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Simvastatin coating of TiO_2 scaffold induces osteogenic differentiation of human adipose tissue-derived mesenchymal stem cells

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ABSTRACT

Bone tissue engineering requires an osteoconductive scaffold, multipotent cells with regenerative capacity and bioactive molecules. In this study we investigated the osteogenic differentiation of human adipose tissue-derived mesenchymal stem cells (hAD-MSCs) on titanium dioxide (TiO_2) scaffold coated with alginate hydrogel containing various concentrations of simvastatin (SIM). The mRNA expression of osteoblast-related genes such as collagen type I alpha 1 (*COL1A1*), alkaline phosphatase (*ALPL*), osteopontin (*SPP1*), osteocalcin (*BGLAP*) and vascular endothelial growth factor A (*VEGFA*) was enhanced in hAD-MSCs cultured on scaffolds with SIM in comparison to scaffolds without SIM. Furthermore, the secretion of osteoprotegerin (OPG), vascular endothelial growth factor A (VEGFA), osteopontin (OPN) and osteocalcin (OC) to the cell culture medium was higher from hAD-MSCs cultured on scaffolds with SIM compared to scaffolds without SIM. The TiO_2 scaffold coated with alginate hydrogel containing SIM promote osteogenic differentiation of hAD-MSCs *in vitro*, and demonstrate feasibility as scaffold for hAD-MSC based bone tissue engineering.

1. Introduction

The use of autologous stem cells has been exploited as a strategy to facilitate healing of bone defects resulting from trauma, disease, surgery or congenital malformations [1]. Among the different cell-types investigated are stem cells from adipose tissue, which appear to be a promising candidate for osseous reconstruction [2–4]. The abundant and easily available hAD-MSCs [5,6] can undergo rapid and efficient osteogenic differentiation *in vitro* [7]. However, undifferentiated hAD-MSCs do not undergo osteogenic differentiation *in vivo* without inductive stimuli [8,9]. Over the past years SIM, widely used to treat hyperlipidemia and arteriosclerosis [10], has gained interest as an appealing candidate for bone tissue engineering because of its osteogenetic properties [11]. Furthermore, SIM has been shown to induce osteogenic differentiation of hAD-MSCs when cultured in a standard two-dimensional culture environment [12]. However, for reconstruction of large-size bone defects a three-dimensional scaffold is needed to support the healing [13]. Several types of synthetic scaffolds have been tested to provide the needed support, but scaffolds lacking osteoinductive signals may not be effective in inducing bone regeneration following hAD-MSC transplantation [14,15]. The scaffolds should therefore be engineered to provide biochemical cues for promoting stem cell-mediated osteogenesis as well as proper physical and mechanical microenvironments. Modification of scaffolds with osteoinductive molecules can compensate for the lack of osteogenic properties. Recently, the development of porous and well interconnected ceramic TiO_2 scaffold coated with alginate hydrogel containing SIM has been described [16]. The TiO_2 scaffold has a high mechanical strength [17] and has been reported as a candidate for use in large-size bone defects [18,19].

The aim of this study was to evaluate the cell viability and osteogenic potential of hAD-MSCs seeded on TiO_2 scaffold coated with alginate hydrogel containing SIM.

Abbreviations: hAD-MSCs, human adipose tissue-derived mesenchymal stem cells; TiO_2, titanium dioxide; SIM, simvastatin; OPN, osteopontin protein; *SPP1*, osteopontin mRNA; OPG, osteoprotegerin protein; *TNFRSF11B*, osteoprotegerin mRNA; OC, osteocalcin protein; *BGLAP*, osteocalcin mRNA; VEGFA, vascular endothelial growth factor A protein; *VEGFA*, vascular endothelial growth factor A mRNA; LDH, lactate dehydrogenase; ALP, alkaline phosphatase protein; *ALPL*, alkaline phosphatase mRNA; qRT-PCR, quantitative RT-PCR.

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2. Materials and methods

2.1. Production of TiO_2 scaffolds coated with alginate hydrogel containing SIM

Porous TiO_2 scaffolds, 8 mm in height and a diameter of 9 mm, were produced as previously described [20]. The scaffolds were subsequently sterilized by autoclaving.

SIM (Krebs Biochemicals & Industries, Andhra Pradesh, India) was dissolved in 100% ethanol to 10 mM before being added to 2% (w/v) Pronova UP LVG sodium alginate (FMC BioPolymer, Sandvika, Norway) in milliQ water at desired concentrations (10 μM, 1 μM, 0.1 μM, and 10 nM) to create TiO_2 scaffolds coated with alginate hydrogel containing SIM as described previously [16]. The scaffolds for the control group were coated with alginate hydrogel without SIM.

2.2. Cell culture and seeding of hAD-MSCs

The hAD-MSCs were isolated from liposuction material from abdominal regions of three healthy female donors (ages 36, 50, 61 years). The donors provided informed consent, and the collection and storage of adipose tissue and hAD-MSCs were approved by the regional committee for ethics in medical research. The isolation and *in vitro* expansion of hAD-MSCs were carried out as previously described [21]. The hAD-MSCs were cultured in culture medium consisting of Dulbecco's modified Eagle's medium/F12 (Gibco/BRL, Carlsbad, CA, USA) supplemented with 10% human platelet lysate plasma, 1000 IU/ml heparin (Wockhardt, Wrexham, UK) and 1% penicillin, streptomycin and amphotericin B. Cells were subcultured at confluence and expanded for 4–5 passages.

Cell seeding on alginate-coated scaffolds with or without SIM was performed using an agitated seeding method in order to ensure a homogenous cell distribution throughout the scaffolds [22]. Scaffolds pre-soaked with culture medium were placed in 48-well plates, and 1 ml cell suspension was added drop-wise on the top of the scaffolds at a density of 4×10^5 cells/ml. After seeding, the plates were agitated on an orbital shaker at 200 rpm for 6 h at 37 °C. Following agitation the cell-seeded scaffolds were transferred to new culture plates in 1 ml culture medium and maintained at 37 °C in a humidified atmosphere of 5% CO_2 for up to 21 days. Cells from one donor (in triplicate) were used for the initial cytotoxicity study of SIM at different concentrations (10 μM, 1 μM, 0.1 μM, and 10 nM). Cells from three individual donors were used for all subsequent studies. Triplicates of each donor, each treatment, and for three harvest time points were included, giving a total of 54 scaffolds. The culture medium was changed every second day and collected for analyses. Scaffolds were harvested after 7, 14 and 21 days of culture for use in qRT-PCR.

2.3. Determination of LDH activity in culture medium

The culture medium was collected every second day up till 14 days, and the cytotoxic effect of SIM containing scaffolds was evaluated based on the LDH activity. The LDH activity was analyzed with a cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The absorbance was measured at 492 nm in a plate reader (Biochrom Asys Expert 96 Microplate Reader, Biochrom, Holliston, MA, USA).

2.4. RNA isolation and qRT-PCR analysis

Total RNA was isolated by the Qiagen RNA mini-kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. The cDNA was synthesized with RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) using random primers. QRT-PCR was performed in the Applied Biosystems 7300 Real-Time System (Life Technologies, Paisley, UK) with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems, Paisley, UK). QRT-PCR was done for *GAPDH*, *RUNX2*, *SOX9*, *PPARG*, *COL1A1*, *SPP1*, *TNFRSF11B*, *ALPL*, *BGLAP* and *VEGFA*. List of probes used in qRT-PCR is provided in Table 1. QRT-PCR analysis was performed in duplicate for SIM 10 nM and control groups at days 7, 14 and 21. Relative mRNA levels were calculated by the comparative CT method [23].

Table 1
List of probes used in qRT-PCR.

Gene symbol	Gene name	TaqMan assay No.
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
RUNX2	Runt-related transcription factor 2	H_s00231692_m1
SOX9	SRY (sex determining region Y)-box 9	H_s00165814_m1
PPARG	Peroxisome proliferator-activated receptor gamma	Hs01115513_m1
COL1A1	Collagen type I alpha 1	H_s00164004_m1
SPP1	Osteopontin	Hs00959010_m1
TNFRSF11B	Osteoprotegerin	Hs00900360_m1
ALPL	Alkaline phosphatase	Hs00758162_m1
BGLAP	Osteocalcin	Hs01587814_g1
VEGFA	Vascular endothelial growth factor A	Hs00900055_m1

2.5. Quantification of secreted proteins

Multianalyte profiling of protein levels in the culture medium was performed on the Luminex 200 system (Luminex, Austin, TX, USA) employing xMAP technology. Acquired fluorescence data was analyzed by the xPONENT 3.1 software (Luminex, Austin, TX, USA). The amount of OPN, OPG, VEGFA and OC in the culture medium after 2, 4, 8, 14 and 21 days incubation was measured using the human bone panel and human cytokine/chemokine kits (Millipore, Billerica, MA, USA). All analyses were performed according to the manufacturer's protocols.

2.6. Determination of ALP activity in culture medium

The ability of ALP to hydrolyze P-nitrophenyl phosphate substrates (Sigma–Aldrich, St. Louis, MO, USA) into the yellow end-product, p-nitrophenol, was used to quantify the ALP activity in the culture medium after 2, 8, 14 and 21 days. The absorbance was measured at 405 nm and the ALP activity was quantified using a standard curve based on calf intestinal ALP (Promega, Madison, WI, USA).

2.7. Staining of DNA and ALP

After 1 and 14 days of culture, scaffolds were cut in half and fixed in 4% PFA/4.6% D-Mannitol for 15 min and subsequently stored in 1% PFA/4.6% D-Mannitol until further processing. Attachment and distribution of cells in scaffolds coated with different concentrations of SIM was evaluated after 1 day of culture by staining the DNA using DAPI at a concentration of 5 mg/ml. The fluorescence was assessed using a Nikon Eclipse E-600 fluorescence microscope equipped with a Color View III digital camera controlled by Cell-B software (Olympus, Center Valley, PA).

The presence of ALP positive cells was assessed in scaffolds after 14 days of culture. Fixed scaffolds were submitted to heat induced epitope retrieval by heating to 95 °C in citrate buffer (pH 6) for

15 min, incubated with monoclonal mouse anti-human ALP antibody (Abcam, Cambridge, UK) diluted to 4 μg/ml in 1.25% BSA in PBS with 0.2% Triton X for overnight at 4 °C, followed by incubation for 30 min at room temperature in goat anti-mouse IgG (Life Technologies, Paisley, UK) diluted in 2.5% BSA/0.05% Tween-20/PBS at a concentration of 2 mg/ml. Cell-seeded whole mount stained scaffolds were counterstained with DAPI, placed on a coverslip and covered with Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Confocal microscopy was performed on a FluoView 1000 (Olympus, Center Valley, PA, USA). Images were analyzed using Image J (NIH, Bethesda, MD, USA).

2.8. Statistics

The data obtained by cytotoxicity, ALP activity, gene expression and protein secretion analyses was compared groupwise by using Holm-Sidak test following a parametric one way ANOVA. Whenever the equal variance and/or the normality test failed, a Kruskal–Wallis one way ANOVA on ranks was performed (Sigma Plot 12.0, Systat Software, San Jose, CA, USA). A probability of $\leqslant 0.05$ was considered significant.

3. Results and discussion

3.1. Cytocompatibility

The distribution of hAD-MSCs and the cytotoxic effect of SIM from alginate-coated scaffolds were tested for a wide range of concentrations (10 μM, 1 μM, 0.1 μM, and 10 nM). The highest number of evenly distributed hAD-MSCs was observed on scaffolds with 10 nM SIM (Fig. 1). SIM was found to be significantly cytotoxic for hAD-MSCs at concentrations above 0.1 μM when cells were seeded on the scaffolds (data not shown). A prolonged 14-day cytotoxicity study of scaffolds with 10 nM SIM did not reveal any cytotoxic effects to hAD-MSCs compared to control (Fig. 1). It was previously shown that concentrations up to 1 μM SIM did not induce cytotoxic response in both human bone marrow-derived stem cells [24] and hAD-MSCs [12]. The previous results were, however, obtained using a two-dimensional cell culture system where the cells were exposed to SIM by overlaid culture medium. In contrast, in the present study hAD-MSCs were cultured within porous three-dimensional scaffolds in direct contact with SIM containing alginate.

3.2. Osteogenic effect of SIM coating

The effect of coating TiO_2 scaffolds with SIM containing alginate was investigated in hAD-MSCs by quantification of mRNA expression and protein secretion of osteoblast-related genes.

3.2.1. QRT-PCR analysis

No significant differences were observed in the expression of the transcription factors *RUNX2*, *SOX9* and *PPARG* mRNA levels after 7, 14 and 21 days of culture (Fig. 2). *RUNX2*, *SOX9* and *PPARG* are considered to be the earliest markers of the osteo-, chondro- and adipogenic lineages [25–27], respectively. Hence, if SIM influences the expression of these genes, a change in gene expression may have taken place before day 7, the earliest time point evaluated

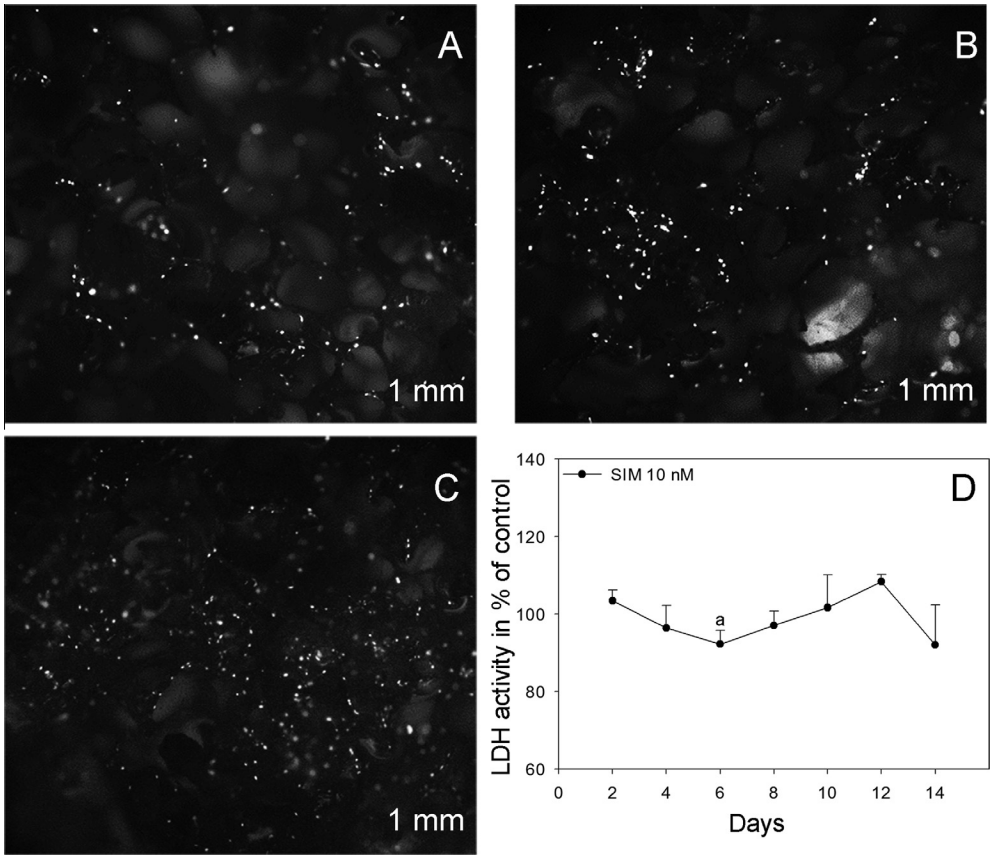

Fig. 1. Distribution of attached hAD-MSCs after 1 day of culture on alginate-coated scaffolds with 1 μM SIM (A), 0.1 μM SIM (B) and 10 nM SIM (C). Cells are visualized by fluorescence microscopy showing nuclei stained with DAPI. The images are representative for the respective groups. LDH activity in culture medium from scaffolds with 10 nM SIM is presented in percentage of control at the same time points. Values represent the mean of three donors (three parallels per donor) +SD (D). Statistical analysis: (a) $p \leqslant 0.05$ versus alginate-coated scaffold without SIM.

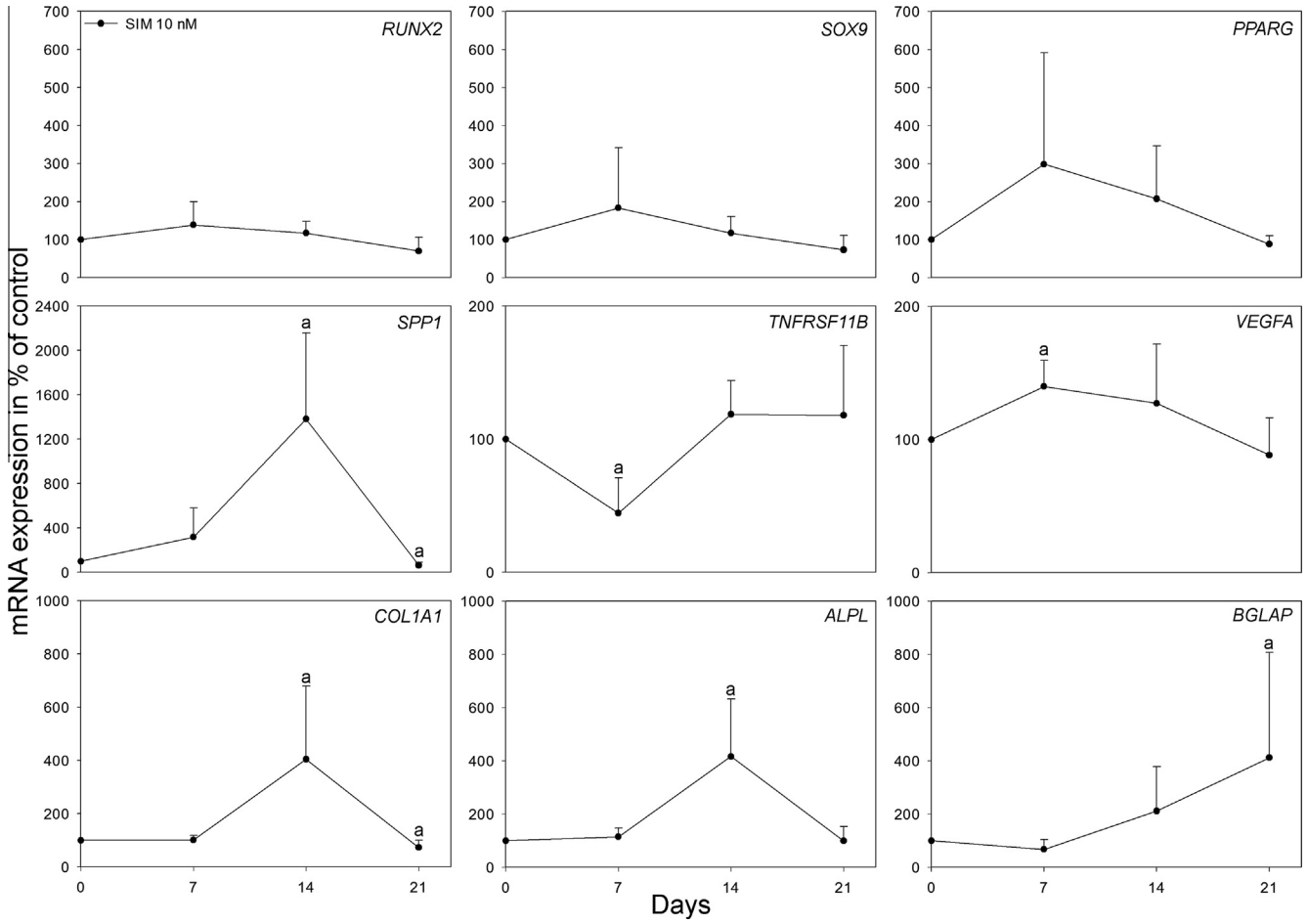

Fig. 2. Relative mRNA expression levels for *RUNX2, SOX9, PPARG, COL1A1, SPP1, TNFRSF11B, ALPL, VEGFA* and *BGLAP* in hAD-MSCs cultured on scaffolds with 10 nM SIM is presented in percentage of control, and normalized to reference gene *GAPDH* at 7, 14 and 21 days. Values represent the mean of three donors (one parallel per donor) +SD. Statistical analysis: (a) $p \leqslant 0.05$ versus alginate-coated scaffold without SIM.

here. *RUNX2* is known to be an osteoblast-specific transcription factor that is essential for differentiation of osteoblasts and formation of bone [28]. Although no significant changes were found in the expression of *RUNX2*, other markers associated with the osteogenic differentiation were up-regulated. After 14 days of culture, the relative expression of *COL1A1*, marker for preosteoblasts or early undifferentiated osteoblast-like cells [29,30], was significantly increased in hAD-MSCs on scaffolds with 10 nM SIM compared to control ($p = 0.029$) (Fig. 2). Collagen type 1 synthesis is known to be a prerequisite for extracellular matrix formation and mineralization in bone [31]. The ability of collagen type 1 to further support osteoblast differentiation was demonstrated by maintenance of the developmental expression of the bone cell phenotype in hAD-MSCs. Also the relative expression of *SPP1* and *ALPL* was significantly enhanced in hAD-MSCs on scaffolds with 10 nM SIM compared to control after 14 days of culture ($p = 0.029$ and $p = 0.029$) (Fig. 2). This also indicates osteogenic differentiation, as the expression of both *ALPL* and *SPP1* increases when hAD-MSCs differentiate into mature osteoblasts [32]. After 21 days of culture the expression levels of *COL1A1, SPP1* and *ALPL* in hAD-MSCs on scaffolds with 10 nM SIM were all at a level similar to the expression in the control group. However, the relative expression of *BGLAP* was increasing from day 7 forth and was significantly higher expressed in hAD-MSCs on scaffolds with 10 nM SIM compared to control ($p = 0.002$) at day 21 (Fig. 2). The enhanced *BGLAP* expression in hAD-MSCs on alginate-coated scaffolds with SIM may indicate terminal differentiation of osteoblasts [29]. Furthermore, the relative expression of *VEGFA* was significantly enhanced after 7 days of culture in hAD-MSCs on scaffolds with 10 nM SIM compared to control ($p = 0.002$). It has been shown that elevated *VEGFA* expression demonstrates osteogenic differentiation of MSCs [33].

Differentiation and maturation of osteoblasts are characterized by a temporary up-regulation of *COL1A1* and *ALPL* combined with a sustained up-regulation of *BGLAP* [29]. Accordingly, the gene expression profile indicates osteogenic differentiation of hAD-MSCs seeded on scaffolds coated with alginate hydrogel containing 10 nM SIM. These results concur with the previous report demonstrating up-regulation of osteogenesis-related gene expression in hAD-MSCs stimulated with SIM [12].

3.2.2. Quantification of proteins

Osteogenesis-related proteins were quantified in the culture medium to further evaluate the osteogenic response of the hAD-MSCs. A significantly higher amount of OPN was detected in the medium from hAD-MSCs on scaffolds with 10 nM SIM compared to control at 14 days of culture ($p = 0.034$) (Fig. 3). OPN is abundantly secreted by MSCs [34] and is further up-regulated during the osteogenic differentiation [35]. Also the content of OPG in the culture medium was significantly increased from hAD-MSCs cultured on scaffolds with 10 nM SIM compared to control at 8 ($p = 0.013$) and 21 ($p = 0.013$) days of culture (Fig. 3). It has been demonstrated that OPG, known as a potent anti-osteoclastogenic protein [36], is also capable of priming undifferentiated human MSCs for enhanced osteogenesis [37]. A

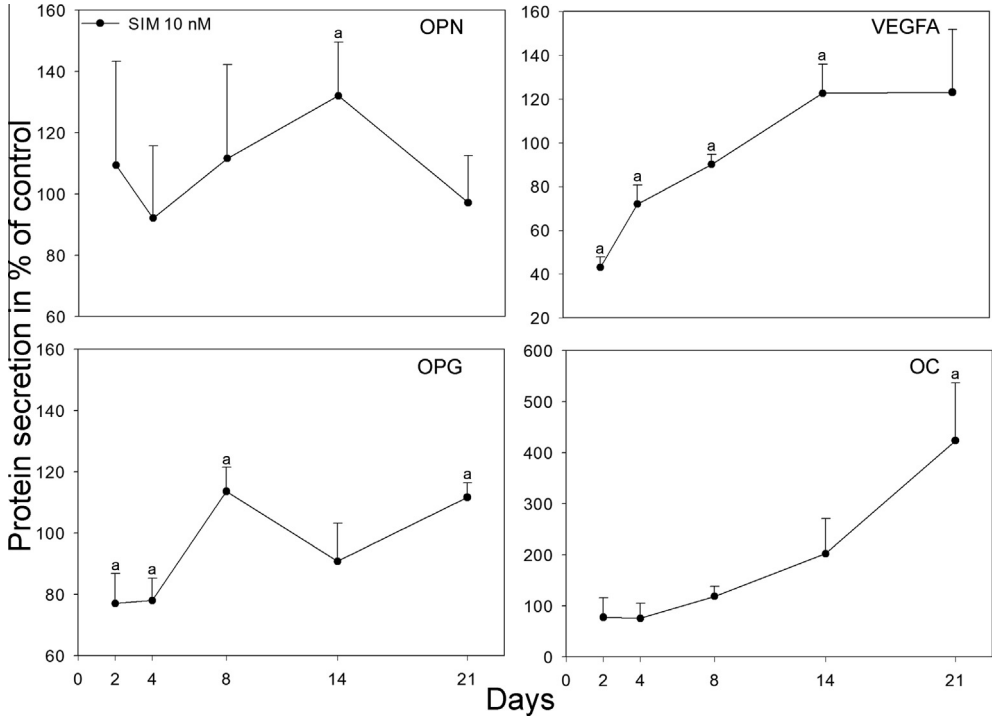

Fig. 3. Secretion of OPN, OPG, VEGFA and OC to culture medium from scaffolds with 10 nM SIM is presented in percentage of control at 2, 4, 8, 14 and 21 days. Values represent the mean of three donors (one parallel per donor) +SD. Statistical analysis: (a) $p \leqslant 0.05$ versus alginate-coated scaffold without SIM.

significantly lower amount of VEGFA was detected after 2 ($p = 0.001$), 4 ($p = 0.005$) and 8 ($p = 0.006$) days of culture in the medium from hAD-MSCs on scaffolds with 10 nM SIM compared to the control group. However, the secretion of VEGFA was continuously increasing in the cultures with 10 nM SIM, and after 14 days of culture a significantly ($p = 0.042$) higher amount was detected compared to control (Fig. 3). VEGF signaling is known to play a central role in angiogenesis and bone formation [38] and it was recently suggested that VEGFA-mediated control of stem cell fate is regulated by intracellular VEGFA and not by paracrine mechanisms [39]. An intracrine mechanism of VEGFA could explain why lower amount of VEGFA was detected in the medium from scaffolds with 10 nM SIM between day 2 and 8 despite similar mRNA expression in the two groups. Finally the secretion of OC, a marker of late osteoblast differentiation [40], was significantly enhanced from hAD-MSCs on scaffolds with 10 nM SIM compared to control at 21 days of culture ($p = 0.008$) (Fig. 3).

Taken together, the osteogenesis-related protein secretion pattern demonstrates the development of the osteoblast phenotype in hAD-MSCs cultured on scaffolds with 10 nM SIM.

3.2.3. ALP localization and activity

ALP, as a marker of osteogenesis in stem cells [41], was identified in the cell membrane of hAD-MSCs at day 14 in the majority of cells, both on scaffolds with 10 nM SIM (Fig. 4) and in the control group (not shown). The ALP activity in the medium was significantly increased from hAD-MSCs cultured on scaffolds with 10 nM SIM compared to control at 8 day of culture ($p = 0.002$) (Fig. 4). The peak in ALP activity observed at 8 day of culture may indicate that the hAD-MSCs have begun osteogenic differentiation. The gene expression of *ALPL* was only found to be higher in the SIM cultures compared to the control at day 14. However, *ALPL* may have been up-regulated earlier than 7 days, which was the earliest time point investigated.

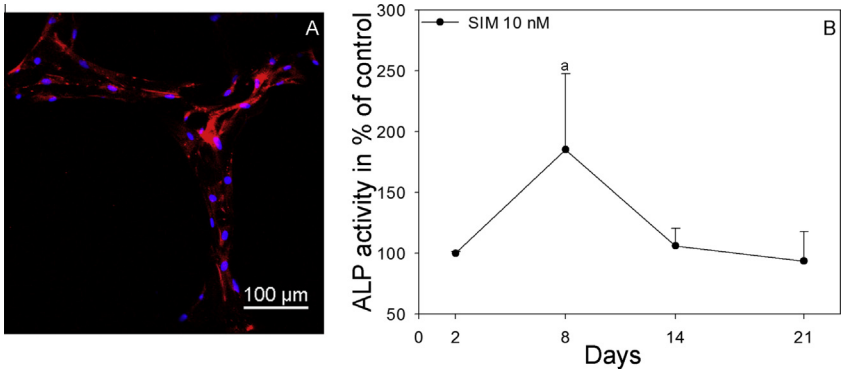

Fig. 4. Immunocytochemical staining of ALP in hAD-MSCs cultured on alginate-coated scaffold with 10 nM SIM. ALP is detected in the majority of the cells. ALP (red), DNA (blue) (A). ALP activity in culture medium from scaffolds with 10 nM SIM is presented in percentage of control at 2, 8, 14 and 21 days. Values represent the mean of three donors (three parallels per donor) +SD. Statistical analysis: (a) $p \leqslant 0.05$ versus alginate-coated scaffold without SIM (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

In summary, the study shows that TiO_2 scaffolds coated with alginate hydrogel containing 10 nM SIM significantly enhanced mRNA expression of *COL1A1, ALPL, SPP1, BGLAP* and *VEGFA* without inducing a cytotoxic response in hAD-MSCs. Furthermore, the secretion of OPN, OPG, VEGFA and OC was significantly increased from hAD-MSCs cultured on scaffolds with 10 nM SIM, confirming the osteogenic differentiation. The combination of the local osteogenic effect of SIM, hAD-MSCs and the physical properties of TiO_2 scaffold may represent a new strategy in stem cell-based bone tissue engineering.

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