids.[1] Statins, particularly simvastatin and atorvastatin, are among the most frequently prescribed pharmacological groups.[2] Besides their lipid-lowering action, they have been reported to exert pleiotropic effects[3] and to possess anti-inflammatory, immunomodulatory and antimicrobial properties

that may be useful against infections associated with bone healing.[4] Statins can activate the AKT1/PI3K pathway, leading to the inhibition of hydroxy-methyl-glutaryl co-enzyme A reductase, while statin-induced protein prenylation can have other important downstream effects, including angiogenesis (increased vascular-endothelial growth factor) and osteogenesis (increased bone morphogenetic protein-2 (BMP-2)) and reduced receptor activator of nuclear factor κB and its ligand (RANK-RANKL).[5] Statin-treated patients were found to have higher serum osteoprotegerin levels[6] and manifested anti-resorption (osteoclast inhibition), and anti-inflammatory (decrease in interleukin-6, C-reactive protein, adhesion molecules and reactive oxygen species) activities.[7] The precise effects of statins can vary according to their type and concentration.[8] The use of local statins as coadjuvants alongside scaling and root planing were reported to improve clinical periodontal parameters and recover bone crest height.[9] Experimental studies of implants in rodents found that simvastatin administration improved the bone contact ratio, bone density and osseointegration,[10,11] which was also found to be enhanced by topical fluvastatin application around implants.[12] In contrast, other authors found no increase in bone density in statin-treated defects,[13] and a recent study reported that simvastatin loading of implant surfaces exerted significant effects for only 2 weeks.[14] Formation of new bone tissue was observed in calvaria of rats after the topical injection of fluvastatin using tricalcium phosphate as carrier.[15] However, high doses of local statin can cause inflammation when used for bone regeneration, as reported by two studies in which simvastatin was applied to calvarial defects in rodents.[13,16] Simvastatin was found to affect osteogenic differentiation in a murine model,[17] while an in vitro study showed that exposure to this drug slightly increased osteoblast expression of osteocalcin, osteoprotegerin, alkaline phosphatase and other bone markers.[18]

The objective of the present study was to determine the in vitro effects of simvastatin on the differentiation and proliferation of the MG63 human tumor osteoblast cell line.

2 | MATERIAL AND METHODS

All procedures in this study were performed in accordance with the 1964 Helsinki declaration and its later amendments. The study was approved by the Ethics Committee of the School of Dentistry of the University of Granada (reference: FOD/UGR/08/2016).

2.1 | Cell line

MG-63 human osteosarcoma cell lines were obtained from the University of Granada Scientific Instrumentation Centre (Spain). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% non-essential amino acid solution in a humidified incubator at 37°C, using a standard mixture of 95% air and 5% CO_2. Cultured cell monolayers were detached with a trypsin-ethylenediaminetetraacetic acid solution (.25%) and seeded into 24-well plates at a density of 15×10^3 cells per well or into T75 flasks (Nunc, Rochester, NY, USA) at a density of 500×10^3 cells per flask, depending on the experiment.

2.2 | Materials

Simvastatin and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich LLC (St. Louis, MO, USA). Simvastatin was resuspended at a concentration of 20 mg/mL in DMSO and stored at −20°C.

2.3 | Proliferation assay

After incubation for 24 hours under culture conditions, cells in the 24-well plates were incubated with simvastatin (.005-25 μmol/L) for 72 hours. The cytotoxicity of this treatment was then evaluated in triplicate by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay, adding 20 μL MTT solution in cell culture medium (5 mg/mL) to each well. After incubation for 4 hours at this temperature, the culture medium was removed and the resulting formazan crystals were dissolved in 200 μL DMSO. The optical density (OD) of the converted dye, which is proportional to the number of viable cells, was measured at 570 nm (with subtraction of background at 690 nm) using a Titertek multisKan colorimeter (Flow Laboratories, Irvine, CA, USA). The percentage of surviving cells was calculated as: (OD treated cells/OD control [untreated] cells)×100. The same procedure was applied to control cells.

2.4 | Simvastatin culture

MG-63 cells were seeded in T75 flasks and grown in the presence of simvastatin for 72 hours at a final concentration of .01 μmol/L (the highest concentration with no effect on cell proliferation after 72 hours of treatment) or in the presence of the same amount of DMSO as control. Cells were then removed from the culture and prepared for microscopic and immunocytochemical studies.

2.5 | Transmission electron microscopy study

Several pellets of treated and control MG63 cells were fixed in 2.5% glutaraldehyde solution and then postfixed in 1% OsO_4 at 4°C for 2 hours, washed in distilled water, dehydrated in increasing concentrations of acetone and embedded in Epon following a conventional protocol. Semithin sections were stained with toluidine blue solution. Ultrathin (~70 nm thick) sections obtained using a Reichert Jung ULTRACUT ultramicrotome (Leica, Wetzlar, Germany) were stained with lead citrate and uranyl acetate and under a Libra 120 Plus transmission electron microscopy (TEM; Zeiss, Oberkochen, Germany).

2.6 | Scanning electron microscopy study

Samples from both groups of cultured MG63 cells were gently removed and immediately immersed in a sodium cacodylate-buffered

formaldehyde-glutaraldehyde fixative for 24 hours at room temperature and post-fixed in 20% osmium tetroxide for 2 hours. Samples were then dehydrated by serial transfer in ascending concentrations of acetone (50%-100%) and infiltrated with liquid carbon dioxide before the critical drying point. Finally, samples were made electrically conductive by mounting them on aluminum slabs with a silver point, followed by sputter coating with gold/palladium to a thickness of approximately 250 Å. After attachment of these specimens to an acrylic plate with glue tape, the plate was vertically divided into two pieces using a diamond disc with chisel and hammer, followed by examination of their cut surfaces with a Quanta 400 SEM (FEI, Hillsboro, OR, USA) at 5-10 kV.

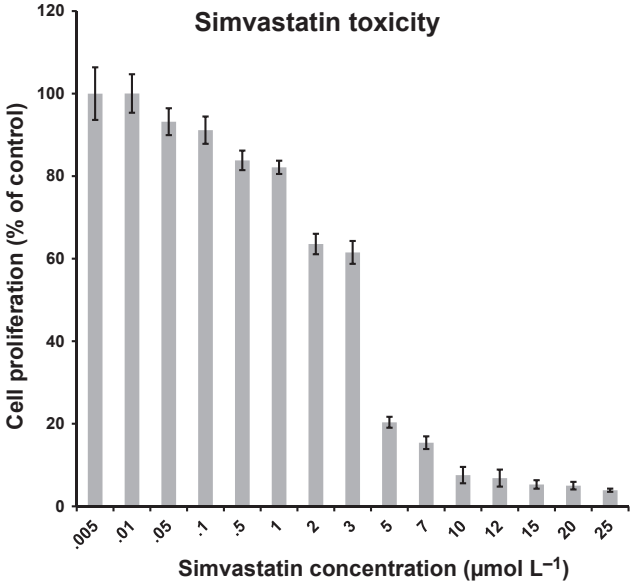

FIGURE 1 In vitro toxicity of simvastatin in MG63 cell line. Growth of MG63 cell was evaluated after 72 h of exposure to a wide range of simvastatin concentrations (.005-25 μmol/L) in comparison to control cells treated with solvent alone. Data expressed as the mean value±SD of triplicate cultures

2.7 | Immunocytochemical analysis

After detachment from the plastic substrate, MG63 osteosarcoma cells from both groups were centrifuged at 302 *g* for 3 minutes in phosphate buffer solution, washed, resuspended in phosphate-buffered saline and centrifuged at 7393 *g* for 30 seconds before embedding the resulting pellets in paraffin. Paraffin-embedded sections were dewaxed, hydrated and heat-treated in 1 mm ethylenediaminetetraacetic acid (pH 8) for antigenic unmasking in an antigen retrieval PT module (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 95°C for 20 minutes. Sections were incubated for 16 hours at 4°C with prediluted polyclonal antibody against Musashi-1 (mesenchymal stem cells, polyclonal; Sigma-Aldrich LLC) and runt related transcription factor 2 (Runx2, clone M-70) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) both at 1:100 dilution, and with prediluted monoclonal antibodies against Ki-67 (proliferative cells, clone SP-6), CD56 (osteoblast differentiation, clone 56C04) and CD44 (osteocyte cells, clone 156-3C11); sections were then incubated for 10 minutes at room temperature to analyze the cell differentiation and antiproliferative response. The immunocytochemical study was conducted using the micropolymer-peroxidase-based method (Master Polymer) with automatic immunostainer (Autostainer 480; Thermo Fisher Scientific Inc., Waltham, MA, USA) followed by development with diaminobenzidine (other monoclonal antibodies and reagents were obtained from Master Diagnóstica, Granada, Spain). Appropriate positive (tonsil) and negative (non-immune serum) controls were run concurrently. Hematoxylin was used for nuclear counterstaining. Results were expressed as percentages of positive cells for each antibody, counting 200 cells per high-magnification field (40× objective) in three independent experiments.

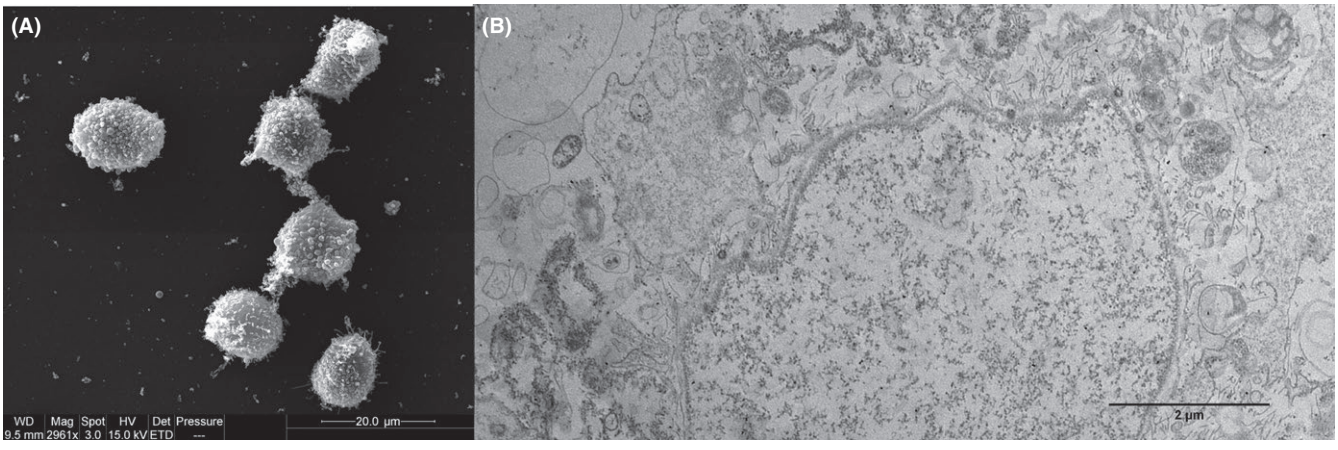

FIGURE 2 Ultrastructural study with scanning and transmission electron microscopy. (A) Spheroid morphology and numerous secretion vesicle accumulations in control MG63 cells. No intercellular-connecting cytoplasmic projections were observed (scale bar in figure). (B) Control cells treated with dimethyl sulfoxide show a clear low-density cytoplasm with appreciable decrease in intracytoplasmic organelles, containing numerous free, unstructured filaments and a large number of lysosomal bodies incorporating abundant phagocytic material

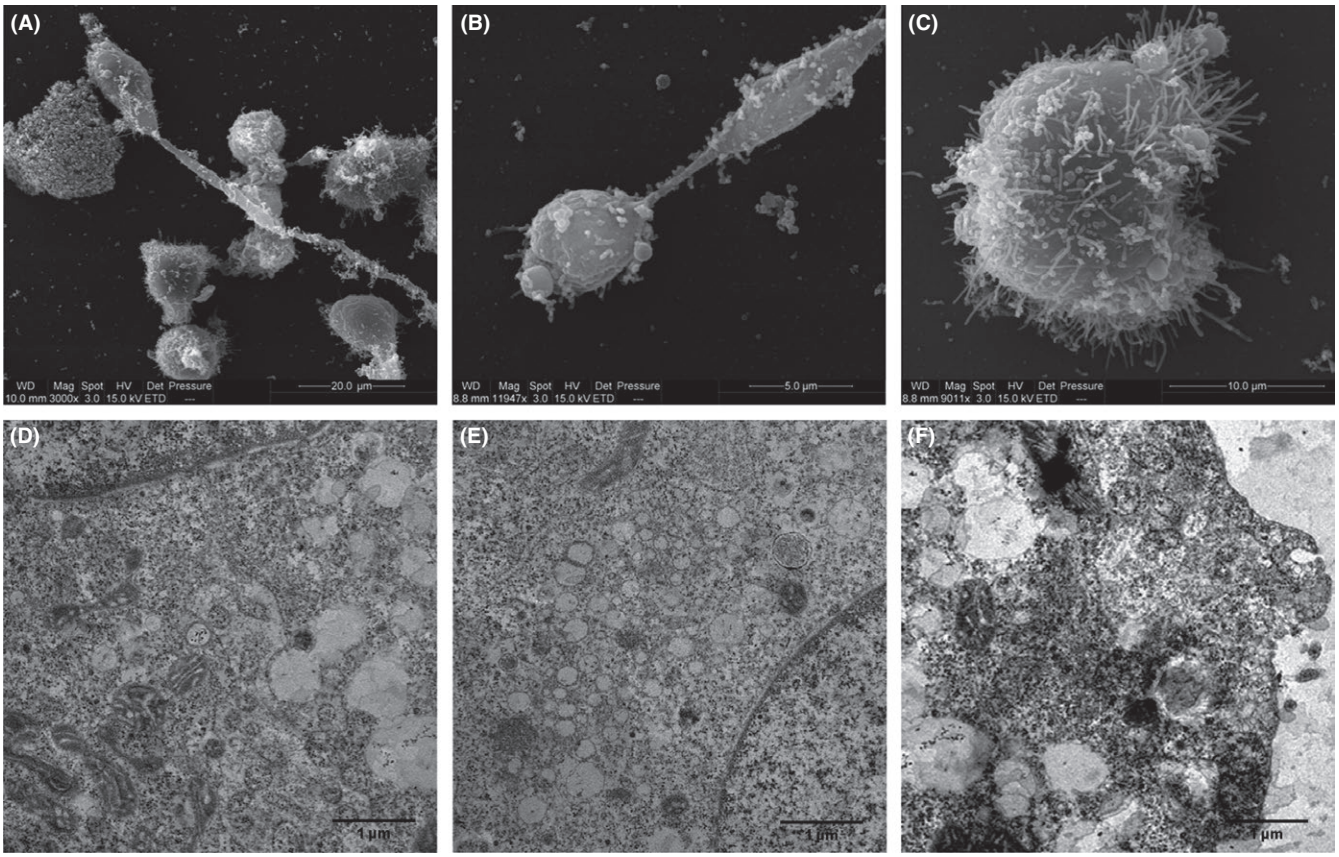

FIGURE 3 Ultrastructural study with scanning and transmission electron microscopy. (A) Morphological heterogeneity (spheroid, polygonal and spindle-shaped) of MG63 cells in cultures treated with simvastatin. (B) Spindle-shaped cell with long projection that makes contact with spheroid cell in the simvastatin-treated culture. (C) Spheroid cell with presence of numerous "microvilli-like" filamentous projections distributed homogeneously over its surface with no defined pattern (scale bar in figures). (D) Simvastatin-treated MG63 cells showing dense cytoplasm with large amount of mitochondria alongside free ribosomes and lysosomes. (E) Significant increase in the accumulation of lipid vacuoles of varied size in the cytoplasm. (F) Microphotograph showing organization of the "clathrin-type" subcellular microvesicular system

2.8 | Statistical analysis

SPSS v. 20.0 (IBM SPSS, Armonk, NY, USA) was used for the statistical analysis. A non-parametric test (Mann-Whitney *U*-test) was applied to compare positive cells between control and treated cells. $P < .05$ was considered significant.

3 | RESULTS

3.1 | Cell viability test

After 72 hours of exposure to simvastatin, the highest dose with no effect on cell proliferation was .01 µmol/L. At higher doses, the proliferative capacity of these cells began to decrease in comparison to control cells (Figure 1).

3.2 | Electron microscopy

TEM study of cultured MG63 cells revealed a similar morphology to that reported by Fernández-Barbero et al.[19] Control cells showed a clear low-density cytoplasm with an appreciable reduction in intracytoplasmic organelles, numerous unstructured free filaments in the cytoplasm and many lysosomal bodies containing abundant phagocytic material (Figure 2). In contrast, simvastatin-treated MG63 cells showed a high density of cytoplasmic organelles, with a large amount of mitochondria and numerous free ribosomes and lysosomal bodies (Figure 3). There was an appreciable increase in lipid vacuoles underlying the cell cytoplasmic membrane, which were grouped, fused and intermixed with smaller vacuoles (Figure 3) and structured vesicular invaginations. The cytoplasmic membrane contained numerous thin projections (see also SEM observations above).

SEM study also showed the morphology of cultured MG63 cells to be similar to that reported by Fernández-Barbero et al.[19] The morphology of control MG63 cells was spheroid, with a large accumulation of secretion vesicles on their surface but no cell-interconnecting cytoplasmic projections (Figure 2). Cells treated with simvastatin were spheroid, polygonal or spindle-shaped (Figure 3), and the spindle-shaped cells had long cytoplasmic projections interconnecting cells (Figure 3). Numerous "microvilli-like" filamentous projections were also distributed over the whole surface of the cells, particularly those with spheroid morphology, but they generally formed no defined pattern (Figure 3).

TABLE 1 Percentage immunocytochemical marker expressions after 72 h of culture

	Control	Simvastatin	*P*-value
CD44 (%)	99.6 ± .78	100 ± .0	NS
CD56 (%)	34.3 ± 2.75	11.0 ± 3.68	<.001*
Ki-67 (%)	87.6 ± 4.86	33.0 ± 5.31	<.001*
Msi1 (%)	97.6 ± 1.69	16.6 ± 3.31	<.001*
Runx2 (%)	.5 ± .54	.5 ± .54	NS

NS, non-significant.Values expressed as mean percentage±SD of three independent experiments.
*Control vs simvastatin, Mann-Whitney *U*-test.

3.3 | Immunocytochemical results

Cytoplasmic membrane expression of CD44 was intense in all MG63 cells and was not modified by statin treatment. After 72 hours of treatment, the CD56, Msi1 and Ki-67 expression of MG63 cells was significantly ($P < .001$, Mann-Whitney *U*-test) decreased in comparison to control cells (Table 1, Figure 4). No nuclear Runx2 expression was detected in MG63 cells.

4 | DISCUSSION

Ultrastructural and immunocytochemical changes observed in this in vitro study suggest that simvastatin exerts differentiating and antiproliferative effects on the MG63 cell line.

Ultramicroscopic study of control MG63 cells revealed groups of cells with cytoplasmic elements characteristic of elevated cell activity and groups with high cytoplasmic destructuration, with a "cytoplasmic void" showing numerous destructured microfilaments and numerous lysosomal bodies containing cell material from different organelles. The abundant apoptotic phenomena observed in TEM images and the high expression of the proliferation marker Ki-67 indicate the presence of major stress in these cells. This cytoplasmic destructuration in MG63 cells was morphologically reverted by simvastatin treatment, which significantly reduced the number of cytoplasmic vacuoles containing cell material and increased the vesicular turnover at cytoplasm membrane level, with the emergence of structured vesicular invaginations and the acquisition of a normal cytoplasmic conformation. According to these results, simvastatin may act against cell stress, stabilizing osteogenic cell renewal processes. In the context of bone production, preservation and remodeling, simvastatin may therefore function as a modulator of osteoclastogenesis/osteogenesis.

TEM findings of a large accumulation of lipid vacuoles in simvastatin-treated MG63 cells would result from statin-induced lipoprotein deprivation of the medium, which in the present case represents physiological normalization through recovery of an adequate lipid cell metabolism. This interpretation is supported by SEM observations of numerous pinocytotic vesicles and abundant microvilli on the surface of treated cells. Simvastatin was previously reported to produce an accumulation of cytosolic lipid droplets in both non-malignant and malignant cells, which may contribute to their antiproliferative effects.[20] In contrast, Martinet et al found little cytoplasm vacuolization of mesenchymal stem cells after treatment with .01 μmol/L

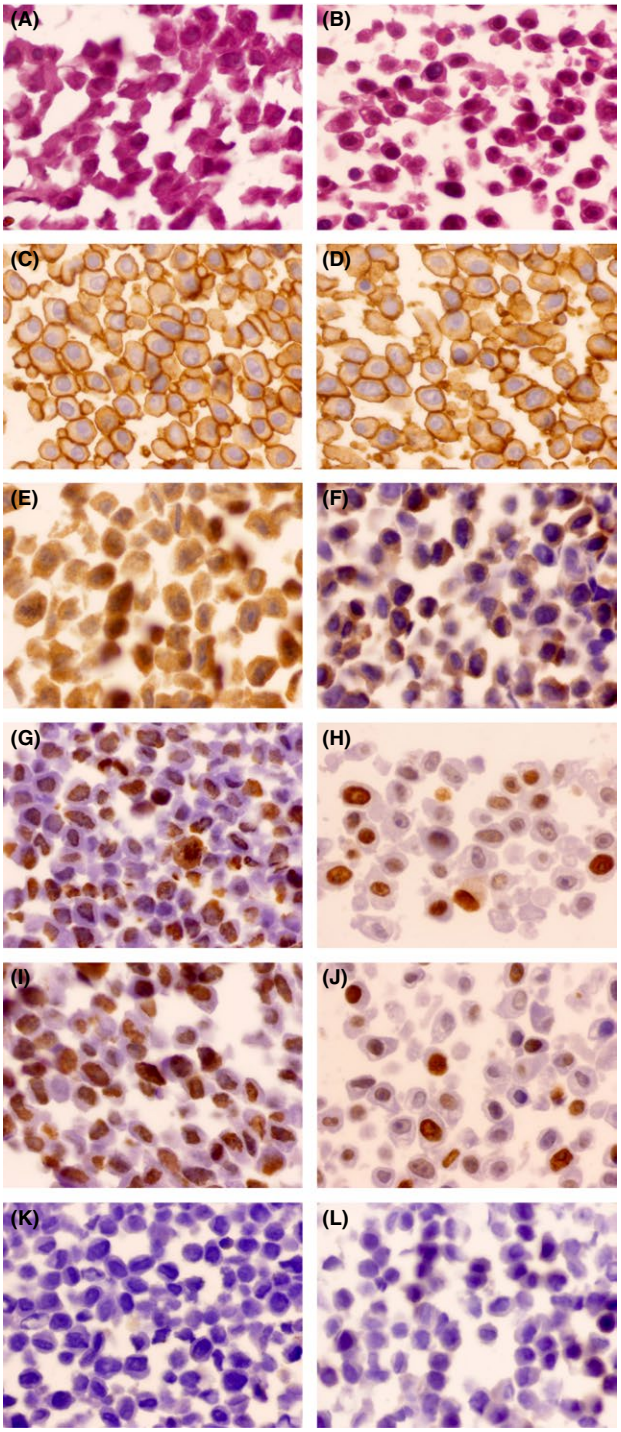

FIGURE 4 Morphological and immunocytochemical study. (A) and (B) Increase in cytoplasmic vacuolization and apoptotic cells in simvastatin-treated MG63 cells. (C) and (D) No modification of CD44 expression after incubation with simvastatin for 72 h; (E) and (F) reduction in CD56 expression; (G) and (H) reduction in nuclear expression of Ki-67 (cell proliferation); (I) and (J) reduction in nuclear expression of MSi-1; (K) and (L) no induction of Runx2 expression (original magnification ×40)

simvastatin,[21] which may be attributable to the difference in baseline differentiation between mesenchymal stem cells and MG63 cells, which have already acquired some degree of osteogenic differentiation. The previously documented absence of Runx2 expression suggests that the mechanism underlying osteogenic differentiation in this cell line is independent of the expression of this transcription factor.[22] These data indicate that the effectiveness of simvastatin may depend upon the initiation of osteogenic differentiation by target cells; in other words, the action of simvastatin may be dependent on the degree of differentiation.

Immunocytochemical analysis showed that simvastatin treatment had no significant effect on the CD44 expression of MG63 cells but significantly reduced their expression of CD56, Musashi-1 and Ki-67 (cell proliferation index). No Runx2 expression was detected in either treated or control cells. Given that CD56 and Musashi-1 expression is characteristic of stem cells and those with a low degree of differentiation,[23,24] these results suggest that simvastatin treatment increases the differentiation degree of MG63 cells, which is consistent with the significant reduction in Ki-67 expression (proliferation-differentiation balance).[25] This mechanism may be dependent on the inhibition of geranylgeranyl pyrophosphate synthase or other upstream isoprenoids.[26] The response of Musashi-1 can be modulated by environmental factors and can regulate physiological or pathological cell renewal.[27] In vivo and in vitro studies have linked high CD56 expression to worse tumor cell behavior in comparison to low or negative expression.[28] Regarding bone tissue, CD56-positive myeloma cells have been linked to the presence of lytic bone lesions.[29] Musashi-1 may play a possible role in osteogenic differentiation, downregulating the Wnt1 pathway and expression of the miR-148 family, because Musashi-1 knockdown was found to increase expression of these two pathways, which are involved in the osteogenic differentiation of stem cells.[30] This reduction in Musashi-1 expression is also compatible with the osteoarticular tissue expression of Musashi-1, reported for the first time by our group, suggesting a possible involvement of this factor in tissue regeneration.[31]

The effects of statins on bone metabolism were first reported by Mundy et al in an in vitro animal model, which revealed higher BMP-2 expression in cultured osteoblast cells and increased medullar bone formation in rodents.[32] Later investigations related statin consumption to a higher bone density and lower incidence of fractures in patients with osteoporosis, although a meta-analysis was unable to confirm this association.[33] Statins were found to favor bone regeneration in an animal model of periodontitis, increasing BMP-2 and reducing RANK-RANKL,[5] while a clinical study by our group observed higher osteoprotegerin levels in simvastatin-treated patients.[6] Statin consumption was recently associated with lower tooth loss over time in a prospective epidemiologic study with 5 year follow-up in a European population.[34] Research is ongoing into the optimal statin concentrations to achieve anti-inflammatory and bone anabolic effects in the local treatment of periodontal lesions; various carriers and drug delivery systems have been used, including the application of statins as bioactive agents on implant surfaces.[35-37]

The proliferation and cell functions exhibited by osteosarcoma cells may not be fully representative of those in human primary osteoblasts, given reported differences between them.[38] Nevertheless, the MG63 cell line has been widely used as an in vitro model for bone research, and findings on the proliferation of these cells have proven comparable to observations in primary human osteoblasts.[39]

Finally, the immunocytochemical and ultramicroscopic results obtained suggest that simvastatin treatment of osteogenic cells may provide a pathway for recovery of the normal morphological and functional status of bone tissue, besides enhancing the differentiation degree of the cells and reducing their proliferation. Further research is warranted to explore the therapeutic potential of this novel approach in periodontal bone preservation and remodeling.

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CONFLICT OF INTEREST

The authors report no conflicts of interest related to this study.

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